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HEPATOLOGY HIGHLIGHTS

Neil Kaplowitz, Liver Biology and Pathobiology Editor

Kill It Before It Kills You

Vesicular stomatitis virus (VSV) is a nonpathogenic RNA virus that is sensitive to interferon suppression in normal but not cancer cells because the latter have defective antiviral pathways. Because of rapid viral replication in cancer cells, a lytic response precedes the development of an antiviral neutralizing immune response which otherwise would undermine the desired effect. Shinozaki et al. tested the efficacy of such an approach in therapy of multifocal HCC in rats. Rats were infused with rat HCC cells via portal vein to establish multifocal HCC. They were then fit with an indwelling hepatic artery cannula and infused with VSV at 0, 2 and 4 days. In serum and normal liver the virus disappeared very rapidly but was sustained for at least 4 days in the tumor tissue. The lytic response after repeated doses was more rapid than the neutralizing immune response (7 days). There was no increase in serum transaminases or histological changes in normal tissue. Repeated low doses of virus were much more effective than a single large dose — completely necrotic tumors in 29% of all nodules at day 5 and overall survival was prolonged with 18% “cure” (long term survival) (see Fig.) Thus, optimization of virotherapy with rapid acting oncolytic agents which are delivered in repeated doses directly to the liver cancer via hepatic artery has considerable promise in prolonging survival and even curing HCC. (See HEPATOLOGY 2005;41:196-203)

The Ups and Downs of LBP

Considerable evidence has accumulated in recent years to support a major role for the innate immune system in modulating the severity of acetaminophen (APAP) hepatotoxicity downstream of hepatocellular metabolism, covalent binding and GSH depletion. A key question is what triggers the participation of the innate immune system. Su et al. address this question by examining the toxicity of APAP in lipopolysaccharide binding protein lipopolysaccharide (LBP) null mice. LPS binds to LBP which then activates Kupffer cells by interacting with CD14 coupled to toll-like receptor-4. The null mice exhibited less necrosis and improved survival. This was accompanied by marked suppression of the upregulation of TNF, IL-10 and IL-6 mRNA. Interestingly, although protection against APAP injury was partial, cytokine expression was near-completely suppressed. No differences in expression of CYP was observed in null mice and GSH depletion and covalent binding were the same as in wild type. Thus, APAP metabolism was not changed in null mice. Finally, after pretreatment of null mice with adenovirus expressing LBP, plasma levels of LBP were restored and APAP-induced liver injury was significantly increased compared to adenovirus expressing β-galactosidase. These studies suggest that LPS, via LBP, plays a role in the activation of the innate immune response which contributes to APAP toxicity. However, since LPS levels in the portal vein did not change, it is not clear if LPS plays a “permissive” role in activating Kupffer cells (possibly sensitized by other effects of APAP) or if LBP exerts unknown effects independent of LPS. (See HEPATOLOGY 2005;41:187-195.)

Chemoprevention of HCC in Cirrhosis

HCC complicates cirrhosis which itself is a proliferative premalignant state. An important strategy in management of cirrhosis would be to prevent the development of HCC. TGF-α activation of EGFR is one potential pathway in promoting mitogenesis in the cirrhotic liver and HCC. Schiffer et al. addressed this issue by examining the effect of gefitinib, an EGFR-tyrosine kinase inhibitor, in a rat model of cirrhosis and HCC. Rats received daily ip diethylnitrosamine (DEN); cirrhosis developed in 12 weeks and multifocal HCC in 16 weeks of treatment (see Fig.) Therefore rats with cirrhosis were randomized to receive gefitinib versus vehicle from 12-18 weeks. TGF-α expression and hepatocyte proliferation were increased in the cirrhotic liver and even further increased in HCC nodules. Gefitinib treatment exerted a significant chemopreventive effect (3.7 vs 18.1 HCC nodules per liver). EGFR and downstream ERK phosphorylation were blocked by the treatment. The residual tumors showed persistent ERK activation along with Akt and IGF-2 which are thus mitogenic pathways independent of EGFR. In conclusion, this is an important study demonstrating that chemoprevention is a feasible strategy in situations where there is established cirrhosis and a stimulus to HCC formation. Clinical applications of this strategy are eagerly awaited. However, one concern is the effect of antiproliferative strategies on the maintenance of the cirrhotic hepatocyte functional mass over longer time intervals. (See HEPATOLOGY 2005;41:307-314.)

Fishing in the Gene Pool

Trovafoxacin, a quinolone antibiotic , is an idiosyncratic hepatotoxin. Postmarketing experience in more than 2 million pa-
tients uncovered reports of 150 cases of hepatotoxicity including 14 cases of acute liver failure. To address the mechanism of this adverse effect, Liguori et al. compared trovafloxacin to four other quinolones. None caused toxicity in animal models or in cultured human hepatocytes at very high doses. Microarray analysis of human hepatocytes, however, revealed changes which distinguished trovafloxacin from the other drugs in this class. A set of genes indicative of oxidative stress was selectively upregulated. Trovafloxacin depleted GSH in HepG2 cells at a much lower concentration than the other quinolones. This was preceded by increased peroxide exposure (DCF fluorescence). Further analysis revealed unique regulation of 142 genes, including some involved in RNA transcription. Since trovafloxacin inhibits bacterial topoisomerase and mammalian topoisomerase II is essential for transcription by RNA polymerase II, gene expression changes caused by trovafloxacin were compared to that caused by the topoisomerase II inhibitor, etoposide. Similar downregulation of genes involved in RNA transcription was observed with both drugs. Interestingly, the gene expression changes were very different in rat hepatocytes, although a few were the same as in human cells. These studies use a toxicogenomic strategy to identify changes in gene expression in human hepatocytes which may provide a unique signature and clues to pathogenesis, even in the absence of toxicity in the experimental system. Although this is a logical and meritorious approach in providing plausible candidate mechanisms contributing to toxicity or to susceptibility, the evidence is circumstantial, there were many alterations (which are important?), and there is a presumption that gene expression changes are involved in toxicity. Thus, the mechanism of trovafloxacin induced hepatotoxicity in rare individuals remains unknown. (See Hepatology 2005;41:177-186.)

The Source Determines the Course

Previous work has strongly implicated oxidative stress in experimental alcohol-induced liver injury. Of the sources of oxidative stress in studies using null mice, NADPH oxidase in nonparenchymal cells was implicated as more important in liver injury while Cyp2e1 did not seem to play a role (although this is controversial). Another consequence of oxidative stress, particularly relevant to HCC development, is DNA damage. Bradford et al. assessed oxidative DNA damage induced by ethanol in the rodent intragastric feeding model and the contribution of the sources of oxidative stress. Feeding ethanol to rats or mice for 4 weeks resulted in increased oxidative DNA adducts, mutagenic apurinic/apyrimidinic sites, and expression of base excision DNA repair genes that are known to remove oxidative DNA lesions. These markers were unaltered in p47 phox-null mice (decreased NADPH oxidase) but were completely absent in Cyp2e1-null mice. Furthermore, treatment with the p450 inhibitor, 1-aminobenzotriazole (ABT), blocked oxidative DNA damage. Interestingly, liver injury still occurred in Cyp2e1-null and ABT-treated mice. The findings suggest that ethanol-induced steatohepatitis and oxidative DNA damage are caused by unrelated mechanisms. Thus, oxidative DNA damage is uniquely dependent on CYP2e1 mediated reactive oxygen generation which may play an important role in the development of cancer in the livers of alcoholic and perhaps nonalcoholic fatty liver cirrhosis. It will be of interest to see if antioxidant treatment is effective in preventing DNA damage which would support a chemoprevention strategy for patients. (See Hepatology 2005;41:336-344.)

No Fret, Better Fate

Recent evidence suggests that EGFR plays a critical role in Fas (CD95) apoptosis. This involves JNK dependent EGFR/CD95 association and EGFR catalyzed tyrosine phosphorylation of CD95 leading to CD95-membrane translocation and DISC (Death-inducing signaling complex)-formation. Eberle et al. used fluorescence resonance energy transfer (FRET) technique to study the interaction between CD95 and EGFR in Huh-7 hepatoma cells which have low endogenous CD95. EGFR and CD95 coupled to different dyes were expressed in the cells allowing their close association to be viewed as a FRET-signal. Hyperosmolarity or CD95L induced EGFR/CD95 association (FRET-signal), CD95 phosphorylation and DISC formation (see Fig. which demonstrates the association of intracellular EGFR and CD95 and mobilization to the plasma membrane and membrane blebs induced by FasL). No intracellular FRET signal was observed in the presence of a JNK inhibitor demonstrating that association of EGFR and CD95 was blocked. On the other hand an inhibitor of EGFR-tyrosine kinase did not block this association but prevented translocation of the complex to the plasma membrane. Microtubule inhibitors also blocked translocation. CD95 mutants (tyrosine phosphorylation incompetent) did not translocate to the plasma membrane even though they associated with EGFR. Thus, EGFR must both associate with and phosphorylate CD95 in order for the complex to undergo microtubule-dependent translocation. Inhibition of any of these steps blocked CD95L induced apoptosis. DISC formation required plasma membrane targeting of the complex and did not occur in the cytosol, thus explaining the mechanism of the previously described protective effect of colchicine. This is an elegant study which elucidates the role of EGFR in promoting the Fas death pathway and offers a number of therapeutic targets to protect the liver. It is of interest that the DISC formation associated with hyperosmolarity was transient and did not propagate the apoptosis cascade, whereas it was sustained and did so in response to CD95L. One aspect requiring further exploration is how CD95L signals to activate membrane translocation of the CD95/EGFR complex. (See Hepatology 2005;41:315-326.)
roglitazone entered the U.S. market in 1997 as the first PPAR-γ agonist available to treat Type 2 diabetes. Within less than 1 year of product launch, the company began receiving reports of liver failure associated with roglitazone treatment. At first, it seemed possible that these events could be unrelated to roglitazone therapy and instead reflect the relatively high prevalence of liver disease in diabetics, particularly nonalcoholic fatty liver disease (NAFLD) and hepatitis C. Review of severe liver event reports, however, revealed a characteristic pattern, or “signature” not compatible with diabetes associated liver diseases. The roglitazone associated injury was generally acute, occurring after weeks or months of treatment. The characteristic injury was hepatocellular in nature with high peak serum aminotransferases. Elevation of serum bilirubin was delayed relative to the aminotransferase elevations, with jaundice typically appearing weeks after the onset. Elevations in serum alkaline phosphatase were generally mild and a late phenomenon. A striking feature of this “signature” was that after stopping treatment with roglitazone, the serum aminotransferases generally continued to rise for many days or weeks. Resolution of liver injury was prolonged, often taking weeks or months.

In a public meeting in March 1999, members of an FDA advisory committee were told that there had been more than 40 reports of acute liver failure in patients receiving roglitazone therapy. The committee suggested some restrictions on the use of roglitazone, but felt that roglitazone’s benefits outweighed its risks. A year later, when the next in class compounds were demonstrated to be safer, roglitazone was withdrawn from the market.

In the 4 years since the withdrawal of roglitazone, there has been a major increase in interest in drug induced liver disease (DILI). The withdrawal of roglitazone followed on the heels of other FDA actions concerning DILI, including the withdrawal of bromfenac, prescribing restrictions placed on felbamate, pemoline, tolcapone, and trovafloxacin, and a string of physician warnings concerning other medications. Troglitazone drew attention to the fact that DILI had become the major single cause for regulatory actions concerning drugs, and to the inability to consistently recognize potential for severe DILI during drug development. Within 1 year of the troglitazone withdrawal, an FDA working group consisting of representatives from the Pharmaceutical Research and Manufacturers Association of America (PhaRMA), and the AASLD released three documents addressing hepatotoxicity: “Nonclinical Assessment of Potential Hepatotoxicity,” “Clinical White Paper,” and “Post-marketing Considerations.” These documents provided “a framework for discussion” for a public workshop held in Chantilly, Virginia in February 2001. The FDA/PhaRMA/AASLD Hepatotoxicity Working Group has had regular meetings since.

The prolonged litigation that has followed the withdrawal of roglitazone has also intensified the interest in DILI research. In addition to amplifying the financial losses and negative publicity that followed the withdrawal, the litigation has created a cohort of plaintiff attorneys well versed on issues regarding DILI, and who have identified expert witnesses with opinions helpful to their clients. There has been concern within the pharmaceutical industry that DILI may become a preferred target for “Big Pharma” litigation in the near future. The roglitazone litigation has therefore contributed to the sense of urgency within industry to improve means of screening out liver liabilities at early phases of drug development. Many companies are hoping to solve the problem with “toxicogenomics.” One common theme has been to look at changes in the rodent liver transcriptome, proteome and/or metabolome in response to treatment with drugs known to cause hepatotoxicity in man (like troglitazone) versus drugs without known liver liabilities. From these studies, a predictive set of messenger RNAs, proteins, or metabolites are chosen to incorporate into compound screening. It appears that these genomic changes can significantly precede biochemical or histological evidence of hepatotoxicity and may therefore speed up preclinical safety screening. However, it is not yet clear whether this
approach will improve prediction for human hepatotoxic potential.

The troglitazone litigation is also beginning to have an impact on the field of hepatology outside the drug development arena. Many of the leading hepatologists and scientists from around the world have been approached to serve as consultants for either the defense or for the plaintiffs. The attorneys generally select the consultants whose opinions most bolster their side’s case. These experts are then asked to write detailed and fully referenced reports addressing specific questions. These reports are then exchanged between the sides. New issues are identified and new reports generated, either by the original experts, or by new experts selected by the attorneys. Key aspects of each side’s legal strategy emerge from this iterative process.

Many of the questions the consultants were asked to address dealt with the mechanisms underlying troglitazone liver injury. As reviewed by Chojkier in this issue of HEPATOLOGY,10 there is not yet an accepted mechanism for troglitazone liver injury. This uncertainty is probably surprising to judges and juries, and causes confusion that can be exploited by each side. It would be beneficial to the plaintiff case if the mechanisms involved (e.g., covalent binding, glutathione depletion, mitochondrial toxicity) could have been detected in assays available in the early 1990s. It could then be argued that the company was negligent in not identifying the problem early in development. Likewise, mechanisms that supported intrinsic rather than idiosyncratic toxicity helped label troglitazone as a “defective product.” In addition to the legal implications of this designation, the status of “intrinsic toxin” could mean to a jury that all treated patients, not just those with the “signature” events, were potentially “poisoned” and therefore entitled to compensation. Along these lines, some plaintiff experts even argued that because troglitazone could cause cells to undergo apoptosis under certain culture conditions, troglitazone might have caused a “silent” liver injury.11 If a jury could be convinced that this was a possibility, the plaintiff pool could potentially include all of the estimated 2 million patients exposed to troglitazone, even those whose physicians dutifully monitored liver chemistries as recommended.

It is important that the perspectives of both defense and plaintiff experts are now appearing in this and other journals. This allows the larger scientific community to obtain the educational benefits of countless hours of literature research. It also invites opinions from a much broader audience.

In spite of the recent increased interest in DILI, very little progress has been made toward understanding mechanisms underlying idiosyncratic hepatotoxicity. The problem is not just that there are no good animal models of idiosyncratic hepatotoxicity, but that the vast majority of humans are not good models. A key to identifying the mechanisms underlying idiosyncratic hepatotoxicity will therefore be to study individuals who have actually experienced idiosyncratic DILI. This is one of the goals of the National Drug Induced Liver Injury Network (DILIN) recently sponsored as a cooperative agreement (U01) by The National Institute of Diabetes and Digestive and Kidney Diseases.12,13 Genomic DNA, serum, and lymphocytes for immortalization will be obtained from patients who have developed idiosyncratic liver injury. These patients will remain in a registry for up to 20 years so they can be offered enrollment in carefully controlled “phenotyping” studies. The creation of this tissue bank and registry should speed progress in DILI research and hopefully prevent another troglitazone.

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References
Biliary atresia (BA) is a progressive, inflammatory cholangiopathy that uniquely presents only during the first few months of life. It is characterized by initial inflammation, fibrosis, and obliteration of the extrahepatic bile ducts, with subsequent sclerosis of intrahepatic bile ducts culminating in biliary cirrhosis sometime during childhood in the majority of patients. Although the etiology of BA is not clearly understood, in most cases a perinatal insult (e.g., a viral infection) appears to trigger an immune (or autoimmune) response causing inflammation and sclerosis of a normally formed biliary tree (the perinatal form of BA).

In approximately 20% of patients, defective development of the extrahepatic bile ducts is suggested by its clinical association with other major congenital anomalies of the gastrointestinal and cardiovascular systems (the embryonic form of BA). Without surgical intervention or liver transplantation, children with BA die from complications of end-stage liver disease in the first 2 years of life. If a child is diagnosed before reaching 3 to 4 months of age, surgical construction of a roux-en-Y portoenterostomy achieves bile flow in a variable percentage of patients and delays the need for liver transplantation, which is eventually required in 70% to 80% of patients in Western countries. Thus, BA accounts for almost 50% of all liver transplants performed in children. Approximately 28% to 35% of BA patients at experienced centers in the United Kingdom, France, Italy, and the United States survive at least 10 years with their native liver, compared with 53% in the Japanese BA Registry. At Tohoku University in Japan, Ohi reports that 75% of long-term survivors with their native liver (92 patients >10 yr of age with almost half >20 yr of age) lead a normal or nearly normal life, although the remaining patients have complications such as easy fatigability, esophageal varices, hypersplenism, hepatopulmonary syndrome, and recurrent cholangitis. Based on the experience in Japan, it appeared that perhaps three quarters of long-term survivors with BA could survive well into adulthood without the need for liver transplantation.

In this issue of Hepatology, Lykavieris et al. report findings that raise questions about whether such favorable long-term, transplant-free survival is likely to occur in BA in Western countries. The group from the Hôpital Bicêtre describe 63 BA patients in France who survived 20 years with their native liver following portoenterostomy, which was equivalent to 23% of patients who underwent BA surgery as infants. Although one third of long-term survivors had normal serum bilirubin concentration, all but two patients had signs of cirrhosis or portal hypertension, and 16 underwent liver transplant or died after 20 years of age. Thus survival without liver transplantation approached 10% at 30 years of age. Long-term complications included ascites, esophageal and gastric variceal hemorrhage, hepatopulmonary syndrome, and pulmonary hypertension, recurrent and severe cholangitis, cholelithiasis, and the risk of alcoholic hepatitis. If these data are applicable to other Western countries, the long-term prognosis for survival with native liver in BA is not as favorable as that reported from Japan. The explanation for this difference in outcome may relate to differences in unknown genetic factors that favor long-term survival in Asian BA patients or to the known determinants for success of the initial portoenterostomy treatment for BA: age at portoenterostomy, the experience of the Center in which the surgery is performed, and differences in postoperative medical care. Close scrutiny of these factors may lead to potential interventions that can be instituted early in life to optimize the outcome of BA patients, leading to longer survival into adulthood without transplantation. In this editorial, we will examine the evidence that supports these interventions.

The age at referral for corrective BA surgery is one of the most important factors determining outcome. Delays in diagnosis of BA remain a considerable problem in many countries. Neonatal jaundice through the first 2 weeks of life is frequently ascribed to “breast milk jaundice” or physiological jaundice. It is the presence of acholic stools, dark urine staining diapers, failure to thrive, enlarged liver or spleen, or persisting jaundice that should prompt evaluation for hepatobiliary disorders. In the United States, normal infants are generally seen by health care providers for routine care at ages 2 and 8 weeks. If evaluation of persisting jaundice is not performed after the 2-week visit but is delayed until after the 8-week visit, the infant is approaching the age limits for...
optimal results of portoenterostomy. It is generally regarded that effective bile drainage with resolution of jaundice can be achieved in up to 70% of BA patients if the portoenterostomy is performed before 60 to 70 days of life, while surgery performed between 70 and 90 days of life yields 40% to 50% of patients with good bile drainage, between 90 and 120 days up to 25% of patients, and after 120 days only 10% to 20% of patients. Long-term survival is similarly associated with age at the time of portoenterostomy. Consequently, it is essential that infants who remain jaundiced after age 2 to 3 weeks be expeditiously evaluated for the presence of conjugated hyperbilirubinemia, and if present, for BA. To identify BA at an earlier age, some countries are evaluating the use of a stool color card as a screen for acholic stools, which would be brought in by the parents at the routine health visit in the first month of life.

The experience of the center performing the portoenterostomy bears a strong influence on the likelihood that BA patients will drain bile following surgery, and, moreover, will survive with their native livers. Three studies have carefully evaluated this “center effect” on outcome. McClement et al. analyzed the surgical outcome for BA in 95 patients in the United Kingdom from 1980 to 1982. The 16 different centers performing portoenterostomies were grouped by the number of cases performed each year. In centers with 1 case per year, 11% of patients were jaundice-free following surgery; in centers with 2 to 5 cases per year, 29% became jaundice-free; and in centers with more than 5 cases per year, 43% were jaundice-free. The age at which surgery was performed was similar in all centers and did not account for the observed differences in outcome. In a large study from France, Chardot et al. analyzed survival data on 440 BA patients from 1986 to 1996 with a mean follow-up of 65 months. Univariate analysis revealed significant improvement in survival (with native liver, after liver transplant, and overall) in the center which performed more than 20 portoenterostomies per year (36.4 ± 3.9% 10-year survival with native liver) compared with centers that performed 3 to 5 portoenterostomies per year (20.9 ± 6.2%) and 2 or fewer per year (18.0 ± 3.5%). Multivariate analysis also demonstrated that the size (i.e., the experience) of the center was a significant predictor of survival. Finally, McKiernan et al. reviewed the outcome of infants with BA in the United Kingdom and Ireland from 1993 to 1995. Cases were identified prospectively by the British Pediatric Surveillance Unit, which distributes monthly reporting cards to all pediatricians. Ninety-one cases of BA were managed and herbal therapy to improve liver health (e.g., kanzou or licorice root). The anti-inflammatory properties of corticosteroids are known to reduce production of inflammatory cytokines (e.g., tumor necrosis factor α, interleukin 1, interleukin 8), prostaglandins, and nitric oxide, all of which have been implicated in the pathogenesis of BA. Corticosteroids also inhibit inflammatory cell migration to sites of inflammation and promote the death of lymphocytes through apoptosis. The results of four open-label studies (without control groups) suggest that postoperative corticosteroids improve the likelihood of bile drainage and survival in BA. However, these studies are severely limited by the lack of randomization to corticosteroids versus placebo in a blinded fashion, the lack of systematic follow-up to assess the safety of corticosteroid therapy, and the confounding effect of different surgeons and postoperative care regimens in addition to the use of corticosteroids. Clearly, a prospective randomized controlled trial of sufficient size will be required to truly assess the potential value and determine the safety of corticosteroids in the management of BA.

The use of perioperative intravenous antibiotics is commonplace at the time of Kasai portoenterostomy; however, the duration of intravenous antibiotics varies widely. Most institutions administer intravenous antibiotics for 2 to 3 days. As stated, many centers in Japan routinely administer intravenous antibiotics for the first 2 to 3 months after surgery. Oral antibiotics are frequently administered as prophylaxis against cholangitis for varying periods, initiated either immediately after surgery or following the first bout of cholangitis. Two oral antibiotics that have been used are...
trimethoprim-sulfamethoxazole and neomycin. Although there are no prospective trials of oral antibiotics, Bu et al. compared the efficacy of trimethoprim-sulfamethoxazole and neomycin used as prophylactic agents against recurrence of cholangitis until 3 years of age in 19 children who had one episode of cholangitis, and compared the results with those of 18 historical control patients who did not receive antibiotic prophylaxis. Patients who received prophylaxis had lower recurrence rates of cholangitis (relative risk of 0.52 and 0.42 for trimethoprim-sulfamethoxazole and neomycin, respectively). This study was limited by the retrospective nature of the control group. Prospective evaluation of timing and dosing of antibiotic prophylaxis is clearly needed.

Ursodeoxycholic acid has been administered to BA patients for variable lengths of time following portoenterostomy, because of its cytoprotective, choleretic, and immunomodulatory effects. Although there are no published randomized controlled trials of ursodeoxycholic acid in BA, it has been shown to be of benefit in adults with primary biliary cirrhosis and in cystic fibrosis. However, BA patients with unsuccessful portoenterostomies may have virtually no bile flow from the liver, raising concern that ursodeoxycholic acid could be retained in the liver in these patients and be potentially toxic. Thus further research on the efficacy and safety of ursodeoxycholic acid in BA is needed.

In summary, optimizing the outcome of patients with BA will require innovative approaches to establishing earlier diagnosis, the possible development of surgical centers of excellence, and improvement of current postoperative therapy. Clearly, large, randomized, controlled trials will be needed to determine the efficacy and safety of such therapies. Despite these efforts, the disappointing truth is that only a fraction of affected infants will survive into adulthood with our current treatments. Thus, there is an urgent need to better understand the etiology, genetics, and pathogenesis of BA so that novel therapeutic and preventative strategies can be developed in the future. The new BA Clinical Research Consortium supported by the National Institutes of Health and similar consortia in Europe and Asia will provide the platform and organizational structure to address many of the issues, with the goal of extending quality survival of children with BA into adulthood.

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References

Ribavirin is a synthetic purine analogue that has a broad spectrum of antiviral activity and is approved for use in combination with interferon for the treatment of chronic hepatitis C. Clinically, it appears to act synergistically with interferon to result in a small increase in end-of-treatment response but more importantly a doubling of the sustained virological response (SVR) rate by preventing virological relapse. In vitro, ribavirin has been shown to act as an immunomodulator, a competitive inhibitor of inosine monophosphate dehydrogenase, a direct inhibitor of the viral RNA dependent RNA polymerase and as a viral mutagen. However, it is unclear which, if any, of these mechanisms is important for its antiviral effect in vivo.

The recommended therapy for patients with chronic hepatitis C is the combination of either peginterferon alfa-2a or -2b once weekly plus daily ribavirin for 48 weeks.\(^1,2\) The recommended dose of ribavirin is dependent on the preparation of peginterferon used. In the United States, ribavirin is approved for use with peginterferon alfa-2b at a fixed dose of 800 mg daily, but in combination with peginterferon alfa-2a, ribavirin is given in a dose of 1,000 or 1,200 mg based on whether body weight is less than or greater than 75 kg, respectively. In Europe, the approved daily dose is based on weight regardless of peginterferon preparation: 800 mg for patients below 65 kg; 1,000 mg for those between 65 and 85 kg; and 1,200 mg for patients heavier than 85 kg. The decision in the United States to approve a lower, fixed dose of ribavirin in combination with peginterferon alfa-2b was based on Food and Drug Administration concerns about the lack of data on efficacy and more importantly, the safety of higher doses of ribavirin.\(^3\) However, in a secondary analysis of the peginterferon alfa-2b plus ribavirin registration trial, body weight was identified as an important predictor of response and the European regulatory agency chose to approve weight-based ribavirin.\(^1\) The approved regimens yield similar SVR rates ranging from 54% to 63%.\(^1,2,4\) In an effort to improve SVR rates, investigators have experimented with different regimens by varying the dose and duration of therapy. A theme emerging from recent studies is that therapy should be tailored to different groups of patients. The current data suggest that patients with genotype 1 should be treated for 48 weeks, whereas those infected with genotypes 2 and 3 might be treated for a shorter duration of 24 weeks and perhaps as short as 14 to 16 weeks in patients who achieve an early virological response (hepatitis C virus RNA negative at treatment week 4).\(^5\)

A controversial issue in chronic hepatitis C therapy is the optimal dose of ribavirin to use in combination with peginterferon and whether higher doses of ribavirin are more effective. The initial evidence supporting higher doses of ribavirin was largely indirect and evolved from a secondary analysis of the large multicenter trial of peginterferon alfa-2b and ribavirin. A logistic regression analysis revealed that, if the ribavirin dose was expressed as mg/kg, patients who received higher doses experienced the highest SVR rates.\(^1\) Using an arbitrary ribavirin dose cutoff of 10.6 mg/kg, genotype 1 patients who received the standard dose of peginterferon alfa-2b (1.5 µg/kg/wk) and higher doses of ribavirin (>10.6 mg/kg) were shown to have higher SVR rates compared with those receiving lower doses (<10.6 mg/kg), 48% versus 38%. Although suggestive that higher doses of ribavirin were better, these data should be interpreted with caution because they were derived from a secondary analysis with the potential for confounding variables and the small numbers of patients within subgroups. More compelling data came from a recent randomized trial of peginterferon alfa-2a and ribavirin which was specifically designed to assess efficacy of low-dose versus standard dose ribavirin. The results clearly showed that for genotype 1 patients treated for 48 weeks the SVR rate was superior in those receiving standard dose (1,000 to 1,200 mg) compared with those receiving low-dose ribavirin (800 mg), 52% versus 41%.\(^4\) In contrast, both studies showed no difference in the SVR rate in patients with genotypes 2 and 3 infections using ribavirin doses ranging from 800 to 1,200 mg daily. Thus, the issue can be narrowed to optimizing therapy for genotype 1 patients.

In this issue of Hepatology, Lindahl and coworkers provide some further data regarding the benefit of higher

**Abbreviation:** SVR, sustained virological response.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20605

Conflict of interest: Nothing to report.
doses of ribavirin for genotype 1 patients. They conducted a small pilot study in which they treated 10 patients with standard doses of peginterferon alfa-2a and individualized the daily dose of ribavirin to achieve a target ribavirin concentration in serum of 15 μmol/mL. The primary goal was to evaluate the safety of using higher doses of ribavirin in patients with genotype 1 and high viral load (the difficult-to-treat patient profile). To achieve the targeted serum concentration, patients required a mean ribavirin dose of 2,540 mg/day (range 1,600-3,600 mg/day), which is more than twice the currently recommend maximum daily dose. However, in an intention-to-treat analysis of 10 patients, the SVR rate was an impressive 90%. Side effects were severe, particularly hemolysis and anemia. Every patient required erythropoietin, and two patients required blood transfusion on two separate occasions.

The results of this study are indeed striking but a note of caution must be struck. Foremost is the issue of safety. One of the major limitations to combination therapy is the high frequency of side effects, some of which may be serious and life-threatening. In the two large multicenter registration trials, 10% to 14% of patients required discontinuation of therapy and 32% to 42% required dose modification for serious or severe side effects.1,2 Higher doses of ribavirin would undoubtedly lead to more side effects. Hemolytic anemia is the major risk associated with ribavirin, and, if defined as a hemoglobin level less than 10g/dL, occurred in 7 of 10 patients. All patients required hemopoetic growth factor, two required blood transfusion and four required dose reduction or temporary discontinuation of the drug to manage side effects. Furthermore, all patients experienced a reduction in ability to work, although, quality-of-life was not specifically assessed. The need for erythropoietin, blood transfusion, and loss of work are not trivial matters particularly in the typical patient with hepatitis C who has few if any symptoms and is largely fully functional. Anemia caused by ribavirin can induce cardiovascular and cerebrovascular incidents in susceptible patients. Thus, even with intensive monitoring this high-dose ribavirin regimen can be life-threatening. Second, we should recognize that the study by Lindahl et al. was a small, nonrandomized pilot one without a control group. Finally, difficult-to-treat patients such as African Americans, those with significant comorbidities and patients with cirrhosis, all of whom have been shown to have lower SVR rates, were not included.3,8

Another concern in today’s practice environment is the cost associated with this treatment regimen. A 48-week course of standard treatment without the need for supplementary therapy or testing costs approximately 24,000 U.S. dollars.9 The additional cost associated with a high-dose ribavirin regimen would not be insignificant and include paying for a greater quantity of ribavirin, the need to monitor ribavirin levels, more frequent laboratory monitoring, use of erythropoietin, blood transfusion, additional clinic visits, hospitalizations, and time missed from work. The obvious question is whether the additional risks and costs are worth the increase in SVR. For genotype 2 and 3 patients who already have high SVR rates with standard peginterferon and low-dose ribavirin, the incremental benefit of higher ribavirin doses is likely to be minimal and not worth the added risks and costs. Similarly, it would be difficult to justify this high-dose ribavirin regimen for treatment naïve genotype 1 patients given a SVR rate of 42% to 47% with standard therapy. Such an approach would unnecessarily expose close to 50% of genotype 1 patients to a potentially more toxic therapy. For the moment, we would suggest that until more data and experience are available, this regimen should not be offered outside the setting of a clinical trial. Ideally, this approach should be evaluated in a prospective controlled trial using patients who have failed to respond to standard therapy.

The finding of Lindahl and colleagues that higher doses of ribavirin may improve the SVR rate suggests that a safer ribavirin preparation is required and that ribavirin-like compounds with better safety profiles may have a role to play in therapy. Viramidine, a prodrug of ribavirin that specifically targets the liver, is associated with less hemolysis than ribavirin, and in preliminary clinical studies, seems to have similar efficacy.10 Levovirin, the L-enantiomer of ribavirin, has been disappointing thus far in clinical trials but higher doses have not been tested.11

The apparent increase in effectiveness of high-dose ribavirin over standard dose is intriguing from a mechanistic view. At higher doses, ribavirin may either augment previously proposed antiviral pathways, act through a novel mechanism or, perhaps, at higher doses more patients achieve a critical threshold of intracellular ribavirin concentration. Unfortunately, the results of Lindahl and coworkers seem to raise more questions than to provide answers as to the mechanism of action of ribavirin. Clearly more research is needed in this area. Future studies should address the dosing, pharmacokinetics, and mechanism of action of ribavirin. The rationale for choosing a target concentration of 15 μmol/L appeared to be arbitrary and not based on good pharmacokinetic and pharmadynamic data. Lower plasma concentrations may have been as effective but it also raises the question as to whether ribavirin should continue to be administered based on body weight or dosed to achieve a target serum concentration. Another possibility is that inter-individual varia-
tions in the metabolism of ribavirin may account for differences in clinical efficacy. Thus, patients who are nonresponders to weight-based therapy may be receiving subtherapeutic doses of ribavirin. This possibility may to a certain extent explain the low SVR rates observed in two trials of peginterferon and ribavirin in human immunodeficiency virus coinfected patients and a recent Spanish Trial of 72 weeks versus 48 weeks of peginterferon plus ribavirin for patients who fail to achieve virological clearance at week four.

In summary, Lindahl et al.’s article is an important study whose results probably provoke more thought about ribavirin’s mechanism of action than provide a practical therapeutic approach for patients with chronic hepatitis C. At present, this strategy should be reserved for the setting of controlled clinical trials and not become part of standard clinical practice.

Acknowledgment: The authors are grateful to Jay Hoofnagle, M.D., for helpful discussion and suggestions during the conceptual phase of this editorial.

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References
Troglitazone (TGZ) (Rezulin; Pfizer, Madison, NJ) was withdrawn from the market by the U.S. Food and Drug Administration (FDA) in March 2000, because it was associated with the development of acute liver failure.1 Approximately 35,000 TGZ injury claims are pending within the United States.2 However, even 4 years later, the mechanisms of TGZ-induced liver injury remain highly controversial and poorly understood. A review of the topic proposed that TGZ hepatotoxicity was mediated through the induction of steatosis and apoptosis and that the injury could be silent (without increased serum aminotransferases, bilirubin, or alkaline phosphatase).3 In addition, it has been suggested that TGZ may induce liver injury as a consequence of mitochondrial damage, oxidative stress, or by inhibiting the bile salt excretory protein (Bsep).3 Furthermore, these effects have been attributed, at least in part, to the accumulation of TGZ in the liver, particularly among patients with liver disease, as well as to the peculiar metabolism of the drug.3 Finally, it has been postulated that liver fibrosis and cancer may result from TGZ hepatotoxicity.3 These presumed chronic complications would constitute an extraordinary medical burden to the approximately 1.92 million patients treated with TGZ. Because these alleged long-term complications of TGZ-induced liver injury have significant medical, social, legal, and financial repercussions, this review evaluates the scientific merits of the proposed mechanisms and suggest alternative explanations.

Abbreviations: TGZ, troglitazone; FDA, Food and Drug Administration; Bsep, bile salt excretory protein; PPAR, peroxisomal proliferator-activated receptor; ALT, alanine aminotransferase; NASH, nonalcoholic steatohepatitis; Cmax, maximal concentration; TNF, tumor necrosis factor.

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Received September 20, 2004; accepted December 2, 2004.

The author’s research is supported by grants from the National Institutes of Health (DK-38652, R37 DK-46971), and the Department of Veterans Affairs (Merit Review).

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DOI 10.1002/hep.20567

Potential conflict of interest: Dr. Chojkier is a consultant and expert witness for Pfizer in the Rezulin litigation.

Troglitazone and Liver Injury: In Search of Answers

Mario Chojkier

Troglitazone-Associated Liver Injury Is Idiosyncratic

Insulin resistance can result from an inherited defect in mitochondrial oxidative phosphorylation in children of patients with type 2 diabetes4 or from acquired defects in mitochondrial biogenesis,5 which leads to reductions in mitochondrial function, in elderly individuals.6

TGZ, a peroxisomal proliferator-activated receptor (PPAR)-γ agonist that improved insulin resistance, was used for the treatment of type 2 diabetes7-8 in the United States by approximately 1.92 million patients from March 1997 to March 2000.9 As a consequence of the improved insulin sensitivity,8 TGZ had beneficial cardiovascular effects,10-13 protected pancreatic β-cell function,14 and corrected metabolic abnormalities associated with polycystic ovarian syndrome15 and lipodystrophy.16

In addition, TGZ antagonized the corticosteroid induction of insulin resistance and abnormal glucose tolerance.17

In clinical trials, 1.9% of the subjects who received TGZ and 0.6% of the subjects who received placebo had elevations (≥3 times the upper limit of normal) of serum alanine aminotransferase (ALT) concentrations.18 In this cohort of 2,510 subjects, TGZ induced overt liver injury and jaundice in two individuals.18 A retrospective analysis also concluded that approximately 2% of the patients developed TGZ-induced liver injury judging by the serum ALT elevations.19 By comparison, serum ALT elevations (≥3 times the upper limit of normal) occurred in approximately 25% of subjects taking tacrine,20 a drug that has not been associated with acute liver failure; however, probably fewer than 100,000 patients have been treated with this drug. Unfortunately, 94 of the 1.92 million patients developed liver failure while taking TGZ.1 Although only 49 of these liver failure cases were considered to be possibly or probably related to TGZ,9 the numbers may be higher because of incompleteness of reporting.1

TGZ treatment was associated with a characteristic hepatocellular injury,18-21-35 with rare instances of either a mixed hepatocellular/cholestatic injury or a predominant cholestatic reaction.36-38 The liver injury associated with TGZ is idiosyncratic39; it is unpredictable, neither time-nor dose-dependent,40 and cannot be reproduced in animals.41-43 Although the mechanism of liver injury induced by TGZ is believed to be a metabolic idiosyncrasy (nonimmunological),39 some of the case reports had

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histological evidences suggestive of an immunological reaction \[16,25,27,36\] or responded favorably to corticosteroids.\[28,38\] One of these patients promptly developed a similar destructive, granulomatous cholangitis when treated subsequently with rosiglitazone, a related PPAR-\(\gamma\) agonist,\[38\] supporting the diagnosis of an immunological reaction and suggesting a class effect.

According to Zimmerman and Ishak\[44\] and Klatskin and Conn,\[45\] the occurrence of numerous eosinophils or granulomatous inflammatory infiltrates in the liver is sufficiently compelling, albeit circumstantial, evidence that hypersensitivity may play an important role in the injury provoked by TGZ in some patients.

**Epidemiology of Troglitazone-Associated Liver Injury**

Although valuable to the medical and scientific community, neither the MedWatch Reports to the FDA nor the published case reports provide statistical support for the causal association between TGZ and acute liver failure. In addition, and except in rare instances (e.g., rechallenge\[38\]), presumed idiosyncratic liver injuries cannot be attributed unequivocally to TGZ or any other drug.\[40,46,47\] As evidence of the diagnostic uncertainties, in the United States, the cases of acute liver failure of undetermined causes (17%) exceed those attributed as probable to all medications combined (13%), excluding acetaminophen.\[48\] Nonetheless, in a prospective study of acute liver failure between 1998 and 2001 at 17 tertiary care centers in the United States, TGZ was prominently implicated, contributing 10% to 15% of the idiosyncratic drug reactions.\[48\] In a small cohort of type 2 diabetic patients and based on a single case of acute liver failure, the incident rate of acute liver failure attributed to TGZ was 240/million person-years,\[49\] compared with the estimated idiopathic acute liver failure of 1 to 2/million person-years.\[49,50\]

Although a study suggested that type 2 diabetes may increase the risk of acute liver failure independently of underlying chronic liver disease or viral hepatitis,\[51\] the study did not eliminate the possible contribution of decompensated chronic liver disease to liver failure. However, in a cohort of diabetic patients treated with hypoglycemic drugs, the incidence of serious liver injury was similar among those treated with sulfonylureas, insulin, metformin, or TGZ (approximately 100/million person-years).\[52\] The estimated risk of unexplained acute liver failure (with coagulopathy and encephalopathy) in type 2 diabetes was approximately 40/million person-years,\[53\] substantially higher than that of 1 to 2/million person-years estimated in the general population.\[49,50\]

Thus, TGZ induces serious liver injury in a large number of patients, but the background incidence rate of acute liver failure among type 2 diabetic patients remains to be determined in prospective studies.

**Troglitazone Pharmacokinetics**

TGZ is metabolized predominantly by sulfation to metabolite 1 (M1) and, to a lesser extent, by glucuronidation to M2 and by oxidation through cytochrome P450 (CYP)3A4 to a quinone M3.\[42,55,56\] TGZ concentration in the liver is essentially the same as that in plasma because these pools are in equilibrium.\[57-61\] In rats, the maximal concentration (CMax) of TGZ in plasma and in the liver was approximately 2.6 \(\mu\)mol/L, whereas the concentration of M1+M2+M3 in the liver was roughly 52 \(\mu\)mol/L.\[58\] Thus, TGZ does not accumulate in the liver, as has been suggested,\[3\] and the actual liver TGZ concentration should be used as a reference for cell culture toxicity studies rather than the combined CMax in the liver for TGZ and its metabolites (approximately 18.5 \(\mu\)g/g, approximately 55 \(\mu\)mol/L).\[57\] Virtually all of the excreted dose in bile was represented by M1, M2, and M3. Indeed, TGZ was rapidly metabolized in the rat to M1 and M2. Thirty minutes after the intravenous administration of TGZ (at a dose approximately 3-fold greater than that given to patients), which is expected to result in much higher plasma TGZ concentrations than when administered orally,\[62\] the concentration of TGZ in the liver was undetectable in male and was approximately 10 \(\mu\)mol/L in female rats.\[50\] The diabetic KK mice had a faster metabolism of TGZ than normal mice, with approximately 66% of the dose being metabolized after 1 hour.\[57\] Experiments in diabetic KK mice showed that the combined CMax for TGZ and its metabolites in the liver was lower (approximately 23 \(\mu\)mol/L) than in normal animals.\[57\]

Studies of up to 52 weeks in monkeys, the closest animal model to humans for the study of TGZ,\[42,43\] showed that at doses 160-fold higher than that given to patients, TGZ was metabolized efficiently but the sulfate M1 accumulated in plasma.\[42\] In humans, after reaching a steady state, the plasma CMax for TGZ (approximately 3-6

\[\text{The molecular weight of TGZ is 445D and the hepatic water content was estimated to be 75\% (\(\mu\)g/g \times 1000/445 \times 100/95 = \(\mu\)mol/L).}\]
µmol/L) was approximately 12% of the CMax for TGZ + M1 + M3. This indicates a very efficient metabolism of TGZ by the liver, supporting the notion that hepatic TGZ in humans is in equilibrium with plasma TGZ. The peak level occurs approximately 4 hours after taking TGZ, and the plasma concentration drops progressively from 4 to 24 hours, which would also decrease the liver concentration concomitantly. Moreover, because TGZ is tightly bound to albumin in plasma (95%-99.8%), only 0.2% to 5% is available as free plasma TGZ capable of a steady-state equilibrium with hepatocytes.

It has been speculated that TGZ levels in the livers of patients with alcohol-induced liver disease and NASH should be higher than in normal individuals, leading to greater rates of liver injury in these patients. However, this hypothesis is not supported by the available experimental data. In patients with type 2 diabetes, the pharmacokinetics of TGZ were normal, and the plasma CMax (and consequently, the liver CMax) for TGZ, M1, and M3 were not different from those of healthy normal individuals. The CMax for TGZ was 16% and 39% lower in cirrhotic patients with moderate and severe liver impairment, respectively. The CMax of the quinone M3 was normal, whereas the CMax for the sulfate M1 was increased in cirrhotic patients. However, the M1 metabolite showed no toxicity in cultured hepatocytes, in HepG2 cells, or in monkeys, even at much higher plasma concentrations. Indeed, the total integrated plasma concentration for M1 among cirrhotic patients was less than 10% of that in monkeys receiving an extremely high TGZ dose for 52 weeks. Likewise, the quinone M3 was not toxic to cultured rat hepatocytes even at concentrations of 100 µmol/L (approximately 50-fold the liver concentration).

### Oxidative Stress and Mitochondrial Damage

Although TGZ has been proposed to exacerbate fatty liver by producing oxidative stress and inflammation in the liver, TGZ actually has potent antioxidant and anti-inflammatory properties.

Increased oxidative stress can lead to mitochondrial damage within the cell, and, in turn, decreased mitochondrial oxidation of fatty acids results in the accumulation of triglycerides. Therefore, increased triglyceride concentration in the liver is an indicator of mitochondrial damage. TGZ treatment induced fatty liver in both diabetic KK mice, and in hyperlipidemic (NZO × NON) F1 mice, but the hepatic triglyceride concentration remained unchanged or was not reported, reflecting the accumulation of unidentified lipids, apparently, unrelated to mitochondrial injury. Moreover, similar fatty changes in the liver occurred after the administration of rosiglitazone or pioglitazone.

Therefore, these mouse models are poor predictors of the liver abnormalities associated with TGZ given that they do not reflect the pathological conditions observed in humans and that the current glitazones, which have not been associated with acute liver failure, induce similar abnormalities.

TGZ, at concentrations relevant to human pharmacokinetics, did not induce changes in mitochondrial oxidation in normal cells. Although TGZ inhibited fatty acid oxidation and esterification in isolated hepatocytes from starved rats, this effect occurred at a concentration approximately 200-fold greater than that achieved therapeutically in the liver.

Furthermore, TGZ prevented mitochondrial abnormalities and apoptosis, as well as decreased liver and pancreatic islet triglyceride concentration in Zucker diabetic rats and Long-Evans fatty rats. More importantly, TGZ decreased the liver size and fat accumulation in the livers of patients with insulin resistance and type 2 diabetes or lipodystrophy syndrome, as determined by magnetic resonance imaging and computed tomography scans. In addition, TGZ improved mitochondrial oxidation in insulin-resistant patients, presumably through PPAR-γ signaling, which is consistent with the stimulation of mitochondrial synthesis and function by PPAR-γ coactivator-1α.

The activation and induction of PPAR-γ caused by TGZ is also observed with the antioxidant vitamin E and anti-inflammatory drugs such as ibuprofen, in the absence of cytotoxicity. TGZ is a potent anti-inflammatory drug because it blocks cytokine- and lipopolysaccharide-induced cytotoxicity, activation of macrophages and nitric oxide synthase and chemokine expression. Like vitamin E, TGZ acts as an antioxidant by inhibiting the oxidation of low-density lipoprotein cholesterol and by blocking the reactive products of oxidative stress. These antioxidant and anti-inflammatory effects may explain the improved patency of both carotid arteries and coronary artery stents in type 2 diabetic patients who are taking TGZ.

These beneficial effects of TGZ in patients and animals with insulin resistance are inconsistent with the hypothesis that TGZ induces oxidative stress and mitochondrial abnormalities, except possibly in rare, susceptible individuals.

### Bile Salt Export Proteins and Cholestasis

The proposal that TGZ and the sulfate M1 may induce intrahepatic cholestasis, contributing indirectly to the development of liver injury in patients taking TGZ, is plau-
sible because in isolated rat liver canalicular preparations, TGZ inhibited Bsep.\textsuperscript{60} Also, impaired biliary excretion of taurocholic acid was observed in isolated perfused livers under albumin-free conditions.\textsuperscript{90,92} However, this cholestatic effect of TGZ was prevented by adding albumin to the perfusate.\textsuperscript{92} This emphasizes the critical role of protein binding in the analysis of TGZ toxicity.\textsuperscript{59,65} Although the intravenous administration of TGZ to rats induced acute cholestasis,\textsuperscript{60} there is no evidence that these effects of TGZ occur after oral administration in chronic studies in animals or in patients.

Comparable experimental results have been observed with other drugs, including cyclosporin A, rifampicin, and glyburide, but there is rarely clinical correlation.\textsuperscript{93} For example, rifampicin inhibits Bsep in experimental studies\textsuperscript{94} but ameliorates pruritus (induced by bile acids) in patients with cholestasis.\textsuperscript{95,96} This effect is achieved through the stimulation of CYP3A4 activity by rifampicin.\textsuperscript{96} In turn, 6-α-hydroxylation of bile acids by CYP3A4 and subsequent conjugation at C6 by UDP-glucuronosyltransferase increases the renal clearance of bile acids.\textsuperscript{97,98} The beneficial effects of rifampicin on bile acid excretion in patients, and the apparent discrepancy between animal and human studies, can be readily explained by the fact that rifampicin is a potent activator of human, but not mouse, pregnane X receptor, which induces CYP3A4.\textsuperscript{96}

In other instances, the correlation between experimental inhibition of Bsep and clinical cholestasis is higher. Because bosentan, an endothelin-1 receptor antagonist that inhibits Bsep in experimental models, can induce cholestasis and liver injury in humans,\textsuperscript{99} it is used with strict monitoring requirements in patients with pulmonary arterial hypertension.\textsuperscript{100}

The cholestatic effects of TGZ have not been confirmed with chronic studies in animals and humans. Increased serum alkaline phosphatase is a sensitive indicator of cholestasis,\textsuperscript{101} because bile acids induce the expression of alkaline phosphatase in the liver by enhancing mRNA translation.\textsuperscript{102} TGZ treatment in monkeys (60- to 120-fold the therapeutic dose) for up to 52 weeks did not increase serum alkaline phosphatase levels.\textsuperscript{42,43} In addition, administration of TGZ to monkeys (approximately 20-fold the therapeutic dose) for 4 weeks did not affect sulfobromophthalein plasma clearance, suggesting that TGZ did not impair the hepatic uptake, transport, and biliary excretion of this organic anion.\textsuperscript{42} Similarly, the serum alkaline phosphatase levels were not increased in the cohort of subjects given TGZ in the clinical trials.\textsuperscript{103}

Alternatively, compensatory mechanisms for the inhibition of Bsep activity by TGZ could exist. These may include the induction of CYP3A4 by TGZ and bile acid sulfation.\textsuperscript{42,43}

Table 1. Hepatocyte Studies Assessing Troglitazone-Induced Apoptosis

<table>
<thead>
<tr>
<th>Species</th>
<th>TGZ (μmol/L)</th>
<th>Albumin (g/100 mL)</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haskins et al.\textsuperscript{121}</td>
<td>Rat</td>
<td>200-600</td>
<td>7.5</td>
</tr>
<tr>
<td>Human</td>
<td>50-100</td>
<td>7.5</td>
<td>No</td>
</tr>
<tr>
<td>Kostubsky et al.\textsuperscript{95}</td>
<td>Human and pig</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Toyoda et al.\textsuperscript{123}</td>
<td>Rat</td>
<td>25</td>
<td>0.1</td>
</tr>
<tr>
<td>Ramachandran et al.\textsuperscript{122}</td>
<td>Human</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Kim et al.\textsuperscript{129}</td>
<td>Monkey</td>
<td>20</td>
<td>0.4</td>
</tr>
</tbody>
</table>

NOTE. Higher troglitazone (TGZ) concentrations than those achieved therapeutically in the liver were used in these hepatocyte studies.

Apoptosis in Cell Cultures

Cell studies are important to understand the effects of drugs on molecular pathways, but the FDA does not require cell studies and considers animal studies sufficient for preclinical assessment of drug toxicity [http://www.fda.gov/cder/guidance/index.htm].

It has been argued that apoptosis may be a mechanism responsible for TGZ-induced liver injury.\textsuperscript{3} Although TGZ has been shown to induce cell death in various cancer cell lines and tumors in animals, it did not affect the corresponding normal primary cells or tissues.\textsuperscript{109-114} Moreover, these studies with tumoral cells cannot be reliably extrapolated to humans because cancer cells have abnormal mitochondrial morphology, protein content, DNA, and metabolism.\textsuperscript{115} In addition, TGZ-induced mitochondrial abnormalities in cancer or transformed cell lines were not associated with either apoptosis or cytochrome c release from mitochondria.\textsuperscript{116,117} An activator of caspase 9 (a mitochondrial apoptotic pathway),\textsuperscript{118} Rather, TGZ facilitated apoptosis initiated by TNF-related apoptosis-inducing ligand, at least in some tumor cell lines, by increasing degradation of FLICE-like inhibitory protein long form (FLIP\textsubscript{L}),\textsuperscript{109} an inhibitor of caspase 8 (a non-mitochondrial apoptotic pathway).\textsuperscript{119}
Induction of toxicity and cell death by TGZ in normal cells also has been demonstrated, but either with concentrations higher than those achieved in animals and patients given therapeutic doses of TGZ or in cells that were cultured in the absence or with very low concentrations of albumin (or fetal calf serum), or in cells that were cultured without albumin, which artificially enhances the TGZ bioavailability by up to approximately 500-fold, was markedly reduced when albumin was added to the media.

TGZ was cytotoxic to cryopreserved human hepatocytes, judging by the decrease in cellular adenosine triphosphate, but the validity of these results is unclear because of the following issues: i) cryopreservation is associated with a loss of hepatocyte viability; ii) TGZ induced hepatocyte toxicity at concentrations approximately 20-fold greater than that achieved therapeutically in the liver; iii) the absence of albumin in the culture media increases TGZ bioavailability 20- to 500-fold; and iv) rosiglitazone also induced cytotoxicity, albeit with a higher median effective concentration.

Predictive model systems for TGZ-induced liver toxicity could include highly differentiated human hepatocytes, cocultured with other liver cells (endothelial, macrophages, or stellate cells), given their modulatory role on hepatocyte injury and survival, as well as mice expressing humanized xenobiotic responses.

Liver Apoptosis and Mitochondrial Abnormalities in Patients

Although apoptosis occurs rarely in normal livers, at least one apoptotic body was found in the proximity of 30% of the terminal hepatic veins in normal human and rat livers. Apoptosis in all tissues, including the liver, can be easily detected by microscopy because of the characteristic pathological features such as pyknotic and fragmented nuclei and shrunken cytoplasm. Hepatocellular apoptosis was detected only in one case report without specifying whether it was increased from background levels, and with normal mitochondria by electron microscopy. None of the apoptotic features have been previously documented in the liver. None of the apoptotic bodies exhibited characteristic pathological features such as pyknotic and fragmented nuclei and shrunken cytoplasm.

Liver Fibrosis and Cancer

The hallmark of chronic liver injury is fibrosis, which eventually evolves into cirrhosis. An apparent increase in fibrosis is expected in biopsies from patients with submassive liver necrosis caused by structural collapse. Therefore, the diagnosis of cirrhosis in patients with TGZ-associated submassive liver necrosis cannot be established. In a pilot study, only minor changes occurred in the liver fibrosis score in NASH patients treated with TGZ, most likely reflecting sampling error in the biopsies.

After their activation, hepatic stellate cells are responsible for the production of excess extracellular matrix. Thus, regardless of the type, severity, or chronicity of a liver injury, activation of quiescent stellate cells into myofibroblastic stellate cells is indispensable for the development of liver fibrosis/cirrhosis. Indeed, this is the basis of potential therapeutic approaches to prevent...
liver fibrosis by blocking stellate cell activation by using PPAR-γ agonists.\textsuperscript{106}

It has been suggested that hepatocyte apoptosis, associated with increased serum ALT and inflammation, may be a link to liver fibrosis.\textsuperscript{145} Moreover, apoptotic body engulfment by a human stellate cell line induced expression of collagen type I.\textsuperscript{146} Whether apoptotic bodies would be engulfed by stellate cells in the liver and stimulate fibrogenesis, in the presence of competing macrophages and in the absence of an inflammatory response, remains to be determined.\textsuperscript{147}

Active fibrogenesis is common in many tumors, and collapse of the architecture after cell death would enhance the fibrotic appearance. For example, the combination of TGZ with all-trans-retinoic acid induced apoptosis and fibrosis in breast tumors implanted into mice.\textsuperscript{110} Treatment with TGZ alone (at a dose approximately 200-fold greater than the therapeutic dose) was not associated with either apoptosis or fibrosis of these tumors.\textsuperscript{110}

Some of the signaling pathways critical for the induction of collagen type I gene expression and stellate cell activation can be blocked with antioxidants.\textsuperscript{144,148,149} TGZ and related PPAR-γ agonists also block the activation of hepatic stellate cells, thereby inhibiting the production of the extracellular matrix proteins that constitutes the wound healing fibrotic response of the liver.\textsuperscript{83,150,151}

The suggestion that TGZ could have induced cancer in patients\textsuperscript{3} is not supported by the experimental data. Increased incidence of liver hemangiosarcomas and hepatocellular carcinomas occurred with TGZ in mice at 20 and 40 times the human therapeutic dose.\textsuperscript{41} No tumors of any type were induced by TGZ in rats at 40 times or in monkeys at 160 times the human therapeutic dose.\textsuperscript{41-43} Furthermore, TGZ was neither mutagenic in bacteria nor clastogenic in bone marrow of mice or rats, indicating no genotoxic risk.\textsuperscript{152} In addtion, none of the analyzed 165 tumors in mice had inactivating p53 mutations, and fewer than 5% had mutations of Ki- and Ha-ras oncogenes.\textsuperscript{153,154}

Thus, there is no evidence that TGZ facilitates the development of liver fibrosis and cirrhosis\textsuperscript{140,141} or the induction of liver tumors in humans.\textsuperscript{41}

**Potential Genetic and Acquired Susceptibility to TGZ**

The metabolism of TGZ by the CYP3A4 in hepatocytes, is a common pathway for approximately 50% of all medications, as well as estrogen, testosterone, and bile acids.\textsuperscript{155} Corticosteroids, St. John’s Wort, and some antibiotics, sedatives, and cardiovascular medications,\textsuperscript{155} for example, share the induction of CYP3A4 with TGZ,\textsuperscript{105} which would increase TGZ metabolism into M3.

Relevant to the understanding of the variable individual susceptibility to TGZ hepatotoxicity, single-nucleotide polymorphisms have been found in three genes critical for the overall activity of CYP3A.\textsuperscript{156} This is important because the hepatic expression of CYP3A4 varies approximately 50-fold, and the *in vivo* CYP3A4 enzymatic function (drug clearance) varies at least 20-fold among individuals.\textsuperscript{157} Depending on the drug studied, 60% to 90% of the individual variability in CYP3A function is caused by genetic factors.\textsuperscript{157,158} Single-nucleotide polymorphisms of members of the CYP3A gene family affect various ethnic groups differently and could have contributed to the individual susceptibility to TGZ.\textsuperscript{156,159} CYP3A activity, which would predict TGZ metabolism into M3, can be accurately determined *in vivo* by a breath test based on N-demethylation of erythromycin.\textsuperscript{160}

The quinone M3, which has a chemical structure similar to that of vitamin E,\textsuperscript{155} has not been found to be toxic after prolonged treatment of monkeys with up to 320-fold the therapeutic TGZ dose.\textsuperscript{82,43} However, in susceptible individuals, M3 conceivably could react with proteins, RNA, or DNA to induce liver injury. Also, M3-derived reactive intermediates are covalently bound to microsomal protein and glutathione,\textsuperscript{67,161} but the significance of these adducts in the pathogenesis of TGZ-associated hepatotoxicity is unknown.

In a cohort of 4,079 patients, combined genetic polymorphisms involving specific genes were associated with increased susceptibility for TGZ-induced liver injury.\textsuperscript{162} They included heterozygous single point mutations for CYP1A1 (increased drug interactions), NQO1 (reduced metabolic activity), GLUT-1 (increased risk for type 2 diabetes), PPARγ-892 (increased insulin sensitivity), and PPARγ-1431 (increased leptin levels).\textsuperscript{162} In a small group of patients, a strong correlation was also observed between TGZ-induced liver injury and the combined glutathione-S-transferase GSTT1-GSTM1 null genotype.\textsuperscript{163}

**Conclusions**

The mechanisms by which TGZ caused severe liver injury remain unknown, but they may include genetic or acquired susceptibilities involving CYP3A, CYP1A1, NQO1, GLUT-1, PPARγ, Bsep, or GST genes. No evidence exists that TGZ induced pathological apoptosis of hepatocytes in culture (under appropriate experimental conditions), in animals or in patients.

In addition, no scientific evidence supports the following notions about TGZ: (i) it accumulates in the liver; (ii) it stimulates oxidative stress in the liver; (iii) that it injures the mitochondria in hepatocytes (except in transformed or cancer cells); (iv) that it induces cholestasis in patients (except in rare cases); (v) that it provokes ste-
atosis in the liver; (vi) that it causes silent liver injury; and (vii) that exposed patients are at risk of developing cirrhosis and liver cancer.

The liver injury induced by TGZ was mainly hepatocellular and attributable to a metabolic idiosyncrasy. Although in some cases evidence exists for an immunological reaction, this mechanism cannot explain most of the cases. Identification of the mechanisms responsible for TGZ-induced liver injury, using tissues from well-characterized cases, might help to minimize the risk of similar toxicities with other drugs.

Acknowledgment: The author thanks Dr. Martina Buck (University of California, San Diego) for her critical review of the manuscript, and to Drs. Urs A. Boelsterli (National University of Singapore), D. Montgomery Bissell (University of California, San Francisco), Joe V. Selby (Kaiser Permanente), Hashem El-Serag (University of Texas, Houston), James Everhart (National Institutes of Health), and Abigail Jacobs (Food and Drug Administration) for their valuable comments. The author thanks Lauren de los Santos for the preparation of the manuscript. Space limitations prevented the citation of many important publications.

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Hepatitis B Virus Mutations Associated With Fulminant Hepatitis Induce Apoptosis in Primary Tupaia Hepatocytes

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Hepatitis B virus (HBV) core promoter mutations have been implicated in the pathogenesis of fulminant hepatitis B. Due to the limited availability of primary human hepatocytes, the functional characterization of HBV mutants has been performed predominantly in transformed cells, which may not represent ideal model systems for studying virus–cell interactions. We and others have shown that primary hepatocytes of the tree shrew Tupaia belangeri support HBV infection and replication. In this study, we used primary Tupaia hepatocytes to analyze the phenotype of two HBV core promoter mutations that have been associated with a clinical outbreak of fatal fulminant hepatitis. Similar to previous findings in human hepatoma cells, the HBV core promoter mutations resulted in enhanced viral replication and core expression. Surprisingly, however, the presence of the mutations had a marked effect on hepatocyte viability not previously observed in hepatoma cells. Reduced cell viability was found to be due to the induction of apoptosis, as evidenced by caspase-3 activation and nuclear fragmentation. In conclusion, HBV mutants exhibit a novel phenotype in primary hepatocytes distinctly different from previous findings in hepatoma cell lines. This phenotype may have important implications for the understanding of the fulminant clinical course associated with HBV mutations. Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html). (HEPATOLOGY 2005;41:247-256.)

Infection with hepatitis B virus (HBV) leads to a wide spectrum of clinical presentations ranging from an asymptomatic carrier state to self-limited acute or fulminant hepatitis to chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma.¹,² Viral factors such as HBV genotypes,³ as well as the host immune response, have been implicated in the pathogenesis and clinical outcome of HBV infection.² Furthermore, evidence has been accumulating that certain HBV mutants lead to particular clinical manifestations, influence the natural course of infection, and modulate the response to antiviral treatment.¹,⁴-⁷

Several independent studies have identified distinct mutations clustered in enhancer II of the HBV core promoter in association with fulminant⁶-¹² and chronic severe hepatitis B.⁶,¹³-¹⁶ A common hallmark of core promoter mutations is the phenotype of enhanced viral replication in hepatoma cell lines transfected with replication-competent HBV constructs.⁶,¹⁰,¹¹,¹³,¹⁵ The most prevalent variant comprises a double mutation (A to T at nucleotide 1764 and G to A at nucleotide 1766, nucleotide numbering according to Raney et al.¹⁷ located at the 3’ end of enhancer II of the basal core promoter).

We have previously identified two mutations in the HBV core promoter (C to T at nucleotide 1768 and T to A at position 1770) of a viral strain associated with a fatal outbreak of fulminant hepatitis B (FH strain¹⁸,¹⁹). This nosocomial outbreak had an unusual clinical course, with fulminant hepatic failure leading to the death of 5 patients
within a few days. Functional characterization of these mutations in human hepatoma cell lines had demonstrated a markedly enhanced viral replication compared with wild-type HBV. The phenotype of enhanced replication was the result of enhanced encapsidation of pregenomic RNA into HBV nucleocapsids by co- and posttranscriptional effects of the core promoter mutations.

Because virus–cell interactions in transformed hepatoma cells may not accurately reflect the phenotype of variants in natural host cells, functional studies in primary hepatocytes are needed for understanding the impact of defined HBV mutations on disease. However, functional studies in primary host cells have been hampered by the limited availability of primary human hepatocytes of sufficient quality. Therefore, we have developed an alternative model to address this important question: primary hepatocytes of the tree shrew Tupaia belangeri. Tupaia belangeri has been shown to be susceptible to infection with a variety of human viruses, including rotavirus, herpes simplex virus, and hepatitis B and C viruses. We and others have demonstrated that primary Tupaia hepatocytes (PTHs) represent a convenient and suitable in vitro model to study HBV infection and replication.

In this study, we demonstrate that HBV core promoter mutants associated with fulminant hepatitis exhibit a phenotype in primary hepatocytes distinctly different from previous findings in hepatoma cell lines. This phenotype may have important implications for the understanding of the fulminant clinical course associated with mutations.

Materials and Methods

Constructs and Recombinant Adenoviruses. Replication-competent constructs of wild-type HBV (adwR9), core promoter mutant strains, and pCDLacZ have been previously described. The HBV constructs contained a 1.2 × genomic length of HBV (Supplementary Fig. 1) and contained the identical genetic background (adwR9 strain). All mutant constructs were sequenced previously to confirm the expected sequence. Control plasmids pGEM7 and pEGFP were obtained from Promega Corporation (Madison, WI) and Clontech Corporation (Palo Alto, CA), respectively. Recombinant chimeric HBV adenoviruses were generated as described recently. Purified parental adenovirus vector was obtained from M. Bartolomé and L. Mohr (Department of Medicine II, University of Freiburg, Freiburg, Germany).

Isolation and Culture of PTHs. Tupaia belangeri specimens were bred and maintained at the animal facilities of the University Hospital Freiburg in accordance with institutionally approved protocols and the National Institutes of Health guidelines for the use of experimental animals. Primary hepatocytes were prepared from adult animals and maintained as described. For inhibition of HBV replication, PTHs were incubated in the presence or absence of adefovir dipivoxil (Gilead Sciences, Foster City, CA) as described previously.

Transfection of Recombinant DNA. PTHs and Huh-7 cells grown on 100-mm or six-well plates were transfected with 4 µg plasmid DNA using liposomes (Lipopectin or Lipopectamin Plus; Invitrogen, Carlsbad, CA) in serum-free William’s E medium or OPTI-MEM (Invitrogen). Huh-7 cells were maintained as described. Transfection efficiency was monitored via cotransfection with construct pCDLacZ, expressing β-galactosidase under the control of the cytomegalovirus promoter. LacZ transcription was monitored via Northern blot analysis using a LacZ-specific probe. LacZ expression was monitored via analysis of LacZ enzymatic activity in cell lysates using a chemiluminescent assay (Galacto-Light, Tropix, Bedford, MA) according to the manufacturer’s protocol.

Adenoviral Transduction. Primary hepatocytes or Huh-7 cells were infected with recombinant or parental adenoviruses. Virus stock aliquots containing the appropriate number of plaque-forming units to obtain a desired multiplicity of infection were mixed with 1 mL culture medium per well in a six-well plate and added to the cells for 6 hours. After the infection period, the viral inoculum was removed and the cells were washed extensively in phosphate-buffered saline (PBS) and incubated again in medium. At various time points, cells and medium were harvested and processed as described in Analysis of HBV Replication and Transcription.

Analysis of HBV Replication and Transcription. PTHs or Huh-7 cells were harvested for viral RNA and DNA analysis 3 or 4 days after transfection. RNA was prepared using the RNeasy System (Qiagen, Hilden, Germany), analyzed via formaldehyde agarose gel electrophoresis, and hybridized with a HBV-specific probe as described. Viral replicative DNA intermediates associated with intracellular core particles were isolated via ultracentrifugation of cell lysate through a 30% sucrose cushion and then analyzed via Southern blot hybridization.

Analysis of HBV Protein Expression. Three to 5 days after transfection or transduction of cells with replication-competent plasmids or adenoviruses, cells were lysed, and HBV core and surface proteins were analyzed via SDS-PAGE and immunoblot of cell lysates using anti-HBe (H800; dilution 1:3,000) or anti–hepatitis B surface antigen antibodies (anti-HBs) (032-A and 4F7; dilution 1:1,000; ViroGen, Watertown, MA) as described recently. For control of transduction efficiency of adenoviruses, blots were reprobed using anti–
green fluorescent protein (GFP) antibody (dilution 1:4,000; Clontech). Hepatitis B surface antigen (HBsAg) synthesis was analyzed in the cell culture medium using commercially available enzyme immunoassays (IMXHBsAg microparticle enzyme immunoassay, Abbott, North Chicago, IL, or Enzygnost HBsAg 5.0 immunoassay, Dade Behring, Marburg, Germany).

For metabolic labeling of small HBsAg (sHBsAg), PTHs (day 5 postinfection) were starved for 1.5 hours in methionine and cysteine-free DMEM medium and labeled for 30 minutes with 250 μCi of [35S]methionine and [35S]cystein (Revideu PRO-MIX L-[35S] In Vitro Cell Labeling Mix, Amersham, Buckinghamshire, England in methionine and cysteine-free DMEM medium) as described.20 The cells were then washed and lysed, and sHBsAg was immunoprecipitated using monoclonal anti-HBs (4F7) as described.20 Immunoprecipitated proteins were analyzed with a phosphoimager (Fuji, Tokyo, Japan). Signals were quantified using MacBas V2.4 software (Fuji).

**Analysis of HBV Virion Synthesis and Secretion.**

PTHs were infected with recombinant adenoviruses as described. Five days following infection, cell culture medium was pooled from one six-well plate of infected hepatocytes. Secreted virions were concentrated via ultracentrifugation of 12 mL medium to a final volume of 0.4 mL using Amicon Ultracentrifugation Devices (Millipore, Bedford, MA). Concentrated virions were subjected to CsCl gradient ultracentrifugation and sedimented virions were detected as described recently.26

**Analysis of Hepatocyte Apoptosis.**

Two to seven days following infection with recombinant adenoviruses, PTHs were removed from culture dishes, resuspended in PBS, and fixed in 4% paraformaldehyde/PBS at 4°C. Apoptosis was assessed via nuclear staining of fixed cells using DNA-binding fluorochrome Hoechst-3325832 and detection of active caspase-3 using immunofluorescence.33–35 Following fixation, cells were permeabilized (0.1% Triton X-100 in PBS) and stained with a phycoerythrin-conjugated rabbit antihuman active caspase-3 antibody (Ab 67345X BD PharMingen, San Diego, CA; dilution 1:500 in PBS). Apoptosis was quantified by counting the average number of cells with nuclear fragmentation or positive staining for active caspase-3 per total cells using a Zeiss Axiophot microscope (Carl Zeiss, Jena, Germany).

**Results**

**Functional Analysis of FH Core Promoter Mutations Associated With Fulminant Hepatitis in PTHs.** To identify the optimal conditions for transduction of PTHs, we compared different methods of viral gene transfer using a GFP expression construct. Although calcium phosphate–mediated transfection resulted only in low transfection efficiency and high toxicity (data not shown), liposome-mediated gene transfer allowed reasonable transfection efficiency at low toxicity (Supplementary Fig. 2). The use of recombinant adenoviruses allowed transgene expression in all hepatocytes. A multiplicity of infection of 1 was sufficient to transduce all PTHs without cell death (see Supplementary Fig. 2).

After having defined the optimal conditions for hepatocyte transfection and transduction, we characterized the biological phenotype of the two previously well-characterized core promoter mutations associated with a fatal outbreak of fulminant hepatitis (FH mutant [MT]; see Supplementary Fig. 1) in PTHs. Using liposome-mediated gene transfer, we first analyzed the replication levels of terminally redundant wild-type and mutant HBV constructs in PTHs. FH MTH resulted in an approximately 10-fold increase of HBV replication (Fig. 1A) as quantified via phosphoimaging. To compare the biological phenotype of the FH MT with another core promoter mutation, we transfected PTHs with a construct containing a second pair of core promoter mutations associated with chronic hepatitis (CHMT; see Supplementary Fig. 1). CH MT exhibited a 1.5- to twofold increase in replication (see Fig. 1A) compared with wild-type HBV. Enhanced replication of mutants was reflected by an increase of relaxed circular and double-stranded linear HBV DNA species (see Fig. 1A). Background strains from the wild-type, FH MT, and CH MT were identical (adwR9 strain10), ruling out the possibility that genotype- or subtype-specific factors were responsible for the observed differences.

In two previous studies, we demonstrated that enhanced viral replication observed in the FH MT is due to a co- and post-transcriptional effect of the core promoter mutations on core protein synthesis. Increased core protein synthesis results in enhanced nucleocapsid assembly followed by enhanced viral encapsidation of pregenomic RNA into HBV nucleocapsids and enhanced viral replication.10,20 In extensive functional studies using transcomplementation assays dissecting the contribution of core, HBx, polymerase, and the pregenomic RNA for enhanced replication observed for the FH MT, we have demonstrated that enhanced core protein expression, nucleocapsid assembly, encapsidation, and replication are largely independent of the pregenomic transcript level.10,20 Therefore, in human hepatoma cells the phenotype of the FH MT is characterized by a markedly enhanced replication and core expression without a concomitant increase in the pregenomic RNA transcript level.10,20

To study whether the FH MT exhibits a similar phenotype in PTHs, we analyzed viral transcription and core
expression in PTHs transfected with terminally redundant R9 constructs containing a wild-type sequence, FH, or CH MT. RNAs were purified and analyzed via Northern blot hybridization. Two species of HBV RNA (3.5 and 2.4/2.1 kb, respectively) were synthesized from all constructs as demonstrated by similar levels of \( \text{LacZ} \) RNA, transcribed from the cotransfected construct \( pCDLacZ \) (Fig. 1B). Although this minor difference in the 3.5-kb RNA level between the wild-type and FH MT appeared small, it was confirmed by two other independent experiments. The construct containing the CH MT produced similar 3.5 kb RNA and 2.1/2.4 kb RNA levels compared to wild-type constructs.

To study whether FH MT results in a similar increase in core protein expression as previously observed in human hepatoma cells \(^{10,20} \) we studied core protein expression of the FH MT in PTHs. As shown in Fig. 1C, the FH MT exhibited a strong increase (tenfold after correction for transfection efficiency) of core protein expression compared with wild-type HBV, paralleling the observed increase in HBV replication. In contrast, the CH MT resulted in a marginal increase (1.5-fold) in core protein expression (see Fig. 1C).

A similar phenotype of FH MT–induced enhanced replication and core expression—which was largely independent of the pregenomic RNA transcript level—was observed when recombinant chimeric HBV adenoviruses were used as a delivery system (Fig. 2A,B).

Taken together, these findings demonstrate that the phenotype of enhanced replication and core expression without a concomitant increase in pregenomic RNA transcription is similar in PTHs (Figs. 1, 2) and human hepatoma cells \(^{10,20} \). This result suggests that the previously characterized molecular mechanism of a combined co- and post-transcriptional effect of the FH core promoter mutations on core expression \(^{10,20} \) is also responsible for the observed FH MT–induced enhanced replication in PTHs.

**Decreased HBsAg Expression in PTHs Transduced With Mutant HBV**

Surprisingly, and in contrast to previous studies performed in hepatoma cells, the FH MT demonstrated a marked decrease of HBsAg in the supernatant of PTHs (Figs. 1D, 2C). This result was not due to differences in transfection efficiency as evidenced by analysis of \( \text{LacZ} \) messenger RNA produced by cotransfected plasmid \( pCDLacZ \) (see Fig. 1B) or analysis of GFP expression of GFP complementary DNA in recombinant adenoviruses (see Fig. 2D). Further, subgenomic messenger RNA levels in PTHs were similar for the wild-type and FH MT, ruling out a significant difference in transcription efficiency (see Figs. 1B, 2B). These results were confirmed by primer extension analysis of viral subgenomic RNA species (data not shown). To study whether decreased HBsAg levels in the supernatant of transduced cells were due to a defect in HBsAg secretion, HBsAg levels were ana-
analyzed via immunoblotting in lysates of PTHs that had been transduced with recombinant HBV adenoviruses as described in Experimental Procedures. Transduction efficiency of PTHs was similar for the mutant and wild-type virus as indicated by similar levels of GFP expression in an immunoblot analysis of hepatocyte lysates (see Fig. 2D). The decrease in sHBsAg expression was confirmed using two different types of anti-HBs (data not shown). In contrast, expression of middle (p33 and p36) and large (p39 and p42) HBsAg was not markedly altered by the presence of the FH MT (Fig. 2D).
To study whether FH MT–induced downregulation of sHBsAg expression was the result of altered protein synthesis, infected PTHs were pulse-labeled with [35S]-methionine and [35S]-cystein, and cell lysates were examined by immunoprecipitation with anti-HBs. HBV adenovirus containing the FH MT displayed a markedly decreased synthesis of sHBsAg as compared with adenovirus containing wild-type HBV DNA (sHBsAg synthesisFH MT / sHBsAg synthesisWT ≈ 30%; Fig. 3A). Subsequent chase in the presence of excess nonradioactive methionine and cystein revealed little or no difference in the turnover rate of sHBsAg in the FH MT and wild-type strains (data not shown). These data clearly indicate that the reduced sHBsAg levels are due to reduced surface messenger RNA translation.

In contrast to these observations in PTHs, we did not observe any significant differences in sHBsAg expression levels between wild-type and mutant constructs in human hepatoma cells (data not shown). Thus, our data reveal an unexpected distinct biological phenotype of the FH MT in nontransformed host cells different from the phenotype observed in human hepatoma cells.10,20

Finally, we studied whether decreased sHBsAg translation resulted in intracellular retention of virions potentially contributing to enhanced viral replication in PTHs. Therefore, we assessed secretion of enveloped virions in PTHs transduced with wild-type or mutant HBV ge-

Fig. 4. Induction of caspase-3 activation in HBV-transduced hepatocytes. Apoptosis was assessed via cytoplasmic staining of fixed cells using a phycoerythrin-conjugated antibody interacting with activated caspase-3 2 days after infection with recombinant adenoviruses.33-35 Representative sections of (A) uninfected hepatocytes and hepatocytes transduced with (B) the parental adenovirus and (C) GFP, (D) HBV wild-type, and (E) FH MT adenoviruses are shown. (F) The percentage of active caspase-3–positive cells was determined by counting the number of cells with positive caspase-3 staining divided by the number of total cells (n = 200). Results show the mean and standard deviation of three experiments. Ad, adenovirus; GFP, green fluorescent protein; HBV, hepatitis B virus; WT, wild-type; FH MT, fulminant hepatitis mutant.

Fig. 5. Nuclear fragmentation as a marker for late-stage apoptosis in HBV-transduced hepatocytes. PTHs were removed from the collagen-coated culture dishes and fixed in 4% paraformaldehyde 5 days after infection with recombinant adenoviruses. Apoptosis was assessed via nuclear staining of fixed cells using DNA-binding fluorochrome Hoechst-33258. (A-E) Fluorescence microscopy of nuclear fragmentation (arrows). Representative sections of (A) uninfected hepatocytes and hepatocytes transduced with (B) the parental adenovirus and (C) GFP, (D) HBV wild-type, and (E) FH MT adenoviruses are shown. (F) Hepatocyte apoptosis was quantified by counting the average number of cells with nuclear fragmentation (apoptotic cells) per total cells (n = 200). Results show the mean and standard deviation of three experiments. Ad, adenovirus; GFP, green fluorescent protein; HBV, hepatitis B virus; WT, wild-type; FH MT, fulminant hepatitis mutant.
nomes. As shown in Fig. 3B, transduction of PTHs with construct containing the FH MT did not show any evidence of an impairment in virion secretion compared with the wild-type construct. Enhanced viral replication resulted rather in an increase in HBV virion secretion. These data indicate that enhanced HBV replication induced by FH MT was not the result of HBV DNA retention in the hepatocyte and decreased sHBsAg translation does not result in intracellular retention of HBV virions.

**Induction of Apoptosis by Mutant HBV in Primary Hepatocytes.** Following the transduction of hepatocytes with FH MT we observed a marked cytopathic effect compared to hepatocytes transduced with HBV wild-type or control vectors (Ad-GFP or the parental adenovirus). Studying hepatocytes transduced with HBV containing the FH MT by light microscopy revealed time-dependent cell death resulting in the detachment of hepatocytes and a marked decrease in the number of adherent cells. This finding was absent in transduced hepatoma cells. To study whether the cytopathic effect was due to enhanced apoptosis, two assays were performed. First, apoptosis was monitored via detection of active caspase-3 in situ; caspase-3 is a key protease that is activated during the early stages of apoptosis. Second, apoptosis was detected using nuclear staining of fixed cells using DNA-binding fluorochrome Hoechst-33258. Nuclear fragmentation occurs at late stages of apoptosis and was observed using fluorescence microscopy.

Using these methods, we observed that hepatocytes transduced with HBV containing the FH MT exhibited a marked increase of caspase-3 activation compared with hepatocytes transduced with HBV wild-type, the control vector Ad-GFP, or the parental adenovirus (Fig. 4). Caspase-3 activation induced by mutant HBV was present already on day 2 (Fig. 4). This finding was confirmed by the analysis of nuclear fragmentation as a marker for late-stage apoptosis on day 5 following transduction (Fig. 5). Interestingly, we also detected an increase of apoptotic cells in wild-type transduced cells at later time points (day 5 following transduction), albeit to a lesser extent than the FH MT (see Fig. 5). Transduction efficiency of hepatocytes was similar as assessed by GFP expression on day 2 (data not shown). These findings were reproduced in several experiments using different

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**Fig. 6.** FH MT-induced hepatocyte apoptosis and HBV replication. Hepatocytes transduced with the parental adenovirus (Ad) or adenovirus containing HBV FH MT (Ad-FH MT) were incubated with or without ADV in a concentration of 100 μmol/L. (A) HBV replication. Viral replication in the presence (lanes 1-3) or absence (lanes 4-6) of ADV was assessed on days 1, 2, and 5 following transduction as described previously. (B) FH MT-induced apoptosis and viral replication. Hepatocytes were transduced and incubated in the presence or absence of ADV as described in panel A, and apoptosis was assessed via cytoplasmic staining of fixed cells using a phycoerythrin-conjugated antibody interacting with activated caspase-3 (day 2) and nuclear staining of fixed cells using DNA-binding fluorochrome Hoechst-33258 (day 5) as described in Figs. 5 and 6. To exclude that apoptosis was induced by the parental adenovirus or ADV, PTHs were incubated with the parental adenovirus and/or ADV in parallel experiments. The percentage of active caspase-3–positive cells was determined by counting the number of cells with positive caspase-3 staining divided by the number of total cells (n = 200). The percentage of apoptotic cells after staining with DNA-binding fluorochrome was quantified by counting the average number of cells with nuclear fragmentation (apoptotic cells) per total cells (n = 200). Results show the mean and standard deviation of three experiments. ADV, adefovir dipivoxil; RC, relaxed circular HBV DNA; DL, double-stranded linear HBV DNA; SS, single-stranded HBV DNA (replicative intermediates); Ad, adenovirus; FH MT, fulminant hepatitis mutant; p.i., post inoculation.
hepatocyte preparations. Taken together, these observations suggest that HBV gene expression and replication can induce apoptosis in primary hepatocytes and that the presence of the FH MT markedly enhances the induction of apoptosis.

To study whether FH MT–induced apoptosis was due to enhanced replication present in the viral genome containing the FH MT, we studied apoptosis in the presence or absence of adeovir dipivoxil (ADV), a nucleotide analogue that efficiently inhibits HBV replication (Fig. 6). Although ADV strongly inhibited viral replication of the HBV genome containing the FH MT (Fig. 6A), FH MT–induced apoptosis (as detected via in situ activation of caspase-3 and nuclear fragmentation) remained unchanged (Fig. 6B). These findings indicate that FH MT–induced apoptosis is mediated by a molecular mechanism independent of enhanced replication.

Discussion

This study describes a detailed analysis of the biological phenotype of two core promoter mutations associated with fatal fulminant hepatitis in primary hepatocytes. Our results reveal two novel phenotypes associated with these mutations in primary hepatocytes but not in hepatoma cells. These phenotypes include mutant-induced apoptosis and downregulation of sHBsAg translation.

Mutant HBV–induced apoptosis was demonstrated by two independent methods assessing early (caspase-3 activation) and late (nuclear fragmentation) markers for apoptosis. Because apoptotic effects were observed at very low multiplicities of infection of recombinant adenoviruses, it is unlikely that apoptosis was mediated by adenovirus transfer vectors and its gene products. This hypothesis is supported by previous observations demonstrating that replication-deficient recombinant adenoviruses do not significantly induce cell death in PTHs or primary human hepatocytes at the multiplicities of infection used in this study.26,36

Although HBV is considered a noncytopathic virus,1 hepadna virus–induced apoptosis and cytopathic effects have been described in three model systems. First, a duck hepatitis B variant containing a single amino acid change in the large surface antigen resulting in accumulation of covalently closed circular DNA has resulted in a strong cytopathic effect in hepatocytes in vitro and in vivo.37-39 In this system, the level of viral replication and covalently closed circular DNA formation correlated with cytopathic effects in infected hepatocytes.37 Second, intracellular retention of the HBV large surface protein has been shown to induce apoptosis in cell lines.40,41 In this model, overexpression of the large surface antigen resulted in cellular vacuolization and apoptosis of transfected hepatoma cells.41 Third, the HBx protein has been suggested to induce apoptosis in both a p53-dependent and p53-independent manner.42-44 Exploring the mechanism of these previous observations, a recent study has elegantly demonstrated that HBx interacts with c-FLIP, a key regulator of the death-inducing signaling complex.45 Recruitment of c-FLIP to the death-inducing signalling complex is inhibited by HBx resulting in hyperactivation of caspase-8 and caspase-3 by death signals.43

Considering these previous findings, the following mechanisms may account for the observation of FH MT–induced apoptosis: First, it is conceivable that enhanced replication contributes to the observed cytopathic effects. However, because inhibition of viral replication by a nucleotide analogue did not reverse FH MT–induced apoptosis, it is unlikely that this mechanism plays a crucial role in mediating this process. The finding of replication-independent apoptosis rather suggests that a viral protein (expressed from the transduced complementary DNA) is responsible for virus-induced hepatocyte cell death. Second, intracellular retention of the large surface protein has been shown to induce apoptosis. Because the FH MT did not result in an impairment of virion secretion (see Fig. 3), and because decreased HBsAg in cell culture medium was not due to HBsAg retention (see Figs. 2, 3), an intracellular retention of surface or other viral proteins contributing to FH MT–induced apoptosis is unlikely. Third, mutations within the HBx open reading frame may contribute to FH MT–induced apoptosis. Because the HBx open reading frame overlaps with the core promoter, the two core promoter mutations result in two amino acid changes of the HBx protein (valine is replaced by isoleucine in position 132 and phenylalanine is changed to tyrosine in position 133). It is therefore conceivable, that the two mutations in the HBx open reading frame may alter the interaction of HBs protein with c-FLIP, thus resulting in enhanced cell death (including caspase-3 activation as shown in Figs. 4 and 6). Studies using recombinant adenoviruses containing wild-type and mutant HBx protein are in progress to answer this important question.

The second hallmark of the mutant phenotype in primary hepatocytes was downregulation of sHBsAg translation. Interestingly, decreased sHBsAg translation did not impair virion secretion, suggesting that the reduced amount of sHBsAg does not limit the synthesis and secretion of enveloped virions.

The precise mechanisms accounting for decreased sHBsAg translation have yet to be elucidated. It is possible that FH MT–induced apoptosis contributes to the alteration of sHBsAg translation. Apoptosis-mediated downregulation of sHBsAg translation may also explain why
HBsAg expression was not altered in transformed Huh-7 cells. Thus, it has been described in detail that apoptosis can result in the alteration of translation of messenger RNA by interacting with translation initiation factors. An example is eukaryotic translation initiation factor 4G being targeted for proteolytic cleavage by caspase-3 in apoptotic cells. It is therefore conceivable that apoptosis-induced modulation of defined transcription factors is responsible for the observed decrease in sHBsAg translation. Notably, in our experimental system, inhibition of translation appeared to be specific to defined viral RNA but did not affect \textit{LacZ} or GFP translation. The factors determining the specificity of apoptosis-induced alteration of translation remain to be further defined.

Whether the observed findings play an important role in the fulminant course of HBV infection associated with the two core promoter mutations has yet to be determined. Interestingly, three out of five patients presenting with fatal fulminant hepatitis following nosocomial transmission of the \textit{FH} strain had undetectable or borderline levels of HBsAg despite the presence of HBV DNA. This finding suggests that FH MT–associated downregulation of sHBsAg expression and HBsAg secretion may occur in the infected human host liver \textit{in vivo}. Furthermore, induction of apoptosis has been a hallmark of fulminant liver failure in various animal models. Therefore, FH MT–induced apoptotic hepatocyte death, as described here, appears likely to contribute to the pathogenesis of fulminant hepatitis B.

In conclusion, our results demonstrate that HBV mutants exhibit a novel phenotype in primary hepatocytes that is distinctly different from previous findings in hepatoma cell lines. This phenotype may have important implications for the understanding of the fulminant clinical course associated with HBV mutations.

\textbf{Acknowledgment:} The excellent technical assistance of Sabine MacNelly and Christine Röslner is gratefully acknowledged.

\textbf{References}


Sources of Variability in Histological Scoring of Chronic Viral Hepatitis

Marie-Christine Rousselet,1,2 Sophie Michalak,1,2 Florence Dupré,1 Anne Croué,1 Pierre Bedossa,3 Jean-Paul Saint-André,1 Paul Calès,2,4 and Hepatitis Network 495

Inter-observer agreement on activity and fibrosis scores used in chronic viral hepatitis has only been studied under selected conditions. The aim of this study was to identify the sources of variability due to specimen characteristics and observers. This study included 254 liver specimens and 15 pathologists and used the Metavir score. In 44 specimens scored by 4 academic pathologists, agreement of Metavir score was good overall, but better for fibrosis (\(\kappa = 0.59\)) than for activity (\(\kappa = 0.43\)) and poor for lobular necrosis (\(\kappa = 0.15\)). The mean agreement was better for senior (0.60 ± 0.24) than junior pathologists (0.52 ± 0.30, \(P < .05\)). Mean intraserver agreement was better than inter-observer agreement (0.77 ± 0.18 vs. 0.58 ± 0.26, \(P < .01\)). In 157 specimens scored by 2 expert pathologists (one senior, one junior), agreement of Metavir score was only good but greatly improved after consensus reading (fibrosis: \(\kappa = 0.48\) and 0.77, activity: \(\kappa = 0.44\) and 0.70, respectively, before and after consensus). Several causes of disagreement were identified: specimen length, fibrosis class number, observer bias, and putative causes related to Metavir score or specimen. In an intercenter evaluation involving 59 specimens, 1 expert and 10 nonacademic pathologists, agreement was very poor and did not improve over 5 years for activity (\(\kappa = 0.22-0.25\)) or fibrosis (\(\kappa = 0.13-0.18\)). In conclusion, the level of experience (specialization, duration, and location of practice) has more influence on agreement than the characteristics of the specimen (length, fibrosis class number, miscellaneous factors). Agreement can be improved by experienced pathologist or consensus reading. (HEPATOLOGY 2005;41:257-264.)

Liver biopsy is usually used to assess the extent of necroinflammatory activity and fibrosis and to diagnose cirrhosis in chronic viral hepatitis. However, the qualitative evaluation of hepatic fibrosis and activity is limited by inter-observer variability. Because of the need for a critical evaluation of the histological features of fibrosis and activity in controlled trials of therapeutic regimens, numerical staging systems have been developed. The most widely used system was initially the Histological Activity Index of Knodell et al.5 The Metavir group has developed a semiquantitative score6 to quantify fibrosis and activity in chronic viral hepatitis C that was shown to be more precise and reproducible in one study.4 However, this pilot study included selection criteria: pathologists were academic experts; the 30 liver biopsy specimens were of good quality and more than 10 mm long. Moreover, disagreement was reduced because of an improved definition during a preliminary workup. In another study, 5 experts evaluated only 20 specimens.5 Thus, inter-observer agreement has only been studied under selected conditions or in relatively few specimens.4-10

The aim of this study was to evaluate inter-observer agreement for the histological interpretation of chronic viral hepatitis under different conditions, especially close to clinical practice, and to identify the sources of observer variability. Variability was evaluated with Metavir score according to elementary lesions, expertise in liver pathology, duration and location of practice, and length of specimens, mainly for inter-observer agreement but also for intra-observer agreement.

Patients and Methods

Observers

This study included different categories of observers who were defined according to 3 criteria: (1) expertise in...
liver pathology, an expert being defined as a pathologist whose main activity is devoted to hepatology; (2) duration of practice: seniors (practice \( \geq 10 \) years) and juniors (practice \(< 5 \) years excluding training); and (3) location of practice: academic centers and nonacademic centers. Overall, 15 pathologists were studied, with 1 senior and 1 junior expert and, among non-experts, 3 pathologists working in an academic hospital and 10 outside.

**Patients**

The 254 liver specimens included in the study were provided by patients admitted to the hepatogastroenterology unit of the University Hospital in Angers, France, for chronic hepatitis B or C infection. Included patients were positive for serum hepatitis B surface antigen or C antibodies, and had had persistently elevated serum aminotransferases greater than 1 N for at least 6 months. None had clinical, biological, echographic, or histological evidence of other causes of chronic liver disease (Wilson’s disease, hemochromatosis, α1-antitrypsin deficiency, biliary disease, autoimmune hepatitis, hepatocellular carcinoma). A transcostal liver biopsy was performed in all patients. The Menghini needle diameter was 1.6 mm in most cases and 1.4 mm in cases with significant coagulation disorders.

**Study Design**

These studies were prospective as part of diagnostic studies about fibrosis markers, and patients were not previously treated with antiviral or antifibrotic drugs. Our study design was based on recommendations for agreement studies.\(^\text{11}\) Thus, methods included standardization of material, identical observation conditions, and independent assessment for the examination to avoid an agreement measurement bias.

**First Study: Intra-center Inter-observer, and Intra-observer Agreements.** In a first part of the study (study 1A) performed in 1997 to 1998, the Metavir score was evaluated in 44 liver specimens by 4 pathologists from an academic hospital (Angers): 2 seniors with 1 expert (M.C.R.) and 2 non-expert juniors. The main aim of this study was to evaluate inter-observer agreement according to the expertise (type and duration of practice) of the observers. Agreement was also evaluated according to characteristics of the specimens: elementary lesions, length and number of portal tracts of specimens, as well as observation reliability; in other words, the ability by the observer to reliably score the liver specimen, for example, non-adequate short or too-fragmented specimens were considered unreliable. Intra-observer agreement was evaluated by a second examination by the senior expert 6 months later.

In a second part of the study (study 1B), performed in 2002 in 157 liver specimens, inter-observer agreement was evaluated between one senior expert and one junior expert from the same academic hospital (Angers). The main aim was to evaluate inter-observer agreement between experts who had been practicing for different durations. The secondary aim was to evaluate the putative causes of disagreement: peripheral lesions, observation reliability, limits of the definition of classification, suspicion of cirrhosis, and others. Moreover, observers were asked to note whether they had any doubts about scoring a lesion of class x in the upper or lower class, noting x minus or plus, and even whether another class was possible (e.g., stage 1 or 2 of fibrosis but cirrhosis was considered possible). Features of fibrosis regression were not noticed. Finally, observers had to reach a consensus in a second and common reading. Thus, the prevalence of lesions could be calculated, and the effect of double reading could be measured.

**Second Study: Inter-center, Inter-observer Agreement in Clinical Practice.** In a first part of the study (study 2A) performed in 1998, agreement was evaluated in 26 specimens between an academic senior expert and 10 nonexpert pathologists working outside the academic hospital. Liver biopsies were performed and scored outside the academic center between 1995 and 1998, and patients were referred later. The expert then reviewed the original slides. Thus, the specific aim was to evaluate inter-observer agreement between pathologists practicing in different locations with the expert as the reference.

In a second part of the study (study 2B), performed in 2003, inter-center, inter-observer agreement was evaluated under the same conditions except that the 33 liver biopsies were performed and evaluated outside the academic center between 2001 and 2003 by 8 of the same 10 pathologists. Because training regarding the Metavir score was provided in France between 1998 and 2001, the main aim was to test whether inter-center, inter-observer agreement in clinical practice had improved over time. The secondary aims were to evaluate the influence of length of liver specimens and number of portal tracts.

**Liver Histological Assessment**

The biopsy specimens were fixed in a formalin-alcohol-acetic acid solution and embedded in paraffin; 3-μm-thick sections were stained with hematoxylin-eosin-saffron, Masson’s trichrome, and 0.1% picrosirius red solution. All slides were evaluated in the same chronological order at the same period and under similar conditions for each study unless otherwise specified.

Independent pathologists (unless otherwise specified) scored liver lesions according to the semiquantitative
Metavir score. The Metavir fibrosis stage of the portal tract was as follows: 0, no fibrosis; 1, enlarged portal tract without septa; 2, enlarged portal tract with rare septa; 3, numerous septa without cirrhosis; 4, cirrhosis. Necro-inflammatory lesions included periportal necrosis and lobular necrosis graded as absent, discrete (only for the former), moderate, and severe. Both lesions are combined in an activity score: 0, no activity; 1, minimal activity; 2, moderate activity; 3, severe activity.

**Statistical Analysis**

Quantitative variables are expressed as mean ± SD. The inter-observer agreement of the qualitative variables was evaluated by using the kappa index, called κ, which excludes chance-expected agreement. Comparisons between kappa indexes were made by using nonparametric tests for paired data (Wilcoxon’s test). The interpretation of κ values is: κ ≥ 0.75, excellent; 0.40 ≤ κ < 0.75, fair to good; 0 < κ < 0.40, poor. Agreement of ordered categorical variables also could be evaluated by weighted κ, which takes into account the proximity of results. The inter-observer agreement for quantitative variables was calculated by using the intra-class correlation coefficient called r_{ic}. r_{ic} combines correlation and similarity but does not take into account chance-expected agreement, unlike κ; therefore, its interpretation is different and should be based on $r_{ic}^2$: $r_{ic}^2 ≥ 0.75$, excellent; $0.71 (r_{ic}^2 = 0.50) ≤ r_{ic} < 0.87$, good; $0.50 (r_{ic}^2 = 0.25) ≤ r_{ic} < 0.71$, fair; $0 ≤ r_{ic} < 0.50$, poor.

Moreover, the proportion of agreement was calculated for qualitative variables according to the method described by Grant. The proportion of agreement for a sign category is an informative index, expressed as a percentage, that measures the number of cases in which all observers agree for a category over the number of patients for whom this category was chosen (by at least one observer). Compared with κ, this index is limited by the inclusion of chance-expected agreement; however, it provides agreement for the presence and absence of a lesion, which is especially interesting when the prevalence of a sign is low.

Finally, the observer bias was investigated. When there were two observers, the observer bias was evaluated by the Stuart-Maxwell test (lesion with more than 2 classes) or the Mac Nemar test (2 classes), and when there were more than 2 observers, by the Cochran’s test. These tests evaluate whether 2 or more observers consistently assess differently from each other or the mean level of scoring as a function of observer(s). An observer bias means that disagreement between observers is not attributable to chance, and that there is a significant trend for overestimation or underestimation by certain observers.

If there were no observer bias—as previously defined—and more than two observers, the presence of one or more poorly trained observer(s) was evaluated by using the Poisson heterogeneity test. A poorly trained observer is defined as an assessment that is in complete disagreement with those of all other observers.

**Results**

**First Study: Intra-center, Inter-observer Agreement for Metavir Score**

In both studies 1A and 1B, the higher the grade of the lesion, the higher the kappa index. Excellent agreement was only observed for cirrhosis. Poor agreement for lobular necrosis was attributable to an observer bias.

In study 1A (Tables 1 and 2) (4 pathologists with several different characteristics), the mean agreement was significantly better in senior than in junior pathologists. Mean intra-observer agreement was significantly better than inter-observer agreement. Except for observation reliability, agreement did not depend on specimen length, whether specimens were divided into 2 classes according to median length (16 mm) or into classes 10 to 19 mm...
and $\geq 20$ mm (data not shown). Observers only disagreed on the observation reliability in small specimens. Agreement was not significantly different between the first and second chronological halves of the slides that were evaluated (data not shown), thus excluding the possibility of a training bias.

In study 1B (1 junior and 1 senior expert pathologist), agreement was fair to good for the activity and fibrosis scores (Table 3). The number of portal tracts and the length of specimens significantly increased as a function of activity and fibrosis scores (data not shown). Agreement ($\kappa$) decreased in relation to specimen length: activity: 0.47, 0.47, 0.41; fibrosis: 0.58, 0.45, 0.37, respectively, for length $< 10$ mm $(n = 18)$, 10 mm $\leq$ length $< 20$ mm $(n = 84)$, length $\geq 20$ mm $(n = 55)$. However, agreement ($\kappa$) did not seem to depend on the number of portal tracts: activity: 0.41, 0.55, 0.10; fibrosis: 0.32, 0.26, 0.60, respectively for number $< 10$ $(n = 49)$, 10 $\leq$ number $< 20$ $(n = 67)$, number $\geq 20$ $(n = 13)$.

The degree of expertise did not seem to influence the results of the consensus reading: activity: 0.74, 0.70; fibrosis: 0.70, 0.77, respectively, agreement ($\kappa$) between consensus reading and junior then senior, suggesting a balanced influence of both experts. As expected, agreement ($\kappa$) was altered by the presence of putative causes of disagreement: activity: 0.51, 0.36; fibrosis: 0.54, 0.42, respectively.

### Table 2. Inter-observer Agreement (Study 1A: 4 Academic Pathologists, Metavir Score): Detailed Results

<table>
<thead>
<tr>
<th>Grades</th>
<th>Lesion Presence</th>
<th>Periportal Necrosis</th>
<th>Lobular Necrosis</th>
<th>Activity</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\kappa$</td>
<td>Agreement Proportion</td>
<td>$\kappa$</td>
<td>Agreement Proportion</td>
<td>$\kappa$</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>Yes 0.01</td>
<td>No 0.99</td>
<td>Yes 0.04</td>
<td>No 0.22</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.55</td>
<td>0.48</td>
<td>0.83</td>
<td>0.105</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.51</td>
<td>0.78</td>
<td>0.46</td>
<td>0.38</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.31</td>
<td>0.20</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td></td>
<td>0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Observers**

<table>
<thead>
<tr>
<th>Observer bias</th>
<th>NS</th>
<th>Poorly trained:§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Senior expert</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Senior non-expert</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>Junior 1</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Junior 2</td>
<td>5</td>
<td>—</td>
</tr>
</tbody>
</table>

* $P < .05$ for grade 2, $P < .001$ for grades 0 and 1.
† Except $P < .05$ for grade 3.
‡ Except $P < .05$ for grades 2 and 4.
§ Figures denote number of complete disagreement with other observers.
||$P < .05$.

### Table 3. Inter-observer Agreement (Study 1B: 2 Academic Experts, Metavir Score)

<table>
<thead>
<tr>
<th>Grades</th>
<th>Lesion Presence</th>
<th>Periportal Necrosis</th>
<th>Lobular Necrosis</th>
<th>Activity</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\kappa$</td>
<td>Agreement Proportion</td>
<td>$\kappa$</td>
<td>Agreement Proportion</td>
<td>$\kappa$</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>0.38</td>
<td>0.25</td>
<td>0.96</td>
<td>0.17</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.34</td>
<td>0.48</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.37</td>
<td>0.51</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>$-0.01$</td>
<td>0.00</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td></td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Observer bias**

| NS | $P < .001$ | NS | $< .01$ |

**Weighted $\kappa$**

| 0.39 | 0.24 | 0.53 | 0.69 |

**Abbreviation:** Pr, prevalence according to consensus.
respectively, when causes were estimated absent (n = 75) and present (n = 82). The most commonly noticed causes of disagreement were: heterogeneous space distribution of lobular activity, uncertain distinction between no portal fibrosis and mild portal fibrosis, difficult assessment of true bridging fibrosis versus some normal large portal tract extension, and incompletely represented septum located in specimen periphery. No training bias was found (data not shown).

The influence that the number of classes of fibrosis had on agreement was tested. The reduction of classes by data management increased agreement: whereas $\kappa$ was 0.48 for the original 5 classes, $\kappa$ increased with two classes to 0.52 for F0 versus F1-4, 0.60 for F0 + 1 versus F2-4, 0.75 for F0-2 versus F3 + 4, 0.86 for F0-3 versus F4. The relationship between the cutoff for original fibrosis Metavir score and agreement for a two-classes Metavir score was linear ($r_c = 1$). Conversely, when classes were increased by adding upper and lower stages during reading (for example, F2+ and F2−) $\kappa$ was reduced to 0.35. There was no significant correlation between the prevalence (Fig. 1) or grade (Fig. 2) of lesions and agreement. However, the curves of the grades plotted against agreement had a U- or V-shape (Fig. 2). This increase at the extremes was expected because the possible error is limited to one way.

**Second Study: Inter-center, Inter-Observer Agreement in Clinical Practice**

In study 2A (first period, Table 4), agreement was poor and not statistically different from 0 for Metavir activity score or fibrosis score. The agreement indexes increased in relation to the length and portal tract number of liver specimens, unlike in intracenter study 1. In both studies 2, there was no significant observer bias for activity but a significant observer bias for fibrosis score with an underestimation by external pathologists.

**Discussion**

**Sources of Variability**

For the interpretation of liver biopsies, there may be several coexistent putative sources of variability: space

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**Table 4. Inter-center Agreement (Study 2A: 1st Period, Metavir Score, Academic Expert and Non-academic Pathologists)**

<table>
<thead>
<tr>
<th>Score</th>
<th>Activity</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$\kappa = -0.06$</td>
<td>$\kappa = -0.16$</td>
</tr>
<tr>
<td>1</td>
<td>$\kappa = 0.19$</td>
<td>$\kappa = 0.07$</td>
</tr>
<tr>
<td>2</td>
<td>$\kappa = 0.30$</td>
<td>$\kappa = 0.25$</td>
</tr>
<tr>
<td>3</td>
<td>$\kappa = 0.25$</td>
<td>$\kappa = -0.08$</td>
</tr>
<tr>
<td>4</td>
<td>$\kappa = 0.42^*$</td>
<td>$\kappa = 0.13 \pm 0.11$</td>
</tr>
</tbody>
</table>

$\kappa^b = 0.47 (0.13-0.73)$ $r_c = 0.69 (0.44-0.85)$

---

$^*P < .05.$  
$^†$Intra-class correlation coefficient (95% CI).
variability, time variability, and observer variability. Space variability may be caused by sampling error. In a recent evaluation in hepatitis C, Regev et al.10 found that 24% of patients had a difference of at least one inflammation grade, and 33% had a difference of at least one fibrosis stage between the right and left lobes with the Metavir score. Siddique et al.15 observed a discrepancy of 45% between two samples from right lobe for activity and fibrosis with the Knodell score.

Influence of Classification and Lesions

There was an inverse relationship between the number of fibrosis classes used in the Metavir score and agreement in the present study. This has already been observed for other scores.9 The decrease in agreement as the number of scoring classes increases can be compared with the poor or fair agreement observed with the Ishak score including 7 classes,7-9 despite the high correlation between Metavir and Ishak fibrosis scores,16,17 whereas the necroinflammatory grades correlated moderately well.17 Interestingly, when two classes were used, κ increased in relation to the fibrosis cutoff. The extent of disagreement was greater among observers for fibrosis, with a difference of 1 to 2 stages (for only F3, F4) than for activity with a difference of 1 grade only.

Influence of Observer

Expertise in Liver Pathology. The influence of the degree of experience in liver disease is summarized in Fig. 3. As expected, agreement decreased in relation to the degree of experience. It should be noted that the same academic expert was included in all of the evaluations and served as a common reference. The reasons for disagreement were by decreasing order: (1) location of practice (i.e., academic vs. non-academic centers); (2) duration of practice—agreement was significantly better in senior pathologists than in junior pathologists. It should be noted that time variability attributable to improvement from learning did not occur for observer disagreement in clinical practice in the present study. Agreement was better for the Metavir score with 2 senior non-expert pathologists than in our intra-center study, even when only senior pathologists were considered. This difference could be attributable to methodological biases11 or a center effect in the former study.

Intra-Observer Agreement. As expected and already observed,9 the mean intra-observer agreement was significantly better than inter-observer agreement. However, this was only evaluated with the common academic expert, which may cause an overestimation. With a modified Scheuer scoring, inflammation grades by each of 2 pathologists on the second examination differed from the

<table>
<thead>
<tr>
<th>Activity</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole group</td>
<td>0.25 ± 0.13</td>
</tr>
<tr>
<td>Stuart-Maxwell test</td>
<td>NS</td>
</tr>
<tr>
<td>Specimen length†</td>
<td>-</td>
</tr>
<tr>
<td>&lt;19 mm</td>
<td>0.29 ± 0.21</td>
</tr>
<tr>
<td>NS</td>
<td>0.04</td>
</tr>
<tr>
<td>≥19 mm</td>
<td>0.24 ± 0.20</td>
</tr>
<tr>
<td>NS</td>
<td>0.03</td>
</tr>
<tr>
<td>Portal tract number†</td>
<td>-0.02 ± 0.18</td>
</tr>
<tr>
<td>&lt;11</td>
<td>0.37 (-0.10-0.73)</td>
</tr>
<tr>
<td>NS</td>
<td>P = .04</td>
</tr>
<tr>
<td>≥11</td>
<td>0.39 ± 0.22</td>
</tr>
<tr>
<td>NS</td>
<td>0.65 (0.19-0.90)</td>
</tr>
</tbody>
</table>

Table 5. Inter-center Agreement (Study 2B: 2nd Period, Metavir Score, Academic Expert and Non-Academic Pathologists)

*Intra-class correlation coefficient (95% CI).
†Cutoff determined by median.
first examination in 0% and 4% of cases, and fibrosis staging differed in 6% and 10%, respectively.\(^\text{10}\)

**Influence of Specimen Characteristics**

**Length.** A specimen length of 15 mm or more was generally considered adequate for diagnosis,\(^\text{18}\) but this statement was not supported by factual data. The relationship between agreement and specimen length is debated.\(^\text{9,19}\) Bedossa et al.\(^\text{20}\) recently suggested that sampling variability of fibrosis was a significant limitation in the assessment of fibrosis with virtual liver biopsy using the Metavir scoring system. They suggested that a length of at least 25 mm was necessary to accurately evaluate fibrosis with a semiquantitative score.\(^\text{20}\) Using the Ishak score, Colloredo et al.\(^\text{21}\) recommended specimens at least 20 mm long and 1.4 mm wide or with at least 11 complete portal tracts,\(^\text{21}\) whereas Petz et al.\(^\text{7}\) showed that intra-observer and inter-observer variabilities were not affected by the needle size using the modified Ishak system. We could not evaluate needle size influence but only specimen length influence. Agreement of the Metavir scoring system did not depend on the length of the specimen in our academic center (study 1A), except that observers disagreed on the ability to score reliably in small specimens. However, this fear was not confirmed, because agreement was very similar whatever the length of the lesion. All of our biopsy specimens were percutaneous, and specimens were at least 10 mm long in all cases but two. However, agreement unexpectedly decreased in relation to specimen length with 2 expert pathologists. In addition, the same study showed that the number of portal tracts and the length of the specimens significantly increased in relation to activity and fibrosis scores, as recently reported.\(^\text{21}\) Conversely, the sampling error was well demonstrated in chronic viral hepatitis.\(^\text{19}\) These data suggest that as the specimen size increases, the probability of various types of lesions increases, explaining the decrease in agreement and the increase in grade, because the higher grade is taken into consideration for the result. Thus, the effect of specimen size is paradoxical because an increase in size results in more lesions and observer variability. However, agreement improved in relation to specimen length in inter-center study. Thus, this paradoxical effect of length on agreement could only be observed for expert pathologists because the baseline agreement level was high. This paradoxical effect raises the question of which lesion degree is representative? The answer would help improve observer reproducibility.

Finally, neither the current study on observer variability nor the previous studies on space variability\(^\text{13,20,21}\) can maximize reproducibility. Adequate size seems to be taken into account, but in addition definitions of elementary lesions must be very precise, and the quantitative aspects are probably less influential. However, consensus scoring with 2 pathologists markedly improved agreement (Fig. 3).

In conclusion, whereas agreement for fibrosis is excellent between academic experts,\(^\text{4}\) it decreases (fair to good) between various pathologists from an academic center. With expert pathologists with different duration of practice, agreement is only good for Metavir score in an academic center. Agreement is poor between pathologists from varied centers, and an academic expert casting doubts about the use of scores in clinical practice without precautions such as double reading,\(^\text{4}\) as in the present study, or reading by a senior expert. Thus, the degree of experience (specialization in liver pathology, duration or location of practice) influences more agreement than specimen characteristics (length interacting with observer). Agreement also can be improved by reducing class number. However, the latter cannot be a recommendation, because this would lose pertinent pathologic information; however, this demonstrates that scores with a high class of numbers are hindered by increased variability. The quality of agreement studies is rather high in liver pathology compared with other area of hepatology,\(^\text{22}\) but this study suggests that factors of variability in pathology of chronic viral hepatitis are more complex than suggested previously.\(^\text{16}\) Because these scores can be extended to other chronic liver diseases,\(^\text{23}\) their limits in terms of reproducibility, especially in clinical practice, have to be taken into account.


**References**

6. Coton T, Matton T, Pecarrere JL, Monchy D, Debonne JM. Interobserver reproducibility of the Knodell score and the Metavir score in chronic viral


Characterization of Host-Range and Cell Entry Properties of the Major Genotypes and Subtypes of Hepatitis C Virus

Dimitri Lavillette,1 Alexander W. Tarr,2 Cécile Voisset,3 Peggy Donot,1 Birke Bartosch,1 Christine Bain,4 Arvind H. Patel,5 Jean Dubuisson,3 Jonathan K. Ball,2 and François-Loïc Cosset1

Because of the lack of a robust cell culture system, relatively little is known about the molecular details of the cell entry mechanism for hepatitis C virus (HCV). Recently, we described infectious HCV pseudo-particles (HCVpp) that were generated by incorporating unmodified HCV E1E2 glycoproteins into the membrane of retroviral core particles. These initial studies, performed with E1E2 glycoproteins of genotype 1, noted that HCVpp closely mimic the cell entry and neutralization properties of parental HCV. Because sequence variations in E1 and E2 may account for differences in tropism, replication properties, neutralization, and response to treatment in patients infected with different genotypes, we investigated the functional properties of HCV envelope glycoproteins from different genotypes/subtypes. Our studies indicate that hepatocytes were preferential targets of infection in vitro, although HCV replication in extrahepatic sites has been reported in vivo. Receptor competition assays using antibodies against the CD81 ectodomain as well as ectopic expression of CD81 in CD81-deficient HepG2 cells indicated that CD81 is used by all the different genotypes/subtypes analyzed to enter the cells. However, by silencing RNA (siRNA) interference assays, our results show that the level of Scavenger Receptor Class-B Type-I (SR-BI) needed for efficient infection varies between genotypes and subtypes. Finally, sera from chronic HCV carriers were found to exhibit broadly reactive activities that inhibited HCVpp cell entry, but failed to neutralize all the different genotypes. In conclusion, we characterize common steps in the cell entry pathways of the major HCV genotypes that should provide clues for the development of cell entry inhibitors and vaccines. (HEPATOLOGY 2005;41:265-274.)
cleotide level, respectively.8 The different genotypes may exhibit differing phenotypic properties. For example, infection by genotype 1 viruses may be associated with development of more severe liver disease9 and more limited response to antiviral therapy.10 Furthermore, high levels of variability are seen particularly in the envelope genes, and such heterogeneity is likely to pose a significant challenge for vaccine design.11

Virus entry is mediated by the viral envelope glycoproteins E1 and E2. These proteins are thought to beanchored in the viral lipid membrane at the surface of the virus, as noncovalent heterodimers12-14 extensively modified by N-linked glycosylation.15 Investigation of the binding and entry of virions has been hampered by the inability to obtain large quantities of virions from patient samples16 and the difficulties in amplifying the virus in vitro.17 Despite this, studies using cell binding assays together with infection assays using HCV pseudo-particles (HCVpp) have shown that HCV entry into isolated primary liver cells and cell lines requires interaction with the cell surface receptors CD81 and Scavenger Receptor Class-B Type-I (SR-BI),13,18-24 at least for those viruses belonging to genotype 1. However, the presence of these receptors alone is not sufficient to allow viral particle entry, indicating that additional factors or cell receptors are required to facilitate entry. A number of recent investigations have suggested that glycoproteins from different genotypes might function differently during the entry pathway. For example, studies with a soluble form of the E2 protein have highlighted genotype-specific differences in CD81 binding affinity,25 even to the extent that genotype 3 of HCV was unable to bind to CD81.25

Thus, to investigate potential differences in the entry pathways used by HCV belonging to different genotypes, here we have generated a panel of full-length HCV glycoprotein clones, representative of genotypes 1 through 6, and assessed whether they were capable of allowing entry in the HCVpp assay. We found that all genotypes of HCV are hepatotropic, with an absolute requirement for CD81 during infection. In contrast, the role of SR-BI in viral entry appeared to vary depending on genotype. Finally, sera from chronic HCV carriers were found to exhibit broadly reactive activities that inhibited HCVpp cell entry, but failed to neutralize all of the different genotypes. Altogether, these findings have implications for the development of cell entry inhibitors and vaccines.

Materials and Methods

Cell Lines. 293T (ATCC CRL-1573), Huh-7, PLC/PRF/5 (CRL-8024), Hep3B human hepatocellular carcinoma (HB-8064), HepG2 (HB-8065), HepG2-CD81,22 SW-13 (CCL-105), 293T SR-BI (22), HOS (CRL-1543), Hela (CCL-2), TE671 (CRL-8805), Molt-4 (CRL-1582), Jurkat (TIB-152), Raji (CRL-86) and Akata, THP-1, U87 (HTB-14), U118 (HTB-15), A431 (CRL-1555), COS-7 (CRL-1651), Vero (CRL-81), CHO (CCL-61) and CHO CD81/SR-BI22 were grown together with infection assays using HCV pseudo-particles (HCVpp) have shown that HCV entry into isolated primary liver cells and cell lines requires interaction with the cell surface receptors CD81 and Scavenger Receptor Class-B Type-I (SR-BI),13,18-24 at least for those viruses belonging to genotype 1. However, the presence of these receptors alone is not sufficient to allow viral particle entry, indicating that additional factors or cell receptors are required to facilitate entry. A number of recent investigations have suggested that glycoproteins from different genotypes might function differently during the entry pathway. For example, studies with a soluble form of the E2 protein have highlighted genotype-specific differences in CD81 binding affinity,25 even to the extent that genotype 3 of HCV was unable to bind to CD81.25

Table 1. Primers Used for Nested Amplification of HCV Glycoproteins, Amino Acids 170–746

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<thead>
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<th>Genotypes</th>
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Materials and Methods

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aligned using ClustalX. Phylogenetic and molecular evolutionary analyses of gap-stripped sequences were estimated by the neighbor-joining method applied to pairwise distances estimated by the 2-parameter method. The reliability of the phylogenetic results was assessed using 1,000 bootstrap replicates. These analyses were performed using MEGA version 2.1.

**Generation of HCVpp and Infection Assays.** HCVpp were produced as previously described from 293T cells co-transfected with a murine leukemia virus (MLV) Gag-Pol packaging construct, an MLV-based transfer vector encoding a marker protein (GFP [green fluorescent protein] or beta-galactosidase), and the E1E2 expression constructs. Infection of target cells was performed as described previously. As shown below, within a given preparation of virions, similar amounts of virion-associated MLV capsid proteins were detected for the pseudo-particles generated with the different HCV glycoproteins. However, important differences in the absolute quantities of virion-associated capsid proteins could be noticed when two independent preparations of pseudo-particles were compared (data not shown), despite comparable infectious titers (see below). Thus, to minimize artifacts caused by differences in the quality of preparations, each subsequent evaluation experiment was conducted using pseudo-particles generated concurrently. Moreover, because the detection of virion-associated capsid proteins did not appear to be a valid indicator of infectious particles and precluded comparison of results, normalization of the pseudotyped vector stocks was performed using the infectious titers determined on Huh-7 cells and expressed as TU (transducing units) per milliliter of producer cell supernatant.

**RNA Interference Assay.** The three siRNAs directed against SR-BI mRNA were 5′-GCAGCAGGGCUUAAGAAC (SR-BI/siRNA-2), 5′-GGACCCCCUUGUGAAUCUC (SR-BI/siRNA-23) and 5′-GGUU-GACUCUGCAGAUCC (SR-BI/siRNA-34). They were expressed in target cells through a VSV-G–pseudotyped bicistronic lentiviral vector, FG12, allowing the expression of the siRNA and a GFP marker protein to control the transduction efficiencies.

**Results**

**Assembly and Host-Range of Infectious HCVpp Harboring E1E2 Glycoproteins of Different Genotypes.** To study the cell entry properties of the E1E2 glycoproteins from different HCV genotypes, we derived HCVpp-harboring E1E2 glycoproteins from subtypes 1a, 1b, 2a, 2b, and 3a, and genotypes 4, 5, and 6. A total of 88 HCV sequences were recovered from sera of patients infected with different genotypes, as determined by the genotyping INNO-LiPA HCV II method (Innogenetics, Ghent, Belgium). The sequences encoding the E1E2 envelope glycoproteins were then amplified from positions corresponding to amino acids 170 to 746 of the HCV polyprotein, cloned into a cytomagalovirus promoter-driven expression vector, and sequenced. Phylogenetic analysis, performed using reference strains, confirmed that genotype definition based on the sequence of the envelope genes concurred with the predicted genotype (Fig. 1). To generate infectious HCVpp, the E1E2 expression vectors were co-transfected into 293T cells with a Gag-Pol expression vector encoding MLV retroviral cores and with an MLV-derived transfer vector encoding the GFP marker protein. Although a limited number of the E1E2 sequences turned out to be functional in infection assays (i.e., 24 of the 88 clones), most expressed E2 protein in transfected cells, and, more importantly, we were able to derive infectious HCVpp for all of the major different genotypes and subtypes. Highly infectious titers, higher than 5 × 10^4 TU/mL, were readily obtained for HCVpp of genotypes/subtypes 1a, 1b, 2a, 2b, 4, 5, and 6 on human hepatoma Huh-7 cells (Table 2). The infectious titers of HCVpp of genotype 3 were lower by more than 1 log on the same target cells, yet we were unable to identify a more permissive cell type (Table 2). All liver cell lines tested, including Huh-7, PLC/PRF/5, and HepG3, could be infected with HCVpp of the different genotypes, with the exception of HepG2 cells, which do not express CD81. Furthermore, none of the nonhepatic human cell lines tested, including osteosarcoma, epithelial adenocarcinoma, rhabdomyosarcoma, glioblastoma, and B- or T-lymphoid cell lines, were permissive to HCVpp (Table 2). Likewise, human T and B primary cells could not be infected (data not shown). To investigate the phenotypic properties conferred by diverse HCV glycoproteins, a subset of 10 infectious clones that were representative of each genotype and main subtype, as determined by their location in the phylogenetic tree (Fig. 1), were chosen for further study. A more limited characterization of the other clones was performed and confirmed that each of the 10 clones selected was representative of its clade (data not shown).

**Cell Entry Pathways.** On receptor binding, entry of enveloped viruses may occur either by direct membrane fusion at the cell surface, for the so-called pH-independent viruses, or after internalization in acid-pH endosomal vesicles, for pH-dependent viruses. To discriminate the cell entry pathway used by the different HCVpp genotypes, we treated Huh-7 target cells with different concentrations of bafilomycin A1, a specific inhibitor of the vacuolar proton pump adenosine triphosphatase involved in acidification of endosomes. As expected, infectivity of
control pseudo-particles generated with the envelope glycoprotein of the RD114 retrovirus that uses a pH-independent membrane fusion mechanism was not affected by bafilomycin A1 (Fig. 2). In contrast, bafilomycin A1 inhibited the infectivity of HCVpp of all genotypes as well as of control pseudo-particles harboring HA or VSV-G glycoprotein that enter the cells through a pH-dependent pathway. Thus, these results suggest that HCV cell entry route proceeds by endocytosis and this process is conserved within the different HCV strains.

**CD81 Is Critically Involved in the Entry Process of All HCV Genotypes.** A functional role for the CD81 tetraspanin and the SR-BI in cell entry was confirmed by using infectious HCVpp of genotype 1,13,22,23,31,32. We sought to investigate the conservation of usage of either receptor in cell entry of other HCV genotypes/subtypes. First, we expressed CD81 in HepG2 human hepatocarcinoma cells. In contrast to the parental cells, CD81-transfected HepG2 cells supported infection by HCVpp of all genotypes/subtypes, indicating that CD81 is involved in the cell entry process of all HCV genotypes (Fig. 3A). Second, to examine the requirement of CD81 at the early stages of HCV cell entry, we performed receptor-competition assays using anti-CD81 monoclonal antibodies that block binding of soluble E2 (Fig. 3C). The infectivity measured on Huh-7 cells was specifically inhibited by the antibodies, with inhibitions ranging between 70% and 99%. No difference in the inhibition of HCVpp from the different genotypes was detected, confirming the conserved usage of CD81 among HCV genotypes.

**Variable Implication of SR-BI in Cell Entry.** To investigate the conservation of SR-BI usage in HCVpp infection, we silenced its expression in Huh-7 cells by RNA interference. Three siRNAs that target different regions of the SR-BI mRNA were designed and expressed in target cells through a vesicular stomatitis virus (VSV)-G-pseudotyped human immunodeficiency virus (HIV)-1-based retroviral vector that also encoded the GFP marker protein. On infection with this vector, more than 99% of the Huh-7 cells expressed the GFP (data not shown), correlating well with SR-BI downregulation in most cells of the populations transduced with two of the three SR-BI siRNAs (Fig. 4A). The reduction of SR-BI expression levels was of approximately 10-fold, as shown both by fluorescence-activated cell sorter analysis (Fig. 4A) and by Western blot analysis (Fig. 4B). We then evaluated the infectivity of HCVpp harboring E1E2 glycoproteins from various genotypes/subtypes on the SR-BI–downregulated Huh-7 cells (Fig. 4C). The SR-BI silencing did not inhibit the infectivity of control pseudo-particles generated with the RD114 envelope glycoprotein, as expected, but induced variable effects on infectivity of HCVpp of different genotypes, even if all were sensitive to SR-BI down-regulation (Fig. 4C). The HCVpp harboring E1E2 glycoproteins derived from strain H77 were the most sensitive to SR-BI down-regulation (titers reduced by more than 90%). Conversely, the infectivity of HCVpp derived from a 2a subtype was three-fold to four-fold less efficiently inhibited by SR-BI silencing than HCVpp of 1a genotype. Like-
Overexpression of SR-BI in 293 cells resulted in up to 10-fold increased infectious titers of HCVpp. Blocking serum in a dose-dependent fashion, and the same pattern of sensitivity to SR-BI blocking could be demonstrated (Fig. 4D).

**Cross-Neutralization of HCVpp Cell Entry by Human Sera.** Antibodies directed against the surface glycoproteins of enveloped viruses can neutralize infection by inhibiting receptor binding and post-binding steps. Such antibodies can be raised during infection but may not inhibit cell entry mediated by variant glycoproteins. Thus, to evaluate the extent of cross-neutralization, we performed neutralization assays using HCVpp and sera from chronic HCV carriers (Fig. 5). No neutralization of control pseudo-particles could be detected with these patient sera. A first serum from a patient infected with a subtype 1b HCV (S1) could neutralize the infectivity of the HCVpp harboring the E1E2 glycoproteins of the different genotypes/subtypes; yet with different efficiencies. However, an alternative genotype 1b serum (S7) did not generate as broad neutralization and raised strain-specific neutralization responses in some cases (e.g., gt3a and gt4). Likewise, a patient serum harboring a genotype 3 (S11) displayed both genotype- and strain-restricted neutralization responses. Sera from other chronic carriers with defined HCV genotypes/subtypes raised similar results (data not shown); that is, broadly reactive neutralization activities that could not always inhibit cell entry of the full range of genotypes and subtypes tested.

**Discussion**

**Generation of Infectious HCVpp Harboring E1E2 Glycoproteins of Different Genotypes/Subtypes.** In this study, we investigated the cell entry properties from different E1E2 clones representative of the diverse HCV genotypes and subtypes. Although approximately 25% of E1E2 sequences appeared to be functional, we were able to isolate functional envelope glycoproteins from genotypes/subtypes 1a, 1b, 2a, 2b, 3a, 4, 5, and 6 that confer infectivity to HCVpp harboring a marker gene. We show that the HCVpp from the different genotypes/subtypes

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**Table 2. Tropism of HCVpp Containing E1–E2 From Genotypes 1a, 1b, 2a, 2b, 3c, 4c, 5, and 6**

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*Note.* The infectivity of HCVpp harboring the E1E2 glycoproteins of the indicated HCV genotypes and subtypes was detected on different cell types. (++, infectious titers higher than 5 \( \times 10^4 \) TU/mL; (+), titers between 5 \( \times 10^3 \) and 5 \( \times 10^4 \) TU/mL; (+/-), titers between 5 \( \times 10^2 \) and 5 \( \times 10^3 \) TU/mL; (-), titers lower than 5 \( \times 10^2 \) TU/mL, which corresponds to the threshold of detection of infected cells by FACs analysis, as determined using pseudo-particles generated in the absence of viral glycoproteins. The results were derived from at least three independent experiments using different virion production batches, and the standard deviations did not exceed 30% of the mean values. The infectivity of control pseudo-particles generated with the VSV-G envelope glycoprotein ranged from 5 \( \times 10^6 \) to 2 \( \times 10^7 \) TU/mL. Overexpression of SR-BI in 293 cells resulted in up to 10-fold increased infectious titers of HCVpp.
displayed a similar tropism for the human hepatoma cells. Interestingly, differences in the infectious titers of the HCVpp were detected. Whereas HCVpp generated with E1E2 glycoproteins of gt1a, gt1b, gt2a, or gt2b had high titers, in the range of $10^5 TU/mL$, the infectious titers of HCVpp harboring glycoproteins of gt3a were generally lower, by more than 1 log. Whether these differences of infectivity were attributable to an inadequate choice of the target cells used in the infection assays, to sequence artifacts incorporated during the reverse transcription polymerase chain reaction (RT-PCR) steps, to retrieval of non-competent variants present in the quasispecies from patients’ samples, to poor viral incorporation of some of the glycoproteins on HCVpp, or to other intrinsic features of these HCV glycoproteins remains to be determined. The recovery of a wider range of isolates will establish whether these differences in infectivity are real. However, in the lines of previous results obtained with E1E2 glycoproteins from prototype 1a and 1b HCV isolates,13,22,23 our results indicate that hepatocytes may be the preferred HCV target cells 	extit{in vitro} because the E1E2 glycoproteins from all of the different HCV genotypes tested efficiently mediated cell entry only into hepatocarcinoma cells. We were unable to detect HCVpp entry into nonhepatic cell lines from several different tissues (including B- or T-lymphoid cell lines) as well as in resting or stimulated peripheral blood mononuclear cells. Therefore, these results are not fully consistent with 	extit{in vivo} data that suggest that HCV may infect a wider range of cell types, including B cells, monocytes/macrophages, and dendritic cells.33-35 as well as nonhematopoietic cell types.36 The HCV clones used in this study were retrieved from sera of HCV-infected patients; this may induce a bias for hepatocytes. Alternatively, the HCV variants that infect extrahepatic targets may poorly replicate in patients and thus may not be easily cloned. The characterization of additional HCV sequences from viruses isolated from alternative cell types, including peripheral blood mononuclear cells and dendritic cells, will help to determine the range of HCV tropism 	extit{in vivo}.

Characterization of a Common Entry Pathway Between Genotypes/Subtypes. We demonstrate that HCVpp-harboring envelope glycoproteins from the different genotypes/subtypes enter the cells by the endocytic pathway and require the acidification of endosomal vesi-
To trigger the fusogenic activity of their fusion proteins. This result is expected owing to the structural homology between the hepacivirus glycoproteins and their classification in Flaviviridae, a family of viruses whose cell entry routes are pH dependent for all members. Moreover, by receptor competition assays with CD81-blocking antibodies as well as by ectopic expression of CD81 in nonpermissive hepatic cells, we found that the CD81 tetraspanin is used by all genotypes and subtypes tested to promote cell entry. Such a conserved usage of CD81 as the cell entry receptor is surprising, because several binding studies using soluble forms of the E2 protein (sE2) indicated considerable discrepancies between genotypes in terms of affinity to bind CD81. Thus, these results show that the strength of the interaction between sE2 and CD81 does not predict the CD81 dependence of infection by HCV genotypes, as also recently pointed out in infection assays using CD81 binding mutants with reduced affinity to sE2. The functional interaction of HCV with CD81 and other cellular molecules seems more complex than the interaction of monomeric sE2 with CD81.

Variations in SR-BI Dependence for Infectivity. The SR-BI dependence for HCVpp infection was evaluated by RNA interference, an approach that has proved powerful to study the function of viral receptors during HIV infection. Negative control experiments were performed using no antibodies or using pseudoparticles generated with VSV-G or RD114 glycoprotein. Similar inputs of viral particles were used in the experiments, allowing infection of approximately 5% to 10% target cells for the lower dilutions of viral supernatants. Results are expressed as the percentages of inhibition of the infectious titers (mean ± SD; n = 4) relative to incubation in the presence of a pool of naive human sera (n = 4). HCVpp were preincubated for 30 minutes at room temperature with sera diluted 1:50 before infection of Huh-7 target cells. The predominant genotype of HCV diagnosed in the patients is indicated in parentheses. Efficient neutralization of the control pseudoparticles (data not shown) was demonstrated using a hyper-immune goat serum raised against the RD114 SU glycoprotein (ViroMed Biosafety Laboratories).
the infection of all HCVpp tested, consistent with the augmented infectivity of all HCVpp in 293 cells overexpressing SR-BI (Table 2). However, we could discriminate the different isolates by the levels of inhibition induced by SR-BI silencing or blocking (Fig. 4). Indeed, whereas the infectivity of HCVpp of subtype 1a was reduced by more than 10-fold, the infectivity of one of the 2a subtype (UKN2A 1.2) was marginally reduced, by less than 30%. Despite this difference, it is clear that CD81 and SR-BI are both required for infection but are not sufficient to mediate HCVpp infection in vitro, because SR-BI/CD81-transfected CHO cells were unable to support infection by all HCVpp genotypes and subtypes (Table 2). Our results also indicate that an unknown hepatocyte-specific component is involved in a receptor complex that encompasses CD81 and SR-BI, because several human cells (e.g., HeLa and HOS cells) co-expressing both receptors are nonpermissive (Table 2). The varying dependence of HCVpp genotypes/subtypes to SR-BI suggests that the stoichiometry of the molecules involved in an optimal receptor complex may differ among HCV genotypes, subtypes, or isolates. Alternatively, these results may indicate that the levels of SR-BI expression in siRNA-treated Huh-7 cells are not sufficient to promote efficient entry of some HCV genotypes. Indeed, variations in SR-BI receptor usage may be inherent to a differential capacity of SR-BI to interact with the E1E2 glycoproteins for some genotypes, so that SR-BI would mediate efficient infection only when expressed at high density. Several reports have established that potentially active receptors fail to mediate γ-retrovirus infections when they are expressed under a certain threshold of expression. Similarly, a mutant HIV-1 co-receptor, CCR5, with a large reduction in affinity for HIV-1 glycoprotein, was found inactive at low concentrations, but was almost as active as wild-type CCR5 at high concentrations, implying the involvement of multiple co-receptors in the HIV-1 receptor complex. Further studies using cell lines expressing different amounts of SR-BI or CD81 receptors will be necessary to determine the number of molecules that are needed to trigger HCV entry. Moreover, identifying the modular element of E1E2 complex that is capable of adapting to changing concentrations of SR-BI will be important. Alternatively, we cannot exclude that the variation in SR-BI dependence for infectivity is attributable to alternative receptor molecules that might be differentially used by different HCV genotypes/subtypes or clones. Several other molecules, for example, DC-SIGN, L-SIGN, and the asialoglycoprotein receptor, have been shown to bind to HCV E1E2 glycoproteins or to pseudo-particles, but it seems clear that their role is limited to the capture of viral particles. Efforts to identify other liver-specific molecules will provide insights in the initial steps of HCV infection. The understanding of the multi-step process of infection should offer good opportunities for therapeutic intervention.

**Cross-Neutralization of HCVpp Cell Entry.** Several epidemiological and experimental studies provide evidence that polyclonal antibody to HCV can protect from hepatitis C. HCV immunoglobulins or hyperimmune sera can indeed delay or even prevent hepatitis C in human and chimpanzees, when the virus is inoculated after or co-inoculated with the antibodies. Such reagents contain high-titer antibodies against E1E2 glycoproteins and can inhibit the infectivity of HCVpp in vitro in a complement-independent manner, hence suggesting neutralization mechanisms at the level of cell entry. Additionally, chimpanzees vaccinated with recombinant HCV glycoproteins that induced high-titer antibodies were partially protected against a subsequent low-dose homologous HCV challenge. However, the demonstration that both experimentally infected chimpanzees and naturally infected humans could be reinfected with heterologous HCV strains and that chronic infection correlated with increased viral genetic evolution, particularly in the HVR1 region, suggested a narrow cross-neutralization capacity. Our results obtained with a limited number of sera from HCV-genotyped chronic carriers confirm the presence of antibodies directed against E1E2 glycoproteins that can neutralize cell entry, most likely by inhibiting receptor binding and post-binding steps, and indicate that whereas some sera display broadly reactive neutralizing activities, others seem to react in both genotype and strain-restricted manners. Further characterization of the neutralization of infection is crucial to understand HCV pathogenesis and to develop vaccines. Thus, the HCVpp described in this report will provide a powerful system to investigate the mechanisms of HCV neutralization and cross-neutralization.

**Acknowledgment:** The authors thank Pascale Nony and Sophie Chappuis from TRANSAT for their help in producing siRNA lentiviral vectors; Pierre-Yves Lozach and Ralf Altmeyer for providing L-/DC-SIGN vectors; Christelle Granier for excellent technical assistance; and all of the laboratory members for stimulating discussions.

**References**


High-Dose Ribavirin in Combination With Standard Dose Peginterferon for Treatment of Patients With Chronic Hepatitis C

Karin Lindahl,¹ Lars Stahle,² Annette Bruchfeld,³ and Robert Schvarcz¹

Improved treatment regimens for patients with chronic hepatitis C, genotype 1 and high viral load are needed. Increasing the dose of ribavirin has increased the response rate, but experience with doses of more than 1,200 mg/day is limited. The aim of this study was to investigate the safety and tolerance to treatment with a high and individualized dose of ribavirin in combination with peginterferon. Ten patients with chronic hepatitis C, genotype 1 and high viral load were treated with peginterferon alfa-2a and ribavirin for 48 weeks in a prospective trial. The initial ribavirin dose was individualized and calculated from a pharmacokinetic formula based mainly on renal function. Ribavirin plasma concentrations were monitored, and the dose was adjusted to reach the target concentration. Hemoglobin was monitored, and patients were treated with erythropoietin and blood transfusions when indicated. After dose adjustments, the mean dose of ribavirin was 2,540 mg/day (range, 1,600-3,600) at week 24. The main side effect was anemia, which was controlled with erythropoietin. Two patients required blood transfusions. One patient was withdrawn at week 24 because of a lack of viral response, and one patient at week 39 because of side effects, primarily interferon associated. At follow-up (≥24 weeks posttreatment), nine of ten patients had undetectable HCV RNA and thus were cured by standard definitions. In conclusion, a high dose of ribavirin according to an individualized schedule is feasible but associated with more frequent and serious side effects such as anemia. The viral response merits further evaluation. (HEPATOTOLOGY 2005;41:275-279.)

See Editorial on Page 234

Although improvements have been made in the antiviral therapy of chronic hepatitis C virus (HCV), patients with genotype 1 infection with a high level of HCV RNA still pose a therapeutic challenge. The standard combination treatment with 12 months of peginterferon combined with ribavirin results in a sustained virological response rate of approximately 40% for this subset of patients.¹⁻³

Abbreviations: HCV, hepatitis C virus; IU, International Units.

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Received October 13, 2004; accepted November 21, 2004.

Supported by an unrestricted research grant from Roche AB, Stockholm, Sweden.

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Potential conflict of interest: Dr. Schvarcz is a recipient of unrestricted grants from Hoffmann LaRoche.

The mode of action of ribavirin is not firmly established.⁴ Furthermore, data regarding the correlation between the dose and the resulting concentration of ribavirin are limited, and the optimal dose is not known. For the treatment of chronic HCV, ribavirin is currently dosed according to body weight, usually between 800 and 1,200 mg daily.⁵ In a recent report by Hadziyannis et al.,⁶ patients with genotype 1 and a high viral load responded better to a ribavirin dose of 1,000 to 1,200 mg daily than to 800 mg/day combined with peginterferon.⁷ In contrast to current recommendations, we have recently shown that renal function is of greater importance than body weight for ribavirin clearance, and we therefore have suggested the use of a pharmacokinetic formula based primarily on renal function as a tool to determine the accurate dose of ribavirin for treatment.⁸

In a previous study, we have shown that with a standard ribavirin treatment dose of 800 to 1200 mg daily, the mean ribavirin concentration was 8.2 μmol/L (range, 0-17.7).⁹ Other studies have shown a correlation between the ribavirin concentration and viral response.⁸,⁹ We propose that concentrations greater than 15 μmol/L may im-
prove the response rate for genotype 1 patients infected with HCV.

The aim of this study was to evaluate safety of and tolerance to a high dose of ribavirin that is selected and adjusted to achieve a high steadystate concentration of ribavirin (>15 μmol/L). We also aimed to prospectively evaluate the use of a pharmacokinetic formula to predict the initial dose of ribavirin required to reach the intended plasma concentration of ribavirin and to measure the viral response to this dose. For this purpose, we conducted an open label study on patients with chronic HCV, genotype 1 and high viral load, with one treatment arm of ribavirin dosed individually, in combination with a standard dose of peginterferon alfa-2a, for 48 weeks.

Patients and Methods

Patients. Ten previously untreated patients with chronic HCV, genotype 1 and a viral load of >800 000 International Units (/IU)/mL, who were attending the outpatient clinic at the Department of Infectious Diseases, Karolinska University Hospital Huddinge, were enrolled in this open-label, prospective trial. The inclusion criteria were as follows; age >18 years, elevated alanine aminotransferase, positive anti-HCV antibody test, detectable serum HCV RNA, and a liver biopsy consistent with chronic HCV but without cirrhosis. Patients with other forms of liver disease, active hepatitis A or hepatitis B infection, hepatocellular carcinoma, human immunodeficiency virus infection, anemia, a previous diagnosis of severe depression or other psychiatric disease, significant cardiac disease, renal disease, seizure disorders, severe retinopathy, or pregnancy were excluded from the study.

Liver biopsies were classified according to inflammation (grade) and fibrosis (stage) on a noncontinuous scale of 0 to 4. Safety Aspects. In patients with moderate to severe anemia, treatment options included blood transfusion and reduced treatment doses. Ribavirin was discontinued if the hemoglobin level fell below 8.0 g/dL. To prevent severe anemia, erythropoietin (Neorecormon, Roche AB) and oral iron supplement were used. Dose adjustments of peginterferon were made according to well-established guidelines.1,2

Results

Ribavirin Dosing. Ten patients, seven men and three women, mean age 51 years (range, 40-64) were enrolled between November 2002 and April 2003. The patient characteristics are shown in Table 1. The initial daily dose of ribavirin was calculated from the pharmacokinetic formula, which aimed to achieve a
steady-state concentration of ribavirin of >15 μmol/L (see Patients and Methods section) and resulted in a mean dose of 1,520 mg/day (range, 1,200-2,200), as shown in Table 2. However, the mean concentration at treatment week 4 was 8.6 μmol/L (range, 6.0-15.7). After a gradual dose escalation, the mean daily dose of ribavirin at treatment week 24 was 2,540 mg/day (range, 1,600-3,600), which in turn resulted in a mean ribavirin concentration of 14.7 (range, 7.8-22.0). The highest individual dose given was 4,000 mg daily.

**Viral Response.** Table 2 shows the viral response. In one patient, the treatment was discontinued after 24 weeks because of HCV RNA levels <600 IU/mL. The remaining nine patients became HCV RNA negative during treatment and remained negative at follow-up ≥24 weeks after the cessation of treatment, including one patient who was HCV RNA positive (<600 IU/mL) at week 24 and one patient who discontinued the treatment at week 39 because of side effects.

**Side Effects.** Hemoglobin levels decreased during the treatment, as shown in Table 2. In two patients, the hemoglobin levels decreased to below 8.0 g/dL (nadir, 7.3 and 7.9, respectively). These patients received blood transfusions on two occasions each, and the dose of ribavirin was reduced or temporarily discontinued for 1 week. An additional 5 patients experienced a decrease in hemoglobin to levels between 8.0 and 10.0 g/dL, and two of these patients had minor dose reductions of ribavirin. For shorter periods, in particular during the first 3 to 6 months of treatment, the hemoglobin levels were measured once per week. Nausea caused a minor dose reduction in one additional patient. During the course of treatment, all patients received erythropoietin with doses in the range of 9,000 to 30,000 IU/week as well as an oral iron supplement.

One patient discontinued ribavirin at week 39 because of side effects, mainly associated with concurrent interferon treatment. In four patients peginterferonalfa-2a was discontinued for a short period or a reduced dose was given because of neutropenia.

Important nonhematological side effects are shown in Table 3. Fatigue, nausea, and dermatological problems were, in our opinion, more frequent and severe problems than those usually observed with standard combination treatment. The working capacity was reduced in all patients. One patient, a former drug addict, relapsed into sporadic intravenous amphetamine use 1 month after

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<th>Table 1. Baseline Clinical Characteristics of the 10 Patients With Chronic Hepatitis C Virus Infection Included in the Study</th>
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<th>Table 2. Ribavirin Doses and Corresponding Ribavirin Concentrations, Viral Load, Hemoglobin Levels, and Erythropoietin Treatment in 10 Patients With Chronic HCV Infection During and After Ribavirin and Peg-Interferon Treatment</th>
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completing the treatment and was initially lost to follow-up, but came for his follow-up visit 10 months after cessation of treatment. The mean weight (range) in kilograms, at baseline, treatment week 12, and treatment week 20, was 80.6 (66.5-109.0), 79.0 (63.5-110.0), and 78.0 (62.9-110.0), respectively.

Discussion

Patients with chronic HCV, genotype 1 and high viral load, who were the target group of this study, have a moderate expected outcome with standard combination treatment; improvements in treatment efficacy are needed.1-3 Our hypothesis was that the fraction of patients with sustained virological response could increase with high-dose ribavirin treatment, resulting in high plasma concentration levels. To investigate this, we conducted this pilot study with the primary goal of evaluating safety and tolerance to treatment.

The optimal target concentration for ribavirin is not well established. In a previous study, we found that patients given standard ribavirin dosing, 800 to 1,200 mg daily, had a mean ribavirin plasma concentration of 8.2 μmol/L.7 The probability of response to treatment has been shown to increase with increasing ribavirin concentrations.8,9 Therefore, we chose a target concentration of >15 μmol/L, based partly on the data of Jen et al.8 and partly on our prior studies in which some patients on standard therapy achieved concentrations above 15 μmol/L. We reasoned that the more effective concentrations achieved in a small proportion of patients on standard therapy should be explored under controlled conditions.

Increases in ribavirin doses from current standard regimens (800-1,200 mg daily) have been discussed. However, the use of high doses of ribavirin as in the current study (1,600-4,000 mg daily) has not to our knowledge been described for treatment of chronic HCV.

Some major limitations of this study were the small number of patients, the lack of controls, and that patients with cirrhosis were excluded. The side effects were more frequent and serious, in particular potentially life-threatening anemia, than those observed with standard combination treatment. However, only minor treatment interruptions occurred among the ten patients who were treated with doses of ribavirin substantially exceeding currently used standard doses.

As expected, anemia was the most challenging side effect to manage. We have previously shown that the degree of anemia is correlated to the plasma ribavirin levels.7 This result was verified in our study, since the patients who were exposed to very high ribavirin concentrations developed severe anemia. In two patients, the hemoglobin dropped below 8.0 g/dL, and they were treated with blood transfusions and discontinuation of ribavirin for a short period. The hemoglobin level dropped to below 10.0 g/L in five patients. Other measures used to limit anemia were minor dose reductions in ribavirin dose and treatment with erythropoietin. Other side effects also seemed to be more frequent and more severe than with standard treatment, in particular fatigue, nausea, and pruritus. Our impression was that this treatment regimen was more demanding for the patients than the standard treatment. Because of the extent and severity of the side effects, closer monitoring and extensive interventions were needed. However, by using these measures to correct anemia and treat other side effects, it was possible, with few exceptions, to maintain the patients on treatment with high doses of ribavirin.

The primary goal of this small pilot study was to determine feasibility and safety of the treatment, and not virological outcome. However, in this difficult-to-treat patient population with genotype 1 and a high viral load, nine of ten patients were cured by standard definitions, which seems to be a better response than that found in studies using standard ribavirin doses.1-3 Absence of patients with cirrhosis in our study population is a potential selection bias. According to current opinion, the major action of ribavirin is to prevent relapse. Interestingly, our impression was that the virological response increased with time and correlated with increasing ribavirin concentrations. Further studies are needed to investigate whether high ribavirin doses could enhance the primary viral response.

An important part of this work was to prospectively analyze the validity of a pharmacokinetic formula, based primarily on renal function, that calculates the dose of ribavirin for any given target concentration.6 Unexpect-
edly, the formula systematically underestimated the dose of ribavirin. Thus, in general, it was necessary to gradually increase the dose of ribavirin during the first part of the treatment to achieve the chosen target concentration. As a consequence, it took considerably longer to achieve steady state in this study than with the common regimen using the same dose of ribavirin over the entire treatment period. The reasons the formula overestimated the expected concentration of ribavirin for a given dose are being investigated. One possibility is that a small number of samples inappropriately stored in the previous study may have biased the model. It is also possible that the absorption of ribavirin is saturable. A third possibility, assuming a difference in the bioavailability of different ribavirin products, is that the use of Copegus in this study instead of Rebetol which was used in the population pharmacokinetic analyses, could contribute to the nonoptimal performance of the formula. However, we find this explanation unlikely—firstly, because a recent small study has shown no difference in bioavailability between the two products (bioequivalence study of ribavirin formulations Rebetol and Ro 20-9963 administered orally to individuals with current or previous chronic hepatitis C infection, 2001, Roche, data on file). Secondly, the first two patients in our study received Rebetol during the first 16 weeks with a similar underestimation of ribavirin dose (calculated by the formula) as in the patients treated with Copegus.

In conclusion, we have shown in this small pilot study that it is feasible to treat patients with chronic HCV with high doses of ribavirin without any major treatment interruptions. Side effects were more frequent and serious, in particular potentially life-threatening anemia, than those usually observed with standard combination treatment. Therefore, we do not recommend this treatment regimen outside clinical trials. In particular, patients with coronary heart disease and patients with cirrhosis would be at great risk. The pharmacokinetic formula used to calculate the initial ribavirin dose systematically underestimated the dose. It is still a useful tool, but it needs to be revised. Considering the high proportion of patients cured in this pilot study, additional studies would be of great value to further evaluate safety and side effects as well as the viral efficacy in a controlled fashion. Nonresponders to conventional therapy may be the appropriate target population for future studies.

Acknowledgment: The professional assistance of research nurses Gunilla Hermann, Anna Hollander, and Mats Christiansen is gratefully acknowledged.

References

Inhibition of NFκB enhances the susceptibility of cancer to TRAIL-mediated apoptosis and is suggested as a strategy for cancer therapy. Because the role of NFκB in TRAIL-mediated apoptosis of hepatocytes is unknown, we investigated the influence of NFκB-inhibition in death ligand-mediated apoptosis in hepatitis. Adenoviral hepatitis resulted in upregulation of NFκB-activity, which could be inhibited by expression of IκBα-superrepressor. We treated mice after the onset of adenoviral hepatitis with adenoviruses expressing FasL (Ad-FasL), TRAIL (AdTRAIL), or GFP (AdGFP). In contrast to apoptosis induced by AdFasL, NFκB inhibition strongly enhanced AdTRAIL-mediated apoptosis of hepatocytes. Expression of IκBα inhibits adenoviral infection-mediated overexpression of bcl-xl, providing a molecular mechanism for TRAIL sensitization. In agreement with this hypothesis, down-regulation of bcl-xl by siRNA enhanced susceptibility of hepatocytes to TRAIL, but not to FasL-mediated apoptosis, resulting in TRAIL-mediated severe liver damage after AdTRAIL application. Our data demonstrate that inhibition of NFκB in adenoviral hepatitis strongly sensitizes hepatocytes to TRAIL-mediated apoptosis. Bcl-xl, in contrast to bcl-2 and c-FLIP, is strongly upregulated after viral infection and represents an essential NFκB-dependent survival factor against TRAIL-mediated apoptosis. In conclusion, inhibition of NFκB or bcl-xl during TRAIL therapy may harbor a risk of liver damage in patients with viral hepatitis. (HEPATOLOGY 2005;41:280-288.)

Hepatotropic viruses induce liver injury by apoptotic cell death. The molecular machinery of apoptosis is triggered by signals released from the cytoplasm or from the cell membrane, leading to the activation of caspase cascades, which execute apoptotic cell death. In viral hepatitis, apoptosis of hepatocytes is mediated by engagement of death receptors belonging to the tumor necrosis factor (TNF) receptor gene superfamily. The mechanisms of TNFα- and FasL-mediated apoptosis in liver damage have been studied in humans and in several animal models.1-6 Recently, involvement of TRAIL-mediated apoptosis has been shown in hepatitis B and C7,8 and in cholestatic liver disease.9,10 The critical role of TRAIL in liver diseases has been confirmed in mouse models of adenoviral hepatitis8 and in Con-A-mediated liver injury.11 Whereas FasL provides a strong constitutional death signal in hepatocytes9,10 TRAIL needs triggering through viral infection or bile acids to activate the caspase cascade. The molecular mechanisms of apoptosis by FasL and TRAIL show similar patterns. Fas and TRAIL-receptors 1 (DR4) and 2 (DR5) recruit the key adapter protein FADD to the cell membrane, thereby inducing the caspase cascade. In contrast to Fas, however, both TRAIL receptors also bind the adapter molecule TRADD,12,13 which explains the more potent activation of NFκB by TRAIL compared with FasL.14 NFκB is a transcription factor that is activated by a large variety of bacteria and viruses and controls expression of many proteins involved in inflammation. In addi-
tion, NFκB is upregulated in other stress conditions, such as treatment with chemotherapy or tissue ischemia, indicating a more general participation of NFκB in stress responses and apoptosis independent of the immune system. However, the role of NFκB in apoptosis of hepatocytes appears to be ambivalent. NFκB has been described to counteract apoptosis and to be an essential strong survival factor in embryonal liver development and during TNF-mediated liver regeneration. Conversely, evidence exists that NFκB activity boosts apoptosis of hepatocytes in the early time course of acute viral hepatitis by transcriptional activation of the Fas receptor.

Our study investigated the role of NFκB in Fasl- and TRAIL-mediated apoptosis of hepatocytes in vivo to elucidate different roles of Fas and TRAIL in viral hepatitis. We showed that inhibition of NFκB activity strongly enhances the susceptibility of hepatocytes to TRAIL- but not to Fasl-mediated apoptosis in hepatitis. We observed a strong upregulation of bcl-xl expression in the liver after adenoviral infection, which was strongly inhibited by 1xBc. We showed that siRNA-mediated inhibition of bcl-xl expression strongly sensitizes the mouse liver to TRAIL-mediated apoptosis in viral hepatitis. These data suggest that NFκB-dependent bcl-xl upregulation confers resistance to TRAIL-mediated apoptosis in hepatocytes in viral hepatitis.

Materials and Methods

Cell Lines, Plasmids, and Detection of Apoptosis in Cell Culture. The human hepatoma cell lines HepG2, Huh7, and the human embryonal kidney cell line 293 were obtained from the American Type Culture Collection. The cells were maintained in growth medium (Dulbecco modified Eagle medium/ Glutamax, Gibco BRL, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 units/mL penicillin, and 100 μg streptomycin at 37°C in 5% CO2. Adenoviral vectors AdGFP, AdTRAIL, and Ad-Fasl were generated as described previously. The adenoviral vectors AdLacZ and AdI-B were provided by Dr. D. Brenner, Chapel Hill, NC.

For detection of apoptosis in hepatoma cells after adenoviral application, cells were harvested 12 hours after infection for photometric histone enzyme-linked immunoassay (ELISA). Histone-ELISA was performed with the Cell Death Detection ELISA Plus® kit (Roche, Basel, Switzerland), according to the manufacturer’s instructions.

Adenovirus Preparation and In Vivo Infection Experiments. To generate high titer viral stocks, 2 × 10⁸ 293 packaging cells at 90% confluence were infected at a multiplicity of infection of 5 to 10 plaque-forming units (pfu) per cell. Adenovirus preparation and viral titering were performed as described previously. Before infection, the virus was dialyzed twice against a solution containing 10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L MgCl₂, 140 mmol/L NaCl at 4°C. Infection of the mice was carried out by administration of 0.25 mL virus solution into the tail vein at total virus loads as indicated in the figures. Virus preparations were stored at −20°C in 25% glycerol, 10 m mmol/L Tris/HCl, pH 7.4, and 1 mmol/L MgCl₂.

Animal Experiments and Preparation of Nuclear and Whole Cell Liver Extracts. Pathogen-free balb/c mice (aged 4 to 8 weeks) or NMRI-nu/nu mice were obtained from the Animal Research Institute of the Medizinische Hochschule Hannover. All experiments were performed in agreement with the German legal requirements.

At the times indicated in the figure legends, mice were killed, and the livers were harvested for the preparation of nuclear extracts, whole cell extracts, total RNA, and cryosections, respectively. Whole cell liver extracts were prepared by homogenizing freshly harvested liver tissue in RIPA buffer (1% Nonider P-40, 0.5% sodiumdeoxycholate, 0.1% SDS in phosphate-buffered saline [PBS], before use, 5 μL protease inhibitor cocktail (Sigma Chemicals, St. Louis, MO) was added per milliliter). The tissue was homogenized on ice with a few strokes in a Elovheim-homogenizer and then centrifuged at 12,000g for 10 minutes (4°C). The supernatant was stored at −80°C. Nuclear extracts were isolated from mouse liver as described by Lichtsteiner et al.

Isolation of Total RNA and Semiquantitative Reverse Transcription Polymerase Chain Reaction Analysis. Total RNA was isolated from liver tissue using the peqGOLD RNApure™ kit (PeqLab, Erlangen, Germany) according to the manufacturer’s instructions. First, strand synthesis for reverse transcription polymerase chain reaction (RT-PCR) was performed by using Oligo(dT)15 primer (Promega, Madison, WI) and the Omniscript RT kit (Qiagen) in combination with 1 μg total RNA per reaction. Amplification of bcl-xl and GAPDH cDNA were carried out in one reaction using the specific primers (bcl-xl: sense: 5’-cgaccacgccacatctcc-3’ and anti-sense: 5’-tgagcttgccctgttcttctc-3’, cDNA length 316bp; GAPDH: sense: 5’-tgatgacatcaagaaggtggagt-3’ and anti-sense: 5’-tggggctcatctgctctc-3’, cDNA length 249bp) and 1 μL of the RT reaction. Briefly, PCR was performed for 10 cycles with first strand-DNA and bcl-xl primers at a Tₘ of 58°C. Then the reaction was interrupted, primers for GAPDH were added, and the reaction was continued for another 25
cycles. PCR products were documented and quantitated by using a Gel Doc 1000 apparatus (Bio-Rad, Hercules, CA) and Molecular Analyst software (Bio-Rad).

**Assessment of Adenoviral Liver Infection by Semi-quantitative Duplex-PCR of Ad5 Fiber and Caspase 8 Gene Locus.** Livers from infected mice were harvested 6 hours after infection. Fifty-milligram portions of liver tissue were digested in a mixture of 750 μL solution A (50 mmol/L Tris, pH 8.0, 100 mmol/L ethylenediaminetetraacetic acid, 100 mmol/L NaCl, 1% SDS), and 50 μL Proteinase K (22 mg/mL, Roche Diagnostics) by overnight shaking at 56°C. Cell debris was pelleted in a tabletop centrifuge, and DNA in the supernatant was isolated by conventional isopropanol precipitation. The DNA was resolved in TE 8.0/RNase by shaking for 2 hours at 37°C, and all DNA preparations from one liver lobe were pooled. Adenoviral infection was determined by using the primer pair fiber-fw (5’-CCCAAAATGTAACCACCTGTGAGC-3’) and fiber-rev (5’-GTGTTTAGGTCGTGTCTGGTACATGC-3’) directed against the coding sequence of the fiber protein of adenovirus serotype 5, resulting in a 417-bp fragment. Mouse genome templates as control were determined by using the primers Intr2-s (5’-CCAGATGTATCTCTGTGCGTTTGC-3’) and exon3-as (5’-GTTATTTTCTGCCAGCATGG-TCC-3’), directed against the murine locus of Caspase-8 (intron 2/exon 3), resulting in a 520-bp fragment. PCR was performed by using the HotStarTaq Master Mix (Qiagen), 50 ng DNA, and murine genomic primers at a concentration of 400 nmol/L each. The PCR reaction was started as follows: 15’—95°C; [30”—94°C; 30”—57°C; 1’—72°C] × 5. The reaction was interrupted, fiber primers were added (same concentration), and a further 27 cycles were performed. PCR products were resolved on a 1.5% agarose gel, and mouse/adenovirus fragment ratio was visually evaluated.

**Assessment of Liver Injury by TUNEL Assay, Haemalaun/Eosin Staining, Histone-ELISA, and Measurement of Serum Alanine Aminotransferase Activity.** Liver tissue of infected mice was embedded in OCT compound (Sakura, Netherlands) and shock frozen in liquid nitrogen. The samples were stored at −80°C. Seven-micron sections were prepared and fixed in PBS-buffered paraformaldehyde solution (4%) for 30 minutes at room temperature. After washing with PBS, endogenous peroxidase activity was blocked by covering the sections with 0.3% H2O2 in methanol for 30 minutes at room temperature. The sections were rinsed with PBS and incubated with 0.1 % Triton X-100 in 0.1% Na citrate for 30 minutes at room temperature. The sections were again washed with PBS and TUNEL-stained with the In Situ Cell Death Detection Kit (Roche Diagnostics), following the instructions of the manufacturer's protocol. The number of TUNEL-positive cells was assessed for each liver by counting TUNEL-positive cells in three sections. For each section, three fields (magnification ×200) were examined.

For hematoxylin-eosin (HE) staining, sections were incubated 10 seconds in Haemalaun solution (Sigma Chemicals), washed in warm water, and then stained 2 minutes in Eosin solution (Sigma Chemicals). After washing, the sections were incubated subsequently in 70%, 96%, and 100% ethanol.

A cell death detection kit (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer’s protocol for the qualitative and quantitative determination of cytoplasmic histone-associated DNA fragments in liver after adenoviral application by measurement of fluorescence. Liver tissue (50 mg) and 450 μL TBE buffer were homogenized and then centrifuged. The supernatants were diluted in incubation buffer (1:10). Ten microliters of this solution and 90 &μL Immunomix (supplied with the kit) were transferred into streptavidine-coated microtiter plates. After incubation for 2 hours at room temperature, we started evaluation according to the manufacturer’s instructions.

Activity of liver-specific alanine aminotransferase was determined by an automated enzyme assay.

**Electrophoretic Mobility Shift Assays.** For electrophoretic mobility shift assay experiments, nuclear extracts from liver tissue were used. Electrophoretic mobility shift assay experiments were performed by using nuclear extracts as indicated and 1 ng end-labeled DNA. The NFκB site ’5-TAG-TTG-AGG-GGA-CTT-TCC-CAG-GCA-3’ was used as the cognate DNA binding sequence for NFκB in electrophoretic mobility shift assays. Super-shift experiments were performed with antibodies directed against p50 and p65 (NFκB p65 (A) sc-109 and NFκB p50 (NLS) sc-114x, Santa Cruz Biotechnology, Santa Cruz, CA).

**SDS-Polyacrylamide-Gel Electrophoresis and Western Blot Analysis.** Protein concentrations of nuclear extracts were measured by Bio-Rad Microassay (Bio-Rad, Munich, Germany). Five to twenty micrograms whole cell extract were separated on a 10% SDS-polyacrylamide gel and blotted onto Hybond N membrane (Millipore, Frankfurt, Germany). As primary antibodies we used anti-Bcl-xL (sc634, Santa Cruz Biotechnology), anti-Bcl-2 (sc7382, Santa Cruz Biotechnology), and anti-FLIP (AF821, R&D Systems, Minneapolis, MN). The antigen–antibody complexes were visualized by using the ECL detection system as recommended by the manufacturer (Amersham, Buckinghamshire, United Kingdom).
SiRNA Duplexes, Transfection of siRNA, and Hydrodynamic Tail Vein Delivery of siRNA. 21-Nucleotide RNA with 3’-dTdT overhangs was synthesized by Dharmacon Research Inc. (Lafayette, CO) in the “ready to use” option C. AA-N19 mRNA Targets 5’-3’ : Bcl-xl target sequence 1: AAA GGA UAC AGC UGG AGU CAG (human and mouse bcl-xl mRNA); Bcl-xl target sequence 2: AAC CGG GAG CUG GUG GUC GAC (mouse mRNA, one mismatch to human bcl-xl mRNA); Bcl-xl target sequence 3: AAG GAU ACA GCU GGA GUC AGU (human and mouse bcl-xl mRNA); Bcl-xl target sequence 4: AAC UGG GGU CGC AUC GUG GCC (mouse mRNA, one mismatch to human bcl-xl mRNA); As scrambled sequences we used AA-N19 mRNA Targets 5’-3’ : AAU UUA ACC GCC AGU CAG GCU or 5’-3’ : AAG CAA AAC ACC AGC AGC AGU.

Transfection of siRNA duplexes was performed using Oligofectamine (Invitrogen, Carlsbad, CA) and Opti-MEM medium (Invitrogen) according to the manufacturer’s recommendations. Huh7 cells grown to a confluence of 40% to 50% in 24-well plates were transfected with 60 pmol siRNA duplex per well.

In vivo delivery of siRNA duplexes was performed via the tail vein by high-volume hydrodynamic injection, using 1 nmol siRNA duplexes per gram body weight. For tail vein injection, siRNA was applied in a total volume of 2.5 mL (0.5 mL dH2O and 2.0 mL NaCl 0.9%). Injection time was 4 to 10 seconds.

Statistical Analysis. Each experiment was repeated 3 times with three animals in each group. The Student t test was used to compare differences between groups. A difference with a P value equal to or less than .05 was considered significant.

Results

Inhibition of NFκB Activity in Viral Hepatitis Strongly Enhances Sensitivity of Hepatocytes to TRAIL-, But Not to FasL-Mediated, Apoptosis. The transcription factor NFκB is activated in many cell types through viral infection and thus may modulate FasL- or TRAIL-mediated apoptosis of hepatocytes in hepatitis. To explore the role of NFκB activity in virally infected livers in vivo, we used a mouse model of adenoviral hepatitis. Infection of the liver with adenovirus (AdLacz or AdIκBα) was performed by administration of different amounts of infectious viral particles (pfu/g) into the tail vein of Balb/c mice. Activation of NFκB DNA binding in nuclear extracts of hepatocytes was evident at a broad range of viral titers (Fig. 1A) and occurred rapidly after adenoviral transduction (Fig. 1B). Using an adenoviral vector expressing the IκBα-superrepressor activation of NFκB after viral infection of the liver could be inhibited significantly (Fig. 1C).

Comparable infection of mice with different adenoviral vectors was achieved by administration of the same particle load regarding the infectious viral titer. Equal infection of the livers by AdLacz and AdIκBα was confirmed by semi-quantitative duplex-PCR comparing the ratio of viral genome (Ad5 fiber) vs. mouse genome (caspase 8 gene locus) as internal control (D).

Fig. 1. Adenoviral hepatitis results in activation of NFκB, which can be suppressed by expression of IκBα. (A) Mice were injected with indicated doses of AdLacz (pfu/g body weight). Nuclear extracts were prepared from livers harvested 10 hours after infection and were investigated by electrophoretic mobility shift analysis. Positions of the NFκB p50-p65 complex and the p50 homodimers were identified in supershift assays with specific antibodies against p50 and p65. (B) The time course of NFκB DNA binding activity was investigated by electrophoretic mobility shift assay after administration of 1 × 10⁹ pfu/g AdLacz. (C) Different activation of NFκB by 1 × 10⁹ pfu/g AdLacz and AdIκBα was investigated 4 hours after adenoviral infection. (D) Equal transduction of the livers by 1 × 10⁹ AdLacz and AdIκBα was confirmed by semi-quantitative duplex-PCR comparing the ratio of viral genome (Ad5 fiber) vs. mouse genome (caspase 8 gene locus) as internal control (D).
infected with adenoviral vectors expressing the death ligands TRAIL or FasL. Inhibition of NFκB by IκBα strongly sensitizes hepatocytes to TRAIL-mediated apoptosis, as shown in Fig. 2. In contrast, inhibition of NFκB has no significant impact on the sensitivity of hepatocytes toward FasL. FasL-mediated apoptosis in mice pretreated with AdIκBα appears to be similar compared with that in mice treated with the control vector AdLacZ (Fig. 2). The same results were observed in nude mice, which indicates that adenoviral expression of the death ligands rather than potential death-ligands expressed by immune cells are responsible for the results (data not shown).

**Bcl-xl Expression in Hepatocytes After Viral Infection Is Dependent on NFκB Activity and Mediates Resistance to TRAIL-Mediated Apoptosis.** NFκB has been implicated in the transcriptional control of several
negative regulators of apoptosis such as cFLIP, bcl-2, and bcl-xl. To evaluate the molecular mechanisms of cellular resistance to TRAIL-mediated apoptosis in viral hepatitis, livers infected with AdLacZ or AdI/H9260B/H9251 were analyzed for expression of cFLIP, bcl-2, and bcl-xl. No significant up-regulation of cFLIP protein expression was observed in viral hepatitis. However, NFκB-inhibition resulted in slightly lower expression of c-FLIP 4 hours after adenoviral infection, but the level of c-FLIP expression remained constant during the further time course of hepatitis (Fig. 3A). In contrast to c-FLIP, bcl-2 expression was upregulated after adenoviral infection. However, inhibition of NFκB resulted only in moderately delayed expression pattern of bcl-2 during the early time course of adenoviral hepatitis without affecting the maximal expression level (see Fig. 3A).

Compared with cFLIP and bcl-2, expression of bcl-xl was significantly upregulated more strongly in viral hepatitis (see Fig. 3B), suggesting a peculiar role of NFκB-dependent regulation of bcl-xl in viral hepatitis. Inhibition of NFκB effectively inhibited bcl-xl overexpression in viral hepatitis, demonstrating that regulation of bcl-xl in viral infection is highly dependent on NFκB activity (see Fig. 3B).

To investigate the role of bcl-xl in TRAIL-mediated apoptosis, we inhibited gene expression by RNA interference. TRAIL- and FasL-resistant Huh7 hepatoma cells were transfected with siRNA duplexes against bcl-xl or with siRNA-scrambled. Interestingly, treatment with siRNA-bcl-xl–sensitized Huh7 hepatoma cells to TRAIL—but not to FasL-mediated apoptosis (Fig. 4).

Because siRNA is effectively delivered into approximately 70% to 80% of hepatocytes by hydrodynamic tail vein injection, RNA interference can also be used for silencing of liver gene expression in vivo.20-23 To investigate the contribution of bcl-xl on TRAIL-apoptosis in vivo, we inhibited bcl-xl expression in the mouse liver by hydrodynamic injection of siRNA-bcl-xl, as shown in Fig. 5A. After silencing of bcl-xl by RNAi, viral hepatitis was triggered by application of AdlacZ. SiRNA-bcl-xl significantly inhibited bcl-xl expression in viral hepatitis and strongly sensitized the hepatocytes to TRAIL-, but not to FasL-mediated apoptosis (see Fig. 5). Application of AdTRAIL into siRNA-bcl-xl pretreated mice suffering from viral hepatitis led to severe liver damage as shown by TUNEL assay (Fig. 5C), HE-stained liver sections (Fig. 5D), histone-ELISA (Fig. 5E), and the strong release of liver transaminases into the serum (Fig. 5F). Compared with the siRNA-scrambled control, hydrodynamic injec-
tion of siRNA-bcl-xl also slightly enhanced liver cell apoptosis in experiments with high-dose (1 × 10^9 pfu/g) AdlacZ/AdGFP, which may be explained by the fact that high-dose adenoviral infection can trigger TRAIL expression in the liver.\(^8\)

Our results demonstrate that downregulation of bcl-xl in viral hepatitis alone strongly sensitizes hepatocytes to TRAIL-mediated apoptosis, suggesting that bcl-xl is an important NFκB-dependent protection factor in TRAIL-mediated apoptosis in the liver.
NFkB is activated by viral infections as a part of the cellular defense mechanism and is important in modulating cell survival and cell death after cellular damage. NFkB protects cells from apoptosis by transcriptional activation of survival factors, such as c-FLIP, XIAP, c-IAP1, c-IAP2, Bfl-1/A1, Bcl-2, and Bcl-xL. NFkB is also capable of transactivating death receptors such as Fas or TRAIL-DR5, providing a molecular explanation for its proapoptotic functions. The ability to induce both—death receptors or antiapoptotic mediators—explains the dual role of NFkB as a mediator or inhibitor of cell death, whereby the modulation of apoptosis by NFkB appears to be largely determined by the nature of the death stimulus.

We investigated the role of NFkB in FasL- and TRAIL-mediated apoptosis of hepatocytes in viral hepatitis. Whereas inhibition of NFkB activity in viral hepatitis resulted in strongly enhanced sensitivity of hepatocytes to TRAIL-mediated apoptosis, no sensitization to FasL-mediated apoptosis was observed.

In some cell lines, TRAIL-mediated apoptosis is inhibited by overexpression of bcl-2 and bcl-xL. Recently, Higuchi et al. showed involvement of c-FLIP in bile acid–induced TRAIL-mediated apoptosis of hepatoma cells.

In adeno- and viral hepatitis, expression of c-FLIP remained unchanged, whereas expression of bcl-2 was moderately affected and expression of bcl-xL was strongly upregulated by viral infection. However, inhibition of NFkB activity by IκBα led only to delayed upregulation of bcl-2, but largely inhibited upregulation of bcl-xL in viral infection.

To investigate the hypothesis that bcl-xL is a crucial NFkB-dependent survival factor in TRAIL-mediated apoptosis in the liver, we used RNA interference to silence bcl-xL gene expression in vitro and in vivo. Inhibition of bcl-xL gene expression resulted in enhanced susceptibility of FasL- and TRAIL-resistant hepatoma cells to TRAIL, but not to FasL-mediated apoptosis, emphasizing the predominant role of bcl-xL in TRAIL-mediated apoptosis. In agreement with the results obtained in vitro, in vivo down-regulation of bcl-xL sensitizes hepatocytes to TRAIL, but not to FasL-mediated apoptosis.

Recently several studies suggested that inhibition of NFkB activity and bcl-xL expression may be used as an attractive therapeutic strategy to sensitize resistant tumor cells to TRAIL-induced apoptosis, provided a molecular explanation for its proapoptotic functions. The ability to induce both—death receptors or antiapoptotic mediators—explains the dual role of NFkB as a mediator or inhibitor of cell death, whereby the modulation of apoptosis by NFkB appears to be largely determined by the nature of the death stimulus.

In summary, viral infection triggers sensitivity to TRAIL-mediated apoptosis as a component of an innate defense mechanism. Overexpression of TRAIL in viral diseases appears to be a defense mechanism of the organism to eliminate infected cells and limit viral replication. The molecular mechanisms involved in sensitizing virally infected cells to TRAIL-mediated apoptosis remain unclear, but protection against apoptosis during the early course of viral infection may be necessary for fine tuning the apoptotic machinery to specifically select damaged cells.

References


Peginterferon Alfa-2a for Hepatitis C After Liver Transplantation: Two Randomized, Controlled Trials

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There is currently no effective treatment for recurrent hepatitis C after orthotopic liver transplantation (OLT). We therefore performed two randomized, controlled trials—a prophylaxis trial and a treatment trial—to evaluate the safety and efficacy of peginterferon alfa-2a in patients who had undergone OLT. The prophylaxis trial enrolled 54 patients within 3 weeks after OLT, and the treatment trial enrolled 67 patients 6 to 60 months after OLT. In each trial, patients were randomized to treatment with once weekly injections of 180 µg peginterferon alfa-2a or no antiviral treatment for 48 weeks and were followed up for 24 weeks thereafter. Peginterferon alfa-2a treated patients had significantly lower hepatitis C virus RNA levels and more favorable changes in hepatic histological features compared with untreated controls. However, only 2 treated patients in the prophylaxis trial (8%) and 3 in the treatment trial (12%) achieved a sustained virological response. In the prophylaxis trial, 8 patients (31%) in the peginterferon alfa-2a group and 9 (32%) in the untreated group were withdrawn prematurely; whereas in the treatment trial, 10 patients (30%) in the peginterferon alfa-2a group and 6 (19%) in the untreated group were withdrawn prematurely. The incidence of acute rejection was similar in the treated and untreated groups in both the prophylaxis (12% vs. 21%; P = .5) and treatment (12% vs. 0%; P = .1) trials. In conclusion, peginterferon alfa-2a treatment for 48 weeks is safe and tolerable and offers some efficacy in the post-OLT setting. Randomized controlled studies are needed to establish the efficacy of pegylated interferon and ribavirin in patients who have undergone OLT. (HEPATOLOGY 2005; 41:289-298.)

Hepatitis C virus (HCV) infection is the leading cause of cirrhosis and liver failure leading to orthotopic liver transplantation (OLT) in the United States. Recurrent infection with HCV after OLT, however, is almost universal and is a significant cause of allograft dysfunction and allograft failure.1-4 Preemptive interferon therapy (prophylaxis) in the early post-transplantation period may reduce the incidence and/or severity of recurrent HCV infection. In one study, 86 patients were randomized within 2 weeks after OLT to 3 million units of interferon alfa-2b or no treatment for 1 year.5 Although interferon therapy did not significantly affect HCV RNA levels, it significantly reduced the incidence of recurrent hepatitis. In another controlled study, 24 patients were randomized within 2 weeks after OLT to interferon therapy or no treatment for 6 months.6 Although interferon treatment did not reduce the incidence or severity of recurrent hepatitis C, it significantly delayed the time to recurrence. Some preliminary reports suggested that preemptive therapy with interferon or a combination of interferon plus ribavirin may lead to less severe HCV recurrence after OLT.7-9 However, there have been no controlled studies evaluating the safety and efficacy of prophylactic pegylated interferon in liver transplant recipients with HCV.

There is also significant interest in treating established recurrent hepatitis C with interferon-related therapies.
Standard interferon monotherapy is associated with a lower sustained virological response (SVR) in the transplantation population. For example, in a recent study in which 52 liver transplant recipients were randomized to interferon plus ribavirin or no antiviral treatment for 12 months, there was no difference in liver histological features between the groups at the end of treatment, but the group treated with interferon and ribavirin had a significantly higher SVR rate than the untreated group (21% vs. 0%; \( P = .036 \)). However, 43% of patients receiving interferon and ribavirin were withdrawn from the study, mostly because of ribavirin-related side effects, including anemia. Recent preliminary reports also have suggested a role for pegylated interferon in the treatment of recurrent hepatitis C,, but these studies were small and were not randomized.

To improve our ability to manage hepatitis C in the post-OLT setting, we conducted two prospective, randomized, open-label, multicenter, phase IIIb trials of pegylated interferon alfa-2a (peginterferon alfa-2a) in liver transplant recipients. Peginterferon alfa-2a is a highly effective treatment for chronic hepatitis C in the non-transplant setting, particularly in combination with ribavirin. The objective of our first trial was to evaluate the safety, efficacy, and tolerability of peginterferon alfa-2a when given preemptively within 3 weeks of OLT to prevent recurrence of hepatitis C (prophylaxis trial). The objective of our second trial was to evaluate the safety, efficacy, and tolerability of peginterferon alfa-2a as a treatment for recurrent hepatitis C in patients 6 to 60 months after liver transplantation (treatment trial).

**Patients and Methods**

**Study Design.** In the prophylaxis trial, eligible participants in whom OLT had been performed 3 weeks previously were randomized to receive weekly subcutaneous injections of 180 \( \mu \)g peginterferon alfa-2a or no antiviral therapy for 48 weeks. In the treatment trial, eligible recipients in whom OLT had been performed 6 to 60 months previously were randomized to receive weekly subcutaneous injections of 180 \( \mu \)g peginterferon alfa-2a or no antiviral therapy for 48 weeks. The 6- to 60-month eligibility interval was chosen to maintain consistency in the practice patterns of immunosuppression and other post-transplantation care. In both trials, liver biopsies were performed before randomization (baseline) and 48 and 72 weeks after randomization. The institutional review boards of the participating centers approved the protocol, and all patients provided written informed consent.

**Eligibility Criteria.** For both trials, eligible participants were male and female adult (\( \geq 18 \) years of age), HCV-infected, post-OLT recipients. All patients had ongoing HCV infection with a positive serum anti-HCV antibody and serum HCV RNA of 1,000 IU/mL or more, as measured by the Roche Amplicor HCV 2.0 assay (Roche Diagnostics, Indianapolis, IN) (lower limit of detection, 50 IU/mL). All participants had documented elevation of serum alanine aminotransferase (\( \geq 1.5 \) upper limit of normal) before OLT (prophylaxis trial) or before enrollment (treatment trial). For both trials, eligible participants must not have received prior interferon therapy. In the prophylaxis trial, where the baseline liver biopsy samples were obtained within 3 weeks after OLT, patients were required to have no histological evidence of acute cellular rejection based on Banff International Consensus Schema. In the treatment trial, patients were required to have histological evidence of hepatitis without evidence of rejection on a liver biopsy sample obtained within 8 weeks before randomization. For both trials, the use of hematopoietic growth factors or mycophenolate mofetil was not allowed.

Patients were excluded from the study if they had a neutrophil count of less than 1,500/\( \mu \)L, a platelet count of less than 75,000/\( \mu \)L, a hemoglobin count of less than 10 g/dL, serum creatinine level of more than 2.0 mg/dL, histological evidence of cirrhosis or cholestatic fibrosing hepatitis, history of uncontrolled seizure disorder, alcohol or drug abuse within 1 year of entry, or severe psychiatric illness. Patients were also excluded if they had a history of significant immune disorder, chronic pulmonary disease, cardiac disease, or poorly controlled thyroid disease.

**Assessment of Efficacy.** The primary efficacy endpoint was SVR, defined as undetectable (<50 IU/mL) HCV RNA at the end of the 24-week treatment-free follow-up period (week 72). Secondary endpoints included the proportion of patients with virological response, defined as undetectable HCV RNA (<50 IU/mL), the proportion with a 2 log\(_{10}\) drop in HCV RNA, the proportion with biochemical response (i.e., with normalized alanine aminotransferase), and mean changes in hepatic activity index (HAI) and fibrosis score from baseline. All liver biopsy specimens were assessed for HAI and fibrosis score by a single central pathologist who was blinded to therapy and to the time at which the biopsy was taken, using the criteria of Ishak et al. Assessment of Safety. Safety was assessed by clinical laboratory testing and by evaluation of adverse events (AEs) at weeks 1, 2, 4, 6, and 8, and every 4 weeks thereafter throughout the 48-week treatment and 24-week follow-up periods. The dose of peginterferon alfa-2a was reduced by 45 \( \mu \)g decrements for clinical or laboratory AEs.

**Statistical Methods.** All efficacy parameters were analyzed on an intention-to-treat basis, and the analyses in-
cluded all randomized patients who had at least one postbaseline observation. The primary and secondary efficacy endpoints were analyzed using the Cochran-Mantel-Haenszel general association test adjusted for genotype and viral load stratum. Safety analyses included all randomized patients who had at least one postbaseline safety assessment. The number and percentage of patients with AE were tabulated by treatment group. A chi-square test or Fisher’s exact test was used to test the difference in the proportion of patients with rejection between treatment groups.

The covariate effects of selected demographic (age [<50 years vs. ≥50 years], sex, race, and weight [≤85 kg vs. >85 kg]) and baseline clinical (viral load stratum, baseline viral load, genotype, alanine aminotransferase [≤60 U/L vs. >60 U/L], and HAI score [≤10 vs. >10]) characteristics on SVR were explored using logistic regression analyses. All statistical analyses were performed using SAS software version 6.12 (SAS Institute, Cary, NC). A P value less than 0.05 was considered statistically significant.

Results

Fifty-four patients participated in the prophylaxis trial, with 26 randomized to peginterferon alfa-2a and 28 to no treatment. Fifteen patients in each group completed the study (Fig. 1A). Selected demographic and clinical characteristics were well matched in the two groups (Table 1). In the treatment trial, 34 of the 67 patients were randomized to peginterferon alfa-2a and 33 to no antiviral therapy. Twenty patients in the peginterferon alfa-2a group and 26 in the control group completed the 72-week study (Fig. 1B). Selected demographic and clinical characteristics in the two groups also were well matched (Table 1).

Virological and Biochemical Responses

Prophylaxis Trial. Baseline serum HCV RNA levels did not differ significantly between the two groups (Table 1). The pattern of virological response over time is shown in Table 2. Compared with the untreated group, patients receiving peginterferon alfa-2a had higher virological response, which was of statistical significance or marginal significance at various intervals (Table 2). SVR was achieved by 2 patients (8%) in the peginterferon alfa-2a group, but by no patient in the untreated group (P = .14). One of the patients who achieved SVR was infected with HCV genotype 1, and the other was infected with HCV genotype 2. Patients in the peginterferon alfa-2a group had a significantly lower viral load than patients in the untreated group (P values from .001 to .01) at each scheduled postbaseline assessment, except week 72 (P = .4; Table 2 and Fig. 2A). Patients receiving peginterferon alfa-2a had a significantly greater drop in HCV RNA at weeks 4 and 24 than the untreated patients (P = .003 and .02, respectively). Biochemical response did not differ significantly between the peginterferon alfa-2a and untreated groups at week 48 (19% vs. 25%, respectively; P = .6) or week 72 (15% vs. 21%, respectively; P = .6).

Treatment Trial. Baseline serum HCV RNA levels did not differ significantly between the two groups (Table 1). The pattern of virological response over time in both groups is shown in Table 2. Compared with the untreated patients, patients treated with peginterferon alfa-2a had a significantly higher virological response rate, both during treatment and at the end of follow-up. SVR was achieved

![Fig. 1. (A) Disposition of patients (Pts) in the prophylaxis trial (n = 54). (B) Disposition of patients in the treatment trial (n = 67).](image-url)
### Table 1. Baseline Demographic and Clinical Characteristics of Patients Enrolled in Both Trials

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<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>White cell count</td>
<td>8.8 ± 0.8</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>Absolute neutrophil count</td>
<td>6.9</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>Platelet count</td>
<td>197.9 ± 17.2</td>
<td>173.4 ± 21.3</td>
</tr>
<tr>
<td>HCV RNA (×10⁶ IU/mL)</td>
<td>2.0 ± 3.8</td>
<td>3.0 ± 7.2</td>
</tr>
<tr>
<td>HCV genotype 1 (%)</td>
<td>73</td>
<td>75</td>
</tr>
<tr>
<td>Baseline ALT (U/L)</td>
<td>95 ± 32</td>
<td>99 ± 30</td>
</tr>
<tr>
<td>Baseline HAI score</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Baseline fibrosis</td>
<td>0.2 ± 0.1</td>
<td>0.0 ± 0.4</td>
</tr>
<tr>
<td>Primary immunosuppression†</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>Tacrolimus (%)</td>
<td>5.8 ± 3</td>
<td>5.4 ± 3</td>
</tr>
<tr>
<td>Cyclosporine (%)</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Mean daily dose ± SD</td>
<td>395 ± 144</td>
<td>278 ± 91</td>
</tr>
<tr>
<td>Prednisone (%)</td>
<td>88</td>
<td>93</td>
</tr>
<tr>
<td>Mean daily dose ± SD</td>
<td>9.8 ± 3.7</td>
<td>8.7 ± 2.8</td>
</tr>
</tbody>
</table>

**NOTE.** Data presented as mean ± SE unless specified otherwise. Abbreviations: GFR, glomerular filtration rate; ALT, alanine aminotransferase. * Calculated using Cockroft-Gault equation. † Primary immunosuppression was interchanged in seven patients in the prophylaxis trial and six patients in the treatment trial.

### Table 2. Patterns of Virological Response Over Time

<table>
<thead>
<tr>
<th></th>
<th>Prophylaxis Trial (n = 54)</th>
<th>Treatment Trial (n = 65)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated (n = 26)</td>
<td>Untreated (n = 28)</td>
</tr>
<tr>
<td>Virological response, n (%)*</td>
<td>3 (12)</td>
<td>0</td>
</tr>
<tr>
<td>Week 4</td>
<td>4 (15)</td>
<td>0</td>
</tr>
<tr>
<td>Week 12</td>
<td>5 (19)</td>
<td>0</td>
</tr>
<tr>
<td>Week 24</td>
<td>4 (15)</td>
<td>0</td>
</tr>
<tr>
<td>Week 48</td>
<td>2 (8)</td>
<td>0</td>
</tr>
<tr>
<td>2 log₁₀ drop in HCV RNA, n (%)</td>
<td>7 (27)</td>
<td>0</td>
</tr>
<tr>
<td>Week 4</td>
<td>5 (19)</td>
<td>0</td>
</tr>
<tr>
<td>Week 24</td>
<td>7 (27)</td>
<td>0</td>
</tr>
<tr>
<td>Week 48</td>
<td>6 (23)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Week 72</td>
<td>2 (8)</td>
<td>0</td>
</tr>
<tr>
<td>HCV RNA titers (×10⁶ IU/mL; mean ± SE)</td>
<td>2.0 ± 0.7</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>Week 0</td>
<td>1.8 ± 0.7</td>
<td>5.2 ± 1.3</td>
</tr>
<tr>
<td>Week 12</td>
<td>2.2 ± 0.7</td>
<td>6.2 ± 1.8</td>
</tr>
<tr>
<td>Week 24</td>
<td>1.3 ± 0.3</td>
<td>5.4 ± 1.8</td>
</tr>
<tr>
<td>Week 48</td>
<td>1.5 ± 0.4</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>Week 72</td>
<td>2.6 ± 0.5</td>
<td>2.4 ± 0.6</td>
</tr>
</tbody>
</table>

* <50 copies/mL of HCV-RNA.
A

Prophylaxis Study
Summary of Mean Viral Load Over time

B

Treatment Study
Summary of Mean Viral Load Over time

Fig. 2. (A) Mean viral loads during the prophylaxis trial. (B) Mean viral loads during the treatment trial. HCV, hepatitis C virus.

by 4 patients (12%) in the peginterferon alfa-2a group, but by none of the patients in the untreated group (P = .03). Of the patients who achieved SVR, 1 was infected with genotype 1, 2 were infected with genotype 2, and 1 was infected with genotype 3. Patients in the peginterferon alfa-2a group had a significantly lower viral load than patients in the untreated group at each scheduled postbaseline assessment (P < .001 each), except week 72 (P = .9; Fig. 2B). In addition, patients in the peginterferon alfa-2a group had a significantly greater decrease from baseline in HCV RNA titers than patients in the untreated group at weeks 4, 12, 24, and 48 (P < .001 each) and at week 72 (P < .03). Biochemical response did not differ significantly between the peginterferon alfa-2a and untreated groups at week 72 (33% vs. 22%, respectively; P = .3), but patients in the untreated group had a higher biochemical response at week 48 (28% vs. 9%; P = .048).

Histological Outcomes

Prophylaxis Trial. Baseline liver biopsies were obtained from 29 patients in the peginterferon alfa-2a group and 24 in the untreated group; week 48 biopsies were obtained from 16 patients in the peginterferon alfa-2a group and 13 in the untreated group; and week 72 biopsies were obtained from 12 patients in the peginterferon alfa-2a group and 11 in the untreated group (Fig. 1A). The changes in liver histological features between baseline and paired week 48 and week 72 biopsies are shown in Table 3 and Fig. 3A. The HAI activity scores at baseline and at week 72 were 0.8 ± 0.4 and 3.5 ± 0.9, respectively, in the peginterferon alfa-2a group and 1.2 ± 0.4 and 5.2 ± 1.0, respectively, in the untreated group. The increase in HAI score over the 72-week period seemed to be lower in the peginterferon alfa-2a group than in the untreated group, but this difference was not statistically significant (2.7 ± 0.8 vs. 4.0 ± 1.0, respectively; P = .3). Fibrosis scores at baseline and at week 72 were 0.2 ± 0.1 and 0.6 ± 0.2, respectively, in the peginterferon alfa-2a group and 0.1 ± 0.1 and 1.1 ± 0.3, respectively, in the untreated group. The increase in fibrosis score over the 72-week study period seemed to be lower in the peginterferon alfa-2a group than in the untreated group, but again, this difference was not statistically significant (0.4 ± 0.2 vs. 1.0 ± 0.3, respectively; P = .3). Between baseline and week 48, HAI inflammatory scores improved
in 20%, stabilized in 13%, and worsened in 67% of patients in the peginterferon alfa-2a group, whereas fibrosis score improved in 7%, stabilized in 80%, and worsened in 13%. In comparison, for untreated patients there was no improvement in fibrosis, stabilization occurred in only 38% of untreated patients, and worsening occurred in 62% of untreated patients.

**Treatment Trial.** Baseline liver biopsies were obtained from all participants, week 48 biopsies were obtained from 21 patients in the peginterferon alfa-2a group and from 24 patients in the untreated group, and week 72 biopsies were available from 20 patients in each group (Fig 1B). The changes in liver histological features between baseline and week 48 and week 72 biopsies are shown in Table 3 and Fig. 3B. The HAI scores at baseline and at week 72 were 5.3 ± 0.6 and 5 ± 0.6, respectively, in the peginterferon alfa-2a group and 4.2 ± 0.5 and 5.2 ± 0.7, respectively, in the untreated group. The change in HAI score over the 72-week period seemed to be lower in the peginterferon alfa-2a group than in the untreated group, but this difference was not statistically significant (−0.3 ± 0.8 vs. 1.0 ± 0.9, respectively; *P* = .2). Fibrosis scores at baseline and at week 72 were 0.8 ± 0.2 and 1.1 ± 0.2, respectively, in the peginterferon alfa-2a group and 0.7 ± 0.2 and 1.3 ± 0.2, respectively, in the untreated group. The increase in fibrosis score over the 72-week study period seemed to be lower in the peginterferon alfa-2a group than in the untreated group, but this difference was not statistically significant (0.3 ± 0.2 vs. 0.6 ± 0.2, respectively; *P* = .2). Between baseline and week 48, HAI score improved in 76%, stabilized in 10%, and worsened in 14% of the patients in the peginterferon alfa-2a group, whereas fibrosis score improved in 10%, stabilized in 63%, and worsened in 24% in these patients. Changes in inflammation for the untreated group were 29% improved, 33% stabilized, and 38% worsened; whereas fibrosis changes did not differ from those in the untreated group at 8%, 63%, and 29%, respectively.

**Predictors of Virological Response**

In a stepwise logistic regression analysis consisting of age, sex, race, weight, alanine aminotransferase level, baseline HCV RNA, genotype, week 12 virological response, and pretreatment HAI or fibrosis score, in both trials only genotype was independently associated with SVR (i.e., patients with HCV genotype 1 were less likely to achieve SVR than those with HCV genotype non-1). In the prophylaxis trial, the association between genotype non-1 and viral response at various time points was: week 12 (OR, 13.5; 95% CI, 1.1-166; *P* = .04), week 24 (OR, 24; 95% CI, 1.9-295; *P* = .01), and week 48 (OR, 19; 95% CI, 1.1-342; *P* = .004). (Week 72 could not be fit into the model because of the small number of patients with SVR.) In the treatment trial, the association between genotype non-1 and viral response at various time points was: week 12 (OR, 22; 95% CI, 1.6-309; *P* = .02), week 24 (OR, 14; 95% CI, 1.4-144; *P* = .02), week 48 (OR, 33; 95% CI, 3.1-353; *P* = .004), and week 72 (OR, 32.5; 95% CI, 1.8-600; *P* = .02).

**Safety Evaluation**

**Prophylaxis Trial.** The number of AEs, serious AEs, rejection episodes, and deaths in the peginterferon alfa-2a and untreated groups were similar. The nature and frequency of common AEs in both groups are shown in

<table>
<thead>
<tr>
<th>Table 3. Changes in Liver Histology Features Over Time</th>
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<tbody>
<tr>
<td><strong>Comparison</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Baseline and week 48</td>
</tr>
<tr>
<td>No. patients</td>
</tr>
<tr>
<td>HAI baseline</td>
</tr>
<tr>
<td>HAI week 48</td>
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<tr>
<td>Change in HAI</td>
</tr>
<tr>
<td>Fibrosis score baseline</td>
</tr>
<tr>
<td>Fibrosis score week 48</td>
</tr>
<tr>
<td>Change in fibrosis score</td>
</tr>
<tr>
<td>Baseline and week 72</td>
</tr>
<tr>
<td>No. patients</td>
</tr>
<tr>
<td>HAI baseline</td>
</tr>
<tr>
<td>HAI week 72</td>
</tr>
<tr>
<td>Change in HAI</td>
</tr>
<tr>
<td>Fibrosis score baseline</td>
</tr>
<tr>
<td>Fibrosis score week 72</td>
</tr>
<tr>
<td>Change in fibrosis score</td>
</tr>
</tbody>
</table>
Table 4. Five patients were judged to have life-threatening AEs during the trial, 2 in the peginterferon alfa-2a group (severe hypoglycemia and severe rejection) and 3 in the untreated group (respiratory failure, severe anemia, and allograft failure). Three patients died during the study: 1 in the peginterferon alfa-2a group as a result of post-transplantation lymphoproliferative disorder with Corynebacterium bacteremia and 2 in the untreated group as a result of sepsis and allograft dysfunction in one patient and as a result of respiratory failure resulting from bronchiolitis obliterans in another patient. All of these life-threatening AEs and deaths were judged by the site investigator to be unrelated to participation in this trial. Three patients in the peginterferon alfa-2a group (12%) and 6 in the untreated group (21%) had acute cellular rejection during the trial (P = .5; Table 4). Three patients in the peginterferon alfa-2a group and 4 in the untreated group received a full course of antirejection treatment (high-dose steroids for 3 consecutive days or more or antithymocyte or lymphocyte globulin or OKT3 for 1 day or more).

The proportion of patients who were withdrawn from the trial during the 48-week treatment period was similar: 8 patients (31%) in the peginterferon alfa-2a group and 9 (32%) in the untreated group (Fig. 1A and Table 5). Eleven patients (42%) in the peginterferon alfa-2a group required a dose reduction because of AEs or laboratory abnormalities, with the most common causes of dose adjustment being thrombocytopenia and/or neutropenia (9 patients).

Table 5. Reasons for Premature Withdrawal Before Week 48

At baseline, mean (± SD) hemoglobin concentrations were low but comparable in the two groups: 10.5 ± 0.4 g/dL in the peginterferon alfa-2a group and 10.7 ± 0.4 g/dL in the untreated group. Mean hemoglobin concentration in both groups increased steadily throughout the trial, with the untreated group reaching values within the normal range earlier (24 weeks) than the peginterferon alfa-2a group (week 72). Mean (±SD) neutrophil counts for patients in both groups were normal at baseline but decreased similarly during the trial period. Absolute neutrophil counts of less than 1.0 × 10⁹/L were observed in 23% of patients in the peginterferon alfa-2a group and in 18% of the untreated patients (P = .3). Mean (±SD) baseline platelet counts in both groups (198,000/µL ± 17,200/µL in the treated and 173,000/µL ± 21,300/µL in the untreated group) were within the normal range (150,000 to 450,000/µL) and showed a reversible decline during treatment. The magnitude of the decline, however, was greater in the peginterferon alfa-2a group. The maximum mean decrease was 111,400/µL ± 80,000/µL in the peginterferon alfa-2a treated group and 29,000/µL ± 101,000/µL in the untreated group, both at week 24.

**Treatment Trial**

The nature and frequency of common AEs in the peginterferon alfa-2a and untreated groups are shown in Table 4. Three patients, all in the peginterferon alfa-2a group, were judged to have life-threatening AEs during the trial (one case each of multiorgan failure, allograft failure, and head and neck cancer with lung metastases). Two patients in the peginterferon alfa-2a group died during the trial period (as a result of hepatic and renal failure associated with severe tacrolimus toxicity in one patient and as a result of head and neck cancer with lung metastases in another patient). None of these life-threatening
AEs or deaths was judged by the site investigator to be related to the study drug. Four patients in the peginterferon alfa-2a group (12%), but none in the untreated group, had biopsy-proven or presumed episodes of acute rejection during the trial \( P = .11 \). No patient in either group, however, required a full course of antirejection treatment. Ten patients (30%) in the peginterferon alfa-2a group were withdrawn from the trial during the 48-week treatment period, compared with 6 (19%) in the untreated group (Fig. 1B and Table 5). Twenty patients in the treatment group required a dose adjustment because of AEs or laboratory abnormalities, with the most common causes of dose adjustment being thrombocytopenia and/or neutropenia (18 patients).

Mean hemoglobin concentration decreased between weeks 1 and 48 in both groups, but returned to near baseline values after treatment was completed. The decrease was greater in the peginterferon alfa-2a group (from 14.0 ± 0.3 g/dL to 11.9 ± 1.45 g/dL) than in the untreated group (from 14.8 ± 0.3 g/dL to 14.1 ± 1.57 g/dL). In the treated group, the mean neutrophil count decreased from baseline (3,600/µL ± 200/µL) to near the lower limit of the normal range (1,980/µL ± 1200/µL; maximum mean decrease, 1,900/µL), but decreased only slightly in the untreated group. Absolute neutrophil counts of less than 1.0 × 10⁹/L were observed in 36% of patients in the peginterferon alfa-2a group and in none of the untreated patients. Mean platelet count decreased in the treated group between weeks 1 and 24 (from 162,000 ± 10,500/µL to 91,000 ± 39,000/µL; maximum mean decrease, 75,700 ± 55,400/µL), but returned to near baseline values by week 72. Mean platelet counts were essentially unchanged for patients in the untreated group.

Discussion

The two trials presented here show the results of prospective, randomized, controlled trials that evaluated the safety and efficacy of pegylated interferon after liver transplantation. Our trials make several important observations. First, they show that peginterferon alfa-2a therapy is safe and reasonably well tolerated in the post-OLT setting when administered prophylactically soon after OLT or later in the post-OLT period to treat recurrent hepatitis C. Second, although SVR was achieved by only a small number of patients, a sizable proportion of patients had significant viral suppression while receiving treatment (up to 27% in the prophylaxis trial and up to 52% in the treatment trial). Third, our results suggest that while on therapy, peginterferon alfa-2a may affect liver histological features favorably in HCV-infected OLT recipients. Last, these results highlight the risks of liver transplantation recipients for the development of serious AEs unrelated to antiviral therapy and show that a sizable proportion of patients are unable to complete the protocol for reasons unrelated to antiviral therapy.

Although many transplantation centers routinely use pegylated interferon to manage recurrent hepatitis C after OLT,¹ nine published reports systematically assessing the safety and efficacy of pegylated interferon in the post-OLT setting are limited.¹¹-¹⁴ In one study, 9 liver transplantation recipients with renal failure and recurrent hepatitis C were treated with peginterferon alfa-2b (1.0 µg/kg weekly), but 8 of these patients were intolerant to treatment and required discontinuation within the first 3 months.¹¹ In a retrospective study, 16 patients, 11 of whom were nonresponders to interferon plus ribavirin therapy, were treated with peginterferon alfa-2b (target dose, 1.5 µg/kg weekly) and ribavirin (target dose, 800-1200 mg/d).¹² Although on-treatment virological response was observed in 6 patients (37.5%), none achieved SVR. In a nonrandomized prospective study, peginterferon alfa-2b and ribavirin were titrated with increasing doses in 19 patients with recurrent hepatitis C for a median duration of 128 weeks after OLT, with therapy continued for 1 year after hepatitis C replication was undetectable by reverse-transcriptase polymerase chain reaction.¹³ Of these 19 patients, 12 (63%) completed the protocol, 7 (37%) had an end-of-treatment response, and 5 (26%) achieved SVR, with the latter showing significant improvement in necroinflammatory scores. A pilot study assessed the safety and efficacy of peginterferon alfa-2b and ribavirin in 20 patients with recurrent hepatitis C for a median duration of 28 months after OLT.¹⁴ Most patients were naïve to prior interferon therapy, and the doses were progressively increased from 0.5 to 1.0 µg/kg weekly for peginterferon alfa-2b and from 400 mg/d to 1,000-1,200 mg/d for ribavirin. Four patients (20%) were withdrawn because of AEs, whereas 6 (37.5%) of the 16 patients who completed the study required a reduction in peginterferon alfa-2b dose to 0.5 µg/kg weekly and 13 (81%) required a reduction in ribavirin dose. On an intention-to-treat basis, end-of-treatment response was achieved by 55% of the patients and SVR by 45%. Importantly, there were significant improvements in mean METAVIR scores (activity, 1.8 vs. 0.3; fibrosis, 2.2 vs. 1.6; \( P < .05 \) for each).

The histological changes we observed during treatment deserve further discussion. Although SVR was infrequent, patients receiving peginterferon alfa-2a exhibited a trend toward reduced exacerbation of liver histological features at week 48, which became less apparent by week 72. For example, in the prophylaxis trial, patients who received peginterferon alfa-2a had a significantly lower increase in...
fibrosis score than the untreated patients at week 48, but this benefit was not evident at week 72. Similarly, in the treatment trial, patients who received peginterferon alfa-2a had a significantly lower increase in HAI score than the untreated patients at week 48, but this benefit disappeared by week 72. In addition, several patients in each trial showed improvement in hepatic histological features in the absence of virological or biochemical response (data not shown). These data suggest that the beneficial histological effects are treatment dependent and that these patients, who are not likely to clear the virus, may require long-term or maintenance antiviral therapy to sustain or improve histological benefits.

Two recently published studies suggest that interferon-based treatment of recurrent hepatitis C may increase the risk of allograft rejection. For example, 8 (35%) of 23 liver transplantation recipients with significant recurrent hepatitis C treated predominantly with pegylated interferon monotherapy for a minimum of 24 weeks showed evidence of acute or chronic rejection on post-treatment biopsy; although most of these patients had no previous history of rejection, two experienced graft loss from chronic rejection. Similarly, in 5 (11%) of 44 liver transplantation recipients receiving interferon-based therapy for recurrent hepatitis C, acute rejection developed during therapy, and two patients experienced graft loss because of severe rejection. Although these results raise concerns about the safety of interferon-related therapies in the post-OLT setting, they should be interpreted cautiously, especially in view of their study design. Studies that systematically examined the risk of allograft rejection from combination therapy are not adequate, but in one randomized controlled study, the risk of acute or chronic rejection was not higher in those who received 48 weeks of interferon alfa-2b plus ribavirin compared with untreated controls. Our two prospective, controlled trials did not show that patients treated with peginterferon alfa-2a experienced a higher incidence of rejection, as assessed by clinical criteria or by sequential liver biopsies.

Our trials used stringent eligibility criteria, making the participants likely to represent only a small proportion of those patients who undergo liver transplantation for hepatitis C-related liver disease. Although these eligibility criteria allowed us to study the efficacy of peginterferon alfa-2a in a relatively homogenous group of patients, they diminish the generalizability of our results. For example, our study excluded patients who had received interferon therapy before liver transplantation. This is problematic because at present, many transplant patients have had prior interferon-based treatments.

Interestingly, we did not observe consistent relationships between biochemical and virological responses at weeks 48 and 72, in that several patients had virological responses, but not biochemical responses, at these intervals. This is not surprising, inasmuch as liver transplant recipients frequently exhibit elevations in liver enzymes for reasons other than HCV, including rejection, drug toxicity, or steatosis. This finding suggests, however, that sustained biochemical response is not an optimal efficacy endpoint for antiviral therapy in liver transplant recipients.

Because our two trials were not designed to be compared against each other, we are unable to comment on which strategy (i.e., prophylaxis or treatment of established recurrent hepatitis C) is better for managing HCV in liver transplant recipients. This can be addressed only by a trial in which liver transplant recipients are randomized to receive prophylactic or therapeutic antiviral therapy. For the present, the timing of antiviral therapy will largely depend on the clinical judgment of physicians and the willingness of their patients to undergo treatment.

Although our results reveal disappointing antiviral efficacy for peginterferon alfa-2a monotherapy in the post-OLT setting, they provide a basis for exploring combination therapy, even within 3 weeks after OLT. One controlled study, and several uncontrolled studies have reported that treatment with conventional interferon plus ribavirin led to higher virological response rates than those achieved by pegylated interferon alone. Based on these results, it is tempting to assume that pegylated interferon plus ribavirin will be more effective than pegylated interferon alone in the post-OLT setting. However, liver transplant recipients may require significant dose reductions of ribavirin because of the higher prevalence in these patients of renal insufficiency, and thus may not derive significant incremental benefit from combination therapy. Randomized controlled studies, with and without hematopoietic growth factors, are urgently needed to establish the efficacy and safety of the combination of pegylated interferon and ribavirin in liver transplant recipients with hepatitis C.

Acknowledgment. We thank Rajinder Sidhu for her contributions to the conduct of this study, Drs. Richard J. Alexander and Jennifer Steeber for medical writing and editing assistance, and Dr. Janet S. Lee for her critical review of the manuscript. Two patients who participated in the treatment trial at the UCLA site were included in another paper published by Dr. Sammy Saab. This was done without the prior knowledge of other members of the Pegasys transplant team or the sponsor. The recognition of this error was promptly shared with the editor.
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References

Oral Administration of Sildenafil Restores Learning Ability in Rats With Hyperammonemia and With Portacaval Shunts

Slaven Erceg, Pilar Monfort, Mariluz Hernández-Viadel, Regina Rodrigo, Carmina Montoliu, and Vicente Felipo

Patients with liver disease with overt or minimal hepatic encephalopathy show impaired intellectual capacity. The underlying molecular mechanism remains unknown. Rats with portacaval anastomosis or with hyperammonemia without liver failure also show impaired learning ability and impaired function of the glutamate-nitric oxide-cyclic guanine monophosphate (glutamate-NO-cGMP) pathway in brain. We hypothesized that pharmacological manipulation of the pathway in order to increase cGMP content could restore learning ability. We show by in vivo brain microdialysis that chronic oral administration of sildenafil, an inhibitor of the phosphodiesterase that degrades cGMP, normalizes the function of the glutamate-NO-cGMP pathway and extracellular cGMP in brain in vivo in rats with portacaval anastomosis or with hyperammonemia. Moreover, sildenafil restored the ability of rats with hyperammonemia or with portacaval shunts to learn a conditional discrimination task. In conclusion, impairment of learning ability in rats with chronic liver failure or with hyperammonemia is the result of impairment of the glutamate-NO-cGMP pathway. Moreover, chronic treatment with sildenafil normalizes the function of the pathway and restores learning ability in rats with portacaval shunts or with hyperammonemia. Pharmacological manipulation of the pathway may be useful for the clinical treatment of patients with overt or minimal hepatic encephalopathy. (HEPATOLOGY 2005;41:299-306.)

Hepatic encephalopathy is a neuropsychiatric syndrome covering a wide range of neuropsychiatric disturbances ranging from minimal changes in personality or altered sleep-and-waking cycle to deep coma and death. Patients with liver cirrhosis with normal neurological or mental status examination results may have minimal forms of hepatic encephalopathy showing intellectual function impairment as revealed by neuropsychological testing.1,2

Hyperammonemia is considered one of the main factors responsible for the neurological alterations in hepatic encephalopathy,3 and the classical clinical treatments are directed toward reducing blood ammonia levels. However, the molecular mechanisms by which hyperammonemia and liver failure lead to neurological alterations and to impairment of intellectual function remain unclear.

The molecular mechanisms involved in different types of learning are not well known. N-methyl-D-aspartate (NMDA) receptors are involved in some types of learning. Activation of NMDA receptors increases calcium in postsynaptic neurons. Calcium binds to calmodulin and activates neuronal nitric oxide (NO) synthase, increasing NO, which activates guanylate cyclase, increasing cyclic guanine monophosphate (cGMP), part of which is released to the extracellular space. Activation of this glutamate-NO-cGMP pathway may be involved in some forms of learning. Some recent reports indicate that guanylate cyclase and cGMP are important in learning and memory. Administration of a membrane permeant analog of cGMP facilitated memory consolidation,4 whereas bilateral intrahippocampal administration of an inhibitor...
of guanylate cyclase caused amnesia for inhibitory avoidance when given immediately after training. Blocking NMDA receptors with dizocilpine or inhibiting NO synthase impaired spatial working memory in mice, suggesting that the reduction in NO and cGMP production in brain may be responsible for dizocilpine-induced learning impairment. These results support the idea that the glutamate-NO-cGMP pathway and cGMP modulate some forms of learning and memory.

Both liver failure and chronic hyperammonemia impair the function of the glutamate-NO-cyclic GMP pathway in rat brain in vivo. Chronic hyperammonemia also impairs the ability of rats to learn a conditional discrimination task.

The step of the glutamate-NO-cGMP pathway altered in rat models of hyperammonemia and hepatic encephalopathy is the activation of soluble guanylate cyclase by NO, which is also altered in brains of patients who died with hepatic encephalopathy.

We hypothesized that the alterations in the function of the glutamate-NO-cGMP pathway in the brain in hyperammonemia and liver disease may be responsible for the impairment in learning ability and intellectual function and that pharmacological modulation of the pathway may restore learning ability in hyperammonemia and hepatic encephalopathy.

The aim of this work was to reverse the impairment in learning ability of rats with portacaval anastomosis and of hyperammonemic rats without liver failure by pharmacological manipulation of the pathway in brain. We assessed whether the learning ability of rats with portacaval anastomosis or hyperammonemia may be restored by increasing cGMP by chronic oral administration of sildenafil, an inhibitor of cGMP-degrading phosphodiesterase. Tests of conditional discrimination learning were performed with control rats, rats with portacaval anastomosis, or hyperammonemic rats without liver failure treated or not with sildenafil.

Materials and Methods

Portacaval Anastomosis

Male Wistar rats were anesthetized with halothane and an end-to-side portacaval anastomosis was constructed as previously described. At the moment of death, the liver was atrophic and the anastomosis was permeable. The rats were subjected to the Y-maze learning test 4 weeks after surgery.

Hyperammonemic Rats Without Liver Failure

Male Wistar rats (120-140 g) were made hyperammonemic by being fed an ammonium-containing diet, as previously described. Animal models of hepatic failure (e.g., portacaval shunt) show, in addition to hyperammonemia, other alterations (e.g., loss of muscular mass and altered metabolism of other compounds in liver) that do not allow identification of the effects of hyperammonemia and of those resulting from other alterations. The model used in this work was developed to make available a rat model of pure hyperammonemia, reproducing the hyperammonemia present in patients with chronic liver disease (e.g., liver cirrhosis) but without the other alterations. This model allows study of the contribution of hyperammonemia to the effects of liver failure and discernment of which effects are the result of hyperammonemia and which are the result of other factors. The model of hyperammonemia used was described in detail previously. The rats were subjected to the Y-maze learning test after 4 weeks of treatment.

Administration of Sildenafil

Hyperammonemic Rats Without Liver Failure.

Male Wistar rats were fed a normal diet (controls) or the diet containing ammonium acetate as indicated above for a period of 28 days before the initiation of the tests and were maintained on these diets during behavioral tests. One group of rats eating the control diet (control + sildenafil) and one group of hyperammonemic rats (ammonia + sildenafil) were treated daily with sildenafil (50 mg/L in the drinking water administered ad libitum). The treatment started 2 days before the learning test, and rats were allowed to drink always at the same time, 1 hour before the test. Rats were treated with sildenafil daily until death. For the other two groups (control group and ammonia group), the rats were treated in the same way with the tap water. Sildenafil was a gift from Pfizer, Inc. (New York, NY).

Rats With Portacaval Shunts.

Four groups of 10 rats were used: the control group; the control + sildenafil group, the portacaval shunted rats group, and the portacaval shunted rats + sildenafil group. The treatment with sildenafil was initiated 28 days after surgery and was carried out as for hyperammonemic rats without liver failure.

The amount of sildenafil ingested by each group of rats is shown in Table 1.

Y-Maze Learning Test

Learning tests were initiated 2 days after the beginning of the treatment with sildenafil. Learning ability was tested as described previously in a wooden Y-maze with three arms. The whole area of the arms was covered by black or white inserts. In each trial, rats were rewarded for choosing the left arm when the inserts were black and the...
Experiment with hyperammonemic rats without liver failure

<table>
<thead>
<tr>
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<th>Sildenafil (µg/kg body weight)</th>
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<tr>
<td>Control rats</td>
<td>19 ± 4</td>
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<tr>
<td>Hyperammonemic rats</td>
<td>19 ± 5</td>
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Experiment with rats with portacaval anastomosis

<table>
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<tr>
<th></th>
<th>Sildenafil (µg/kg body weight)</th>
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</thead>
<tbody>
<tr>
<td>Control rats (sham)</td>
<td>22 ± 5</td>
</tr>
<tr>
<td>Rats with portacaval anastomosis</td>
<td>20 ± 5</td>
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NOTE. The volume of water containing sildenafil and the total amount of sildenafil ingested by each rat were measured every day for 30 days starting 2 days before the beginning of learning tests. The value given is the mean of the 30 days. Body weight of each rat was determined every 3 to 4 days. Eight measurements were obtained during the 30 days. To calculate the ingestion of sildenafil per kilogram of body weight, we divided the total amount of sildenafil ingested in 24 hours by the mean of the eight determinations of body weight. In all cases, values are the mean ± SD of six rats per group. No significant difference was found in the ingestion of sildenafil between the different groups of rats.

### Analysis of the Function of the Glutamate-NO-cGMP Pathway and Extracellular cGMP in Rats With Portacaval Anastomosis

Rats with a portacaval shunt showed a reduced learning ability (Fig. 1) and needed 96 ± 19 trials to learn a conditional discrimination task, which was significantly higher ($P < .001; F = 17.49$) than the number of trials needed by control rats (67 ± 8). Treatment with sildenafil restored the ability of rats with portacaval anastomosis to learn, reducing the number of trials required to 57 ± 11, which was significantly lower ($P < .001; F = 17.49$) than the number of trials for rats with portacaval anastomosis and was not different from the number of trials required by control rats. The number of trials for control rats treated with sildenafil (76 ± 10) was not significantly different from control rats drinking tap water.

### Determination of cGMP

cGMP was measured by using the BIOTRAK cGMP enzyme immunoassay kit from Amersham (Amersham Pharmacia Biotech, Buckinghamshire, UK).
samples of the extracellular fluid were taken to measure cGMP.

The five initial fractions were taken before the rats were allowed to drink sildenafil or water. The concentration of cGMP in these samples is shown in Fig. 3A. The basal concentration of extracellular cGMP before drinking was calculated as the mean ± SEM of the values for fractions 1 through 5. (B) Extracellular cGMP after drinking was calculated as the mean ± SEM of the values for fractions 6 through 9. Values are the mean ± SEM of six animals per group. Values that are significantly different from controls are indicated by a. Values that are significantly different from rats with portacaval anastomosis are indicated by b. The statistical significance is indicated by asterisks: **P < .01, F = 17.49 (one-way ANOVA after Newman-Keuls multiple comparison test). +, treatment with sildenafil, rats with portacaval anastomosis; −, control rats.

After taking these five initial fractions, rats were allowed to drink sildenafil or water, starting at the beginning of fraction 6. As shown in Figs. 2 and 3B, drinking either water or sildenafil increased extracellular cGMP in all groups of rats, but the differences between the concentrations of cGMP in the cerebellum of rats with portacaval anastomosis (446 ± 90 pM) and did not affect extracellular cGMP in the cerebellum of control rats (476 ± 105 pM).

After taking these five initial fractions, rats were allowed to drink sildenafil or water, starting at the beginning of fraction 6. As shown in Figs. 2 and 3B, drinking either water or sildenafil increased extracellular cGMP in all groups of rats, but the differences between the concentrations of cGMP in the cerebellum of the different groups of rats were maintained. For rats drinking tap water, the concentration of cGMP in rats with portacaval anastomosis (446 ± 90 pM) and did not affect extracellular cGMP in the cerebellum of control rats (476 ± 105 pM).
water (2068 ± 519 pM). Treatment with sildenafil did not affect extracellular cGMP in control rats, remaining similar to rats drinking water (2026 ± 333 pM).

To assess the effect of treatment with sildenafil on the function of the glutamate-NO-cGMP pathway, NMDA receptors were activated by administering NMDA (0.3 mM) through the microdialysis probe for 20 minutes during fraction 10. As shown in Fig. 2, administration of NMDA activated the pathway and increased extracellular cGMP in the cerebellum in all groups of rats. The increase in extracellular cGMP was significantly lower in rats with portacaval anastomosis drinking water than in control rats. The increase in extracellular cGMP in the samples collected during the 90 minutes after application of NMDA reached 39-fold basal levels in control rats, but only 10-fold in rats with portacaval anastomosis, indicating a reduction of 74% in the function of the glutamate-NO-cGMP pathway.

Treatment with sildenafil significantly enhanced the function of the glutamate-NO-cGMP pathway in rats with portacaval anastomosis, reaching levels (29-fold increase) similar to that of control rats. The increase in extracellular cGMP in control rats treated with sildenafil (44-fold increase) was not significantly different from that of control rats drinking water (Fig. 2).

**Treatment With Sildenafil Does Not Affect Ammonia Concentration in Extracellular Fluid**

Ammonia was measured in the same microdialysis samples used to measure cGMP. The concentration of ammonia was significantly higher (*P < .01; F = 8.52) in rats with portacaval anastomosis drinking water (218 ± 33 µM) or sildenafil (183 ± 13 µM) than in control rats drinking water (86 ± 15 µM) or sildenafil (108 ± 17 µM). Treatment with sildenafil did not affect ammonia concentration neither in control rats nor portacaval shunted rats.

**Treatment With Sildenafil Restores Learning Ability in Hyperammonemic Rats Without Liver Failure**

Hyperammonemia is one of the main factors contributing to the neurological alterations in hepatic encephalopathy. To assess the role of hyperammonemia in the alterations in learning ability, extracellular cGMP, and the function of the glutamate-NO-cGMP pathway observed in rats with portacaval anastomosis, we carried experiments similar to those reported above using rats with chronic moderate hyperammonemia without liver failure.

Chronic hyperammonemia significantly reduced the ability of rats to learn the conditional discrimination task, increasing the number of trials required to learn from 58 ± 3 in control rats to 83 ± 6 in hyperammonemic rats (Fig. 4). Treatment with sildenafil restored the ability of hyperammonemic rats to learn the conditional discrimination test, decreasing the number of trials required to 52 ± 6, which was significantly lower (*P = .01; F = 10.89) than for hyperammonemic rats and was not significantly different from that of control rats. The administration of sildenafil to control rats significantly increased the number of trials required to learn the test to 101 ± 10.

**Treatment With Sildenafil Normalizes the Function of the Glutamate-NO-cGMP Pathway and Extracellular cGMP in Hyperammonemic Rats Without Liver Failure**

The effects of hyperammonemia without liver failure and of treatment with sildenafil are shown in Fig. 5. The experiments were performed after 28 days of treatment with sildenafil. A microdialysis probe was inserted in the cerebellum, and samples of the extracellular fluid were taken to measure cGMP.

The five initial fractions were taken before the rats were allowed to drink sildenafil or water. The basal concentration of extracellular cGMP was significantly reduced (*P < .05; F = 9.84) in the cerebellum of hyperammonemic rats (228 ± 30 pM) compared with that of control rats drinking water (396 ± 36 pM). Treatment with sildenafil increased basal extracellular cGMP in hyperammonemic rats (331 ± 32 pM), reaching levels that were not significantly different from those in control rats drinking water. Extracellular cGMP in control rats treated with sildenafil was 536 ± 72 pM, which was significantly higher than in control rats drinking water.
were 22-fold the basal levels in control rats and were significantly increased in hyperammonemic rats after treatment with sildenafil: cGMP increased 20-fold, which was not significantly different from control rats drinking water. The increase in cGMP was 29-fold in control rats treated with sildenafil (Fig. 5).

**Treatment With Sildenafil Does Not Affect Ammonia Concentration in Extracellular Fluid**

Ammonia was measured in the same microdialysis samples used to measure cGMP. The concentration of ammonia was significantly higher ($P < .001$; $F = 14.78$) in rats with hyperammonemia that drank water ($220 \pm 12 \mu M$) or sildenafil ($210 \pm 17 \mu M$) than in control rats that drank water ($147 \pm 27 \mu M$) or sildenafil ($127 \pm 18 \mu M$). Treatment with sildenafil did not affect ammonia concentration neither in control rats or hyperammonemic rats.

**Discussion**

Our results show that portacaval anastomosis reduces the ability of rats to learn a conditional discrimination task as well as the concentration of cGMP in the extracellular fluid and the function of the glutamate-NO-cGMP pathway in brain in vivo. Chronic treatment with sildenafil normalizes the function of the pathway, the extracellular concentration of cGMP, and the ability of rats to learn the task. These results indicate that the impairment in learning ability in rats with portacaval anastomosis is a consequence of the reduced cGMP formation and that learning ability may be restored by pharmacological manipulation of the glutamate-NO-cGMP pathway to increase cGMP.

The glutamate-NO-cGMP pathway shows similar patterns of activation in the hippocampus, striatum, and cerebellum. Also in an acute paradigm, ammonia activates the pathway similarly in the cerebellum and striatum. This study was performed in cerebellum because the expression of the pathway is high in this area, allowing more accurate quantitative determinations, which are more difficult to obtain in hippocampus (considered the main memory structure of the brain) because it has lower basal levels of extracellular cGMP. The changes in the function of the pathway and in extracellular cGMP in cerebellum may be considered a marker of the effects of sildenafil in other brain areas, including the hippocampus and striatum. Moreover, it should be noted that recent studies show that cerebellum also plays a role in spatial learning that is not the result of motor components but of cognitive components of spatial learning.
direct contribution of the glutamate-NO-cGMP pathway in cerebellum to modulation of learning therefore cannot be excluded.

As is the case for patients with chronic liver disease, rats with portacaval anastomosis present a series of alterations that include hyperammonemia, loss of muscular mass, liver atrophy, and other alterations in liver-dependent, ammonia-independent parameters such as metabolization of compounds reaching the liver from gut, and so forth. Hyperammonemia is considered the main factor contributing to the neurological alterations in hepatic encephalopathy. To assess whether hyperammonemia is responsible for the impairment of learning ability in rats with portacaval shunts, we also used an animal model of pure hyperammonemia without liver failure that presents hyperammonemia but not the other alterations associated with chronic liver failure.

The results reported confirm previous reports showing that, as is the case for rats with portacaval anastomosis, rats with hyperammonemia without liver failure also show reductions in learning ability, extracellular cGMP, and function of the glutamate-NO-cGMP pathway. Moreover, we show herein that chronic treatment of these rats with sildenafil also restores the function of the pathway, extracellular cGMP, and learning ability.

The fact that rats with portacaval anastomosis or with hyperammonemia without liver failure show the same alterations in the function of the pathway, extracellular cGMP, and learning ability indicates that hyperammonemia, which is the only common alteration in both models, is responsible for the alteration in the function of the pathway and, subsequently, of the impairment of learning ability. The effect of hyperammonemia on the function of the pathway may be a direct effect of ammonia or an indirect effect mediated by other ammonia-induced alterations. For example, Jones et al. showed that activation of GABA<sub>A</sub> receptors reduces the function of the glutamate-NO-cGMP pathway in cerebellum. Ammonia may lead to increased GABAergic tone, and this could mediate the effects of ammonia on the pathway.

In the experiments with hyperammonemic rats, sildenafil increased extracellular cGMP by 51% in control rats and reduced its ability to learn (Fig. 4), suggesting that an excessive increase in cGMP may impair learning and that cGMP must be kept high but below a certain threshold to reach maximum learning ability.

The fact that increasing extracellular cGMP by pharmacological manipulation is able to restore learning ability in hyperammonemic rats and in rats with portacaval anastomosis may have important clinical implications for the treatment of the impairment of intellectual function present in patients with evident hepatic encephalopathy but also in patients with minimal (subclinical) hepatic encephalopathy with reduced performance in psychometric tests.

The step of the glutamate-NO-cGMP pathway altered in brain in vivo in rats with portacaval anastomosis or with hyperammonemia without liver failure is the modulation of soluble guanylate cyclase by NO. This modulation is altered in patients who have died of hepatic encephalopathy, as it is in rats with portacaval anastomosis. Therefore, the function of the glutamate-NO-cGMP pathway also should be altered in the brains of these patients as in rats with portacaval anastomosis and also should be responsible for the impairment of some intellectual functions.

The results also indicate that concerning learning ability, the relevant alteration in hyperammonemia and hepatic failure is the reduced content of cGMP. Independently of other steps of the glutamate-NO-cGMP pathway, it is enough to normalize cerebral cGMP levels to restore learning ability by pharmacological manipulation using sildenafil or other inhibitors of the phosphodiesterases (e.g., vardenafil) or by other means. Prickaerts et al. showed that both sildenafil and vardenafil improve object recognition memory in rats and attributed the effect to increased levels of cGMP in brain. This indicates that cGMP may modulate different types of learning and memory processes.

Patients with liver cirrhosis or hepatitis C show cognitive impairment. They also show reduced memory function, mainly attributed to deficits in attention and visual perception. Although caution must be taken considering the possible deleterious increase in the existing vasodilatation in liver disease by sildenafil, pharmacological manipulation of cGMP in brain by safe procedures may be a useful treatment to restore cognitive and intellectual functions in patients with overt or minimal hepatic encephalopathy.

**References**


Hepatocellular carcinoma (HCC) accounts for more than 5% of all cancers and more than 500,000 deaths per year worldwide. The vast majority of patients have pre-existing cirrhosis at the time they develop HCC. Because current therapies are rarely able to achieve complete tumor ablation, chemoprevention in high-risk patients with established cirrhosis has been envisioned as a promising alternative option. Yet, data indicating that chemoprevention may be effective in patients at risk for HCC, are scarce. A preventive effect of an acyclic retinoid on the development of a second tumor after ablation of the original tumor has been shown by one group. Conflicting results regarding a potential preventive effect of interferon have also been reported. To validate and expand the concept of chemoprevention to other therapeutics, the molecular events that contribute to hepatocarcinogenesis in the liver with cirrhosis need to be identified and targeted.

Epidermal growth factor and transforming growth factor \(\alpha\) (TGF-\(\alpha\)) stimulate mitogenesis in hepatocytes through their binding to the epidermal growth factor receptor (EGFR). TGF-\(\alpha\) is produced by hepatocytes and may act as an autocrine factor in the regenerating liver. Diverse lines of evidence suggest that activation of the TGF-\(\alpha\)/EGFR signaling pathway may contribute to hepatocarcinogenesis: TGF-\(\alpha\) messenger RNA (mRNA) is overexpressed in cirrhosis and in HCC.
histochemical studies of human HCC have shown local-
izations of TGF-α and EGFR in HCC cells, consistent
with autocrine and paracrine mitogenic actions of TGF-α in
HCC;\(^{15}\) and enhanced expression of TGF-α in hepato-
cytes is sufficient to induce tumor formation in trans-
genic mice\(^{16}\) and dramatically accelerates the appearance
of HCC in hepatitis B surface antigen and TGF-α bi-
transgenic mice.\(^{17}\) However, in these different animals, as
in virtually all previously reported models of liver carci-
nogenesis, tumors arise from liver tissue without cirrhosis.

In the present study, a rat model of diethylnitrosamine
(DEN)-induced liver injury that reproduces the progres-
sion of cirrhosis toward HCC was established. The pro-
files of TGF-α and EGFR expressions at different stages
of liver injury in this model have raised the possibility that
the administration of an EGFR inhibitor at the stage of
cirrhosis may prevent the subsequent development of
HCC. Gefitinib (AstraZeneca, Macclesfield, UK), an
adenosine triphosphate mimic anilinoquinazoline that
is currently under clinical evaluation in the treatment of
patients with lung cancer and other tumors, is an orally
active EGFR-tyrosine kinase inhibitor that reduces epi-
dermal growth factor–stimulated tumor cell growth.\(^{18}\)
Based on the assumption that gefitinib would disrupt ac-
tivation of the TGF-α/EGFR pathway, we herein tested a
potential antitumoral effect of this drug in rats that had
cirrhosis.

Materials and Methods

Experimental Design. All animal care and experi-
mentation conformed to the Guide for the Care and Use of
Laboratory Animals from the National Academy of Sci-
ences. Male Wistar rats weighing 200 g received intraperi-
toneal injections of DEN (Sigma-Aldrich, St. Louis, MO)
at 50 mg/kg body weight once a week. Rats received 12
(n = 8) or 16 (n = 16) weekly injections of DEN, and
were killed 2 weeks after the last injection (to allow recov-
er from acute necrosis). Three age-matched normal rats
were used as controls. The 16 rats that were submitted to
the 16-week DEN regimen received daily intraperitoneal
injections of either gefitinib (n = 8), an EGFR-tyrosine
kinase inhibitor (AstraZeneca), at 2 mg/kg body weight or
of vehicle (n = 8) between weeks 12 and 18 (Fig. 1). At
the time of sacrifice, animals were anesthetized with an
intraperitoneal injection of thiopental. Blood was col-
cected for analyses of serum aminotransferase activities
and bilirubinemia. The count of malignant nodules was
performed at macroscopic examination of the liver by two
independent investigators based on the following criteria:
nodules with a diameter of 3 mm or more and a dysmor-
phic or dyschromic aspect. Samples of both tumoral and
nontumoral liver tissue were frozen and stored at –80°C
or fixed in 10% buffered formalin and embedded in par-
affin. Tissue samples were also homogenized in TRIzol
lysis solution (Invitrogen SARL, Cergy Pontoise, France)
and in protein lysis buffer as described later for subse-
quent analyses.

Histology and Immunohistochemistry. Four-micro-
meter-thick tissue sections of formalin-fixed, paraffin-em-
bedded liver samples were stained with hematoxylin-
phloxin-safran for standard histology. Hepatocyte
proliferation was assessed by Ki67 immunolabeling using
an immunoperoxidase method. In brief, tissue sections
were incubated sequentially with an anti-Ki67 antibody
(Novocastra Laboratories, Newcastle, UK) at 1:100 for
30 minutes, with peroxidase-conjugated rabbit anti-
mouse immunoglobulins (Dako, Glostrup, Denmark) at
1:40 for 40 minutes and with peroxidase-conjugated
swine anti-rabbit immunoglobulins (Dako) at 1:20. Per-
oxidase activity was revealed by 3-amino-9-ethyl carba-
zole and counterstaining was performed using Mayer’s
hematoxylin. An eyepiece with a net micrometer (Carl
Zeiss, Jena, Germany) at high magnification (\(\times400, 0.96\)
mm\(^2\)) was used to count Ki67-positive hepatocyte nuclei
in 10 successive fields.

Reverse-Transcriptase and Real-Time Polymerase
Chain Reaction. Total RNA was extracted from liver
tissue homogenates in TRIzol solution. The first-strand
complementary DNA (cDNA) was generated from 5 µg
of total RNA using the Moloney Murine Leukemia Virus
Reverse transcriptase (Invitrogen SARL) and pd(N)\(_6\)
primers (Amersham Biosciences, Orsay, France). Differ-
ent amounts of calibrated mRNAs (e.g., 1.25 pg to 1.25 ×
10\(^5\) pg) (Applied Biosystems, Applera France S.A.,
Courtaboeuf, France) were submitted to reverse transcription with the Moloney Murine Leukemia Virus Reverse transcriptase and pd(N)_6 primers and, subsequently, used in real-time polymerase chain reaction (PCR) for standardization of 18S transcripts. For standardization of target gene transcripts (TGF-α, EGFR, and vascular endothelial growth factor [VEGF]), a PCR product was amplified, sequenced, purified, and calibrated as a number of copies. The procedure used to generate VEGF-calibrated cDNA was reported in a previous study. To generate TGF-α and EGFR-calibrated cDNA, the primers were designed according to published rat cDNA sequences in the GenBank database within the respective calibrated PCR products, were previously defined for VEGF. For TGF-α, EGFR, and insulin-like growth factor 2 (IGF-2), the primers were designed according to published rat cDNA sequences in the GenBank database, using Primers Express software version 1.5 (Applied Biosystems): TGF-α forward 5'-TATGTATTAGGTGGATGACG-3', reverse 5'-GGGAAACAAAAAACAAG-3', generating a 500-bp fragment; EGFR forward 5'-GA-CACCTGCCCACCACTCAT-3'; Reverse 5'-CTC-CCTGCCCCCTGCTCACAT-3', generating a 799-bp fragment. The cDNA prepared from 200 ng RNA was added to 50 μL PCR buffer containing 150 μmol/L of deoxyribonucleoside triphosphate and 20 pmol of each forward and reverse primers. For real-time PCR, the primers, which were designed according to published rat cDNA sequences in the GenBank database using Primer Express software version 1.5 (PE Applied Biosystems): TGF-α forward 5'-TCAGTATCGGGCATCCATGTT-3' and reverse primer and 50 nmol/L of each 18S reverse transcriptase and pd(N)_6 primers and, subsequently, generating a 500-bp fragment; EGFR forward 5'-GA-CACCTGCCCACCACTCAT-3'; Reverse 5'-CTC-CCTGCTGGCTCACAT-3', generating a 799-bp fragment. The cDNA prepared from 200 ng RNA was added to 50 μL PCR buffer containing 150 μmol/L of deoxyribonucleoside triphosphate and 20 pmol of each forward and reverse primer. For real-time PCR, the primers, which were designed according to published rat cDNA sequences in the GenBank database within the respective calibrated PCR products, were previously defined for VEGF. Statistical Analysis. The Mann-Whitney U test was used to compare mean values between two groups; the Kruskall-Wallis test was used to compare mean values between more than two groups. The calculation of Spearman's rank correlation coefficient was used to assess the relationship between quantitative parameters. Data are expressed as the mean ± SEM. All reported P values are two-sided, and a P value less than .05 was considered statistically significant. Kendall's coefficient of concordance was calculated for evaluation of macroscopic tumor count between the two investigators.

**Results**

**Sequential Development of Cirrhosis and HCC in a Rat Model of DEN-Induced Liver Injury.** In all rats submitted to the present protocol of DEN administration (50 mg/kg/wk), cirrhosis developed after a short-term administration and HCC nodules arose from livers with cirrhosis after a long-term administration (Fig. 1). After the short-term administration of DEN (12 weeks plus a 2-week wash-out period), macronodular cirrhosis was evident at macroscopic examination of the liver and was confirmed by histological analyses (data not shown). No HCC nodule was detected either at macroscopic examination or at histological analysis at this stage. In contrast, after the long-term administration of DEN (16 weeks plus a 2-week wash-out period), malignant nodules that fulfilled the predefined criteria (a diameter ≥3 mm and a dysmorphic or dyschromic aspect) were detected on the surface of the liver (Fig. 2A). All malignant nodules were identified as HCC at histological examination (Fig. 2B-C). The surrounding peritumoral liver exhibited cirrho-
sis, and no additional tumor was detected in any case on full sections of the liver with cirrhosis either at macroscopic or histological examination. After the long-term administration of DEN, the mean number of malignant nodules detected at the surface of the liver was 18.1 ± 2.4.

**Deregulation in Cell Proliferation and in the TGF-α/EGFR Signaling Pathway in Rats With Cirrhosis.** After short-term DEN treatment, hepatocyte proliferation was markedly increased (approximately 14-fold) in the liver tissue of rats with cirrhosis compared with normal liver, as assessed by Ki67 immunolabeling (Fig. 3A). In keeping with a stimulation of proliferation, the mitogen-activated protein kinase pathway was activated. The levels of phospho-ERKs were markedly increased, while the levels of total ERKs were unchanged (Fig. 3B). This stimulation of cell proliferation coincided with an increase in the expression of TGF-α mRNA (Fig. 3C), while the expression of EGFR mRNA was lower (Fig. 3D).
that the TGF-β signaling pathway may contribute to increased proliferation of hepatocytes in the liver with cirrhosis and to the development of HCC in this setting (Fig. 3E). A decreased expression of EGFR in proliferating hepatocytes has been previously reported and attributed to ligand-mediated EGFR downregulation. TGF-β mRNA expression and hepatocyte proliferation were further increased in HCC nodules compared with the liver tissue exhibiting cirrhosis at 18 weeks, suggesting that the TGF-β/EGFR signaling pathway may contribute to increased proliferation of hepatocytes in the liver with cirrhosis and to the development of HCC in this setting (Fig. 3F).

**Antitumoral Effect of Gefitinib Treatment in Rats With Cirrhosis.** The above results led us to examine whether disruption of the TGF-β/EGFR signaling pathway could have a prophylactic effect on carcinogenesis in animals with cirrhosis. To test this possibility, we used gefitinib, a specific EGFR tyrosine kinase inhibitor, in these animals. Some of the rats under long-term DEN administration received daily intraperitoneal injections of gefitinib or vehicle for 6 weeks between weeks 12 and 18. In each group of animals (n = 8), HCC tumors were counted at the surface of the liver by two independent investigators according to predefined criteria (a diameter ≥3 mm and dysmorphic or dyschromic aspect). Data are expressed as the mean ± SEM. *P < .05.

than in normal liver tissue. Consistent with this latter result, total EGFR, at the protein level, was decreased in liver tissue exhibiting cirrhosis compared with normal liver (Fig. 3E). A decreased expression of EGFR in proliferating hepatocytes has been previously reported and attributed to ligand-mediated EGFR downregulation. TGF-β mRNA expression and hepatocyte proliferation were further increased in HCC nodules compared with the liver tissue exhibiting cirrhosis at 18 weeks, suggesting that the TGF-β/EGFR signaling pathway may contribute to increased proliferation of hepatocytes in the liver with cirrhosis and to the development of HCC in this setting (Fig. 3F).

To verify that the antitumoral effect of gefitinib was the result of a disruption in the EGFR-dependent signaling cascade, we examined the phosphorylation status of EGFR and ERKs in rats treated with gefitinib. Even though TGF-β mRNA expression was similar in livers with cirrhosis from gefitinib-treated and untreated animals (Fig. 5A), the amounts of both phospho-EGFR (Fig. 5B) and phospho-ERKs (Fig. 5C) were dramatically decreased in the liver tissues exhibiting cirrhosis from rats treated with gefitinib compared with those from untreated rats. These results confirmed the strong inhibitory effect of gefitinib treatment on EGFR tyrosine kinase activity and downstream mitogenic signals.

In the residual tumors that developed in rats despite gefitinib treatment, the level of phospho-EGFR was also dramatically decreased compared with tumors from untreated rats (Fig. 6A). However, the activation of ERKs was maintained (Fig. 6B), suggesting that the dose of gefitinib sufficient to suppress EGFR kinase activity may not be sufficient to completely inhibit downstream EGFR-dependent signaling pathways or that alternative proliferation and/or cell survival pathways might be activated in these tumors. The serine/threonine kinase Akt, a major component of survival pathways, was also activated in residual tumors from gefitinib-treated animals as in tumors from untreated animals (Fig. 6C). Residual tumors from gefitinib-treated animals contained lower levels of TGF-β mRNA than tumors from untreated animals (32.1 ± 5.3 vs. 96.4 ± 22.5 transcripts × 10^5/μg RNA) (Fig. 6D). This result suggested that gefitinib treatment might have an additional inhibitory effect on TGF-β mRNA expression in HCC nodules. Because EGFR activation also regulates the expression of VEGF,
a major angiogenic factor that may contribute to the progression of HCC, we examined in parallel the effect of gefitinib treatment on VEGF expression. Similarly, we found that VEGF mRNA levels were lower in tumors from gefitinib-treated animals compared with untreated animals (102.1 ± 15.3 vs. 164.6 ± 30.6 transcripts × 10^3/µg RNA) (Fig. 6E). Such inhibition in VEGF expression might also have contributed to the antitumoral effect of gefitinib in the present model. Finally, because IGF-2—a potent growth and survival factor for hepatocytes—has been implicated in liver carcinogenesis,25 we next evaluated its expression level in the model using quantitative reverse-transcriptase PCR. We found that IGF-2 mRNA expression was increased in HCC nodules compared with livers exhibiting cirrhosis with or without gefitinib treatment (Fig. 6F). Altogether, these results suggested that IGF-2 overexpression together with persistent activation of ERKs and Akt signaling pathways may contribute to the formation of residual tumors in animals with cirrhosis under EGFR inhibitory treatment.

**Discussion**

The protocol of DEN-induced liver injury used in the present study caused the sequential formation of cirrhosis and HCC. In this model, we show that TGF-α mRNA expression increases in the liver with cirrhosis compared with normal liver and even further in HCC nodules compared with the surrounding liver with cirrhosis. This increase in TGF-α expression coincides with a parallel increase in hepatocellular proliferation. Consistent with a causal relationship between the activation of the TGF-α/EGFR pathway and the emergence of HCC, treatment of the animals at the stage of cirrhosis with the EGFR inhibitor gefitinib caused a dramatic reduction in the number of HCC nodules arising from the liver with cirrhosis. Of particular interest with respect to therapeutic perspectives, this prophylactic effect was obtained without liver toxicity.

Different models of DEN-induced liver carcinogenesis have been described previously.26-28 However, in contrast to previous models, the present model reproduces the sequence of cirrhosis and of HCC—as is most often the case in human liver disease—and thus provides a unique tool to test preventive treatments of HCC. The upregulation of TGF-α expression that occurs in this model may result both from the effect of cytokines produced by inflammatory cells and from the local regenerative response to cell loss in the cirrhotic liver.29 TGF-α, which is produced mainly by hepatocytes,10 acts both as a paracrine and autocrine factor, induces its own expression,30 stimulates hepatocyte proliferation, and may cause the onset of liver tumors at least partly through activation of the ERK signaling pathway. In keeping with these data, we observed a decrease in the phosphorylation levels of both EGFR and ERKs in liver tissues with cirrhosis from animals under gefitinib. This inhibitory effect was associated with a decrease in HCC occurrence in gefitinib-treated animals. Other mechanisms might also be involved in the prophylactic effect of gefitinib such as an inhibition of angiogenesis. Indeed, TGF-α is also a mitogen for endo-
thelial cells and the blockade of the EGFR signaling pathway with gefitinib leads to apoptosis in endothelial cells and almost completely inhibits angiogenesis. Therefore, the antitumoral effect of gefitinib might be partly mediated by an inhibition of angiogenesis. Consistent with this possibility, we observed that gefitinib treatment induced a decrease in VEGF mRNA expression in tumors.

Gefitinib-induced EGFR inhibition, although effective in HCC nodules and surrounding tissues exhibiting cirrhosis, did not completely prevent the onset of HCC in our experimental conditions. In residual tumors from gefitinib-treated animals, ERKs and Akt were phosphorylated similar to tumors from untreated animals. Possible resistance of ERKs and Akt signaling pathways to EGFR kinase inhibitors has been previously reported. Further activation of these pathways by other growth factors such as IGF-2 could explain the emergence of residual tumors. Deregulation in insulin and IGF signaling pathways including re-expression of fetal IGF-2 mRNA, which may contribute to hepatocarcinogenesis, have been reported in human and murine HCC. The possibility that IGF-2 overexpression contributed to the formation of gefitinib-resistant tumors is supported by recent in vitro findings showing that IGF-dependent signaling may antagonize the growth inhibitory effect of trastuzumab (Herceptin), an anti-c-erbB2 receptor monoclonal antibody, in human breast cancer cell lines. Other mechanisms might also be implicated in gefitinib-acquired resistance. For example, the loss of function of the phosphatase and tensin homolog phosphatase has been recently reported to counteract the antitumor action of EGFR tyrosine inhibitors in vitro. Other members of the erb-B receptor family, such as c-erbB3 and c-erbB2—which are expressed in 84% and 21% of human HCC, respectively—might also contribute to liver carcinogenesis. The effect of gefitinib on these receptors is poorly known. However, c-erbB2 does not appear to be involved in gefitinib resistance, because its association with Herceptin did not improve the antitumoral effect of gefitinib in our model (data not shown).

In conclusion, the present study provides a demonstration that a specific inhibitor of EGFR reduces the onset of HCC tumors in animals with cirrhosis. Further studies are now required to determine the optimal conditions for gefitinib alone or in combination with other drugs to completely overcome resistance and inhibit liver carcinogenesis upon cirrhosis. Altogether, our results suggest that inhibition of the TGF-α/EGFR loop with gefitinib could prevent or at least delay the emergence of HCC in patients with cirrhosis.

References


Fluorescence Resonance Energy Transfer Analysis of Proapoptotic CD95–EGF Receptor Interactions in Huh7 Cells

Andrea Eberle, Roland Reinehr, Stephan Becker, and Dieter Häussinger

Hyperosmolarity- and CD95 ligand (CD95L)-induced interactions between CD95 (Fas/APO-1) and the epidermal growth factor receptor (EGFR) involve EGFR-catalyzed CD95 tyrosine phosphorylation. Such interactions were studied by means of fluorescence resonance energy transfer (FRET) and CD95 receptor mutagenesis in Huh7 hepatoma cells. In cells cotransfected with EGFR–cyan fluorescent protein and CD95–yellow fluorescent protein, FRET studies showed a rapid, hyperosmolarity-induced, c-Jun-N-terminal kinase–dependent CD95–EGFR association in the cytosol with subsequent microtubule-dependent translocation of the protein complex to the plasma membrane. Inhibition of EGFR tyrosine kinase activity by AG1478 and cyclic adenosine monophosphate had no effect on hyperosmotic CD95–EGFR association in the cytosol but prevented CD95 tyrosine phosphorylation, targeting of the protein complex to the plasma membrane, and formation of the death-inducing signaling complex (DISC). The requirement of EGFR-mediated CD95 tyrosine phosphorylation for hyperosmotic and CD95L-induced CD95 membrane targeting and DISC formation was also shown in CD95 mutagenesis experiments. CD95 mutants with tyrosine–phenylalanine exchanges at positions 232 and 291 failed to translocate to the plasma membrane and to recruit Fas-associated death domain and caspase 8, although these mutants still associated with the EGFR in the cytosol in response to hyperosmolarity and CD95L. Cells transfected with these mutants were also resistant to CD95L-induced apoptosis. Single mutations of tyrosine 91, 232, and 291 failed to inhibit CD95 membrane targeting, DISC formation, or CD95L-induced apoptosis. In conclusion, we identify EGFR–CD95 interaction and phosphorylation of critical CD95 tyrosine residues as important early events in hyperosmotic and CD95L-induced CD95 activation and apoptosis induction. Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html). (HEPATOLOGY 2005;41:315-326.)

Activation of the CD95 system (also known as Fas/APO-1) and subsequent apoptosis induction play an important role in the pathogenesis of liver injury induced by the CD95 ligand (CD95L), hydrophobic bile acids, or ethanol.\(^1\)\(^{--}\)\(^1\)\(^1\)\(^1\) In addition, hyperosmotic hepatocyte shrinkage triggers translocation of CD95 to the plasma membrane and formation of the death-inducing signaling complex (DISC) and sensitizes hepatocytes to CD95L-induced apoptosis.\(^1\)\(^4\) Such phenomena may contribute to liver damage in response to dehydration, hypernatremia, and drugs such as monoamines that induce hepatocellular K\(^+\) release and shrinkage.\(^1\)\(^2\) CD95 activation is a complex process that involves ligand-independent activation of the epidermal growth factor receptor (EGFR) through the Src family kinase Yes, followed by a c-Jun-N-terminal kinase (JNK)-dependent EGFR–CD95 association and EGFR-catalyzed CD95 tyrosine phosphorylation, which triggers CD95 membrane translocation and DISC formation.\(^4\)\(^3\)\(^\text{13-15}\) Thus, the EGFR plays a decisive role in CD95-dependent hepatocyte apoptosis. On the other hand, EGFR activation is also involved in cell proliferation and carcinogenesis in a variety of tissues. These dual roles played by the EGFR are important for the understanding of cell proliferation and cell death.
To obtain further insight into EGFR–CD95 interactions in response to hyperosmolarity and CD95L, the fluorescence resonance energy transfer (FRET) technique was employed. FRET offers a powerful tool for investigating protein–protein interactions in the intact cell. It occurs when two proteins that are coupled to corresponding fluorescent proteins—yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP)—interact and thereby get as close as 3 to 10 nm. Under these conditions, the light emitted upon excitation of the CFP moiety will excite the YFP moiety and yield the so-called “FRET signal.”

The present data show that within 30 minutes, hyperosmolarity and CD95L induce a JNK-dependent stable EGFR–CD95 association in the cytosol with subsequent EGFR-catalyzed CD95 tyrosine phosphorylation and a microtubule-dependent targeting of this protein complex to the plasma membrane within 2 hours. Inhibition of EGFR tyrosine kinase activity by AG1478 prevents CD95 tyrosine phosphorylation, trafficking to the plasma membrane, and DISC formation but not the formation of the CD95–EGFR complex in the cytosol. Wild-type CD95 contains three tyrosines at amino acid positions 91, 232, and 291, with tyrosines 232 and 291 being located in the so-called “death domain” of the receptor. CD95 mutagenesis experiments revealed that tyrosine residues 232 and 291 are essential for CD95–EGFR trafficking to the plasma membrane, DISC formation, and apoptosis but not for EGFR–CD95 association in the cytosol. The data are relevant for the understanding of CD95-mediated apoptosis in the liver, potential antiapoptotic strategies, and the understanding of the dual roles played by the EGFR in cell proliferation and death.

Materials and Methods

Materials. The following materials were used: Dulbecco’s Modified Eagle Medium and fetal calf serum from Gibco Life Technologies (Gaithersburg, MD); soluble CD95L (employed with enhancer protein as provided by Prof. Arndt-Jovin, Ph.D.21) by CFP of pECFP-N1 (Clontech); the EGFR-CFP construct was obtained by replacing GFP of F7 erb B1-EGFP (kindly provided by Prof. Arndt-Jovin, Ph.D.21) by GFP of F7 erb B1-EGFP (kindly provided by Prof. Arndt-Jovin, Ph.D.21) by CFP of pECFP-N1 (Clontech). The CD95-YFP construct was used as a template for polymerase chain reaction–based site-directed mutagenesis according to standard procedures. All constructs and mutants were confirmed with sequencing (MWG Biotech, Ebersberg, Germany).

Cell Culture and Transfection. Huh7 cells were cultured in Dulbecco’s Modified Eagle Medium/nut. mix F12 supplemented with 10% fetal calf serum and 1% penicillin/streptomycin resulting in a final osmolarity of 305 mosmol/L as measured with a cryoscopic osmometer (Osmomat 030; Gonotec, Berlin, Germany). Cells were grown to 70% confluency before transient transfection using expression vectors for CFP and YFP fusion proteins in equal amounts supplemented with Lipofectamine 2000 (Invitrogen). Confocal Fluorescence Microscopy and Fluorescence Resonance Energy Transfer Technique. Confocal pictures were taken using the LSM 510 META laser scanning microscope (Zeiss, Oberkochen, Germany). All YFP/CFP cotransfections were detected using a META scan avoiding bleed-through of CFP in the YFP channel. CFP was excited with 405 nm, YFP with 514 nm.22 Membrane translocation was detected in living cells plated on glass-bottom dishes 24 hours after transfection. At least 100 cells from three independent experiments were counted. To detect FRET, after the respective experiment cells were first fixed using Zamboni’s fixative applied for 15 minutes at room temperature. Cells were washed twice with phosphate-buffered saline (PBS) and then mounted with vectashield (Vector Laboratories, Burlingame, CA). FRET efficiency was determined using LSM Image Examiner 3.1 software (Zeiss); FRET pictures were normalized for the FRET efficiencies in the respective setting as indicated by the accompanying scale (FRET efficiency is given from blue [0] to red [255]).
Western Blot Analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described previously.4,14

CD95–YFP Immunoprecipitation. Huh7 cells were cultured on culture plates (10 cm in diameter; Falcon, Heidelberg, Germany) and harvested, and samples were taken as described previously.4,14 Equal protein amounts (500 μg) of the respective samples were taken, added to an identical volume of lysis buffer, and incubated for 2 hours at 4°C with an anti-GFP antibody (dilution 1:100) to immunoprecipitate CD95–YFP. Then 10 μL of protein A–agarose and 10 μL of protein G–agarose (Santa Cruz Biotechnology) were added and incubated at 4°C overnight. Immunoprecipitates were washed three times and then transferred to Western blot analysis.4,14

Immunocytochemistry. Huh7 cells were cultured on glass coverslips. After the respective incubation, cells were washed briefly with PBS, fixated in methanol (−20°C for 2 minutes), then permeabilized using Triton-X100 (0.1% v/v in PBS) for 10 minutes at room temperature. Cells were exposed to an anti-EGFR antibody (1:250 in PBS; Upstate Biotechnology, Lake Placid, NY) applied for 1 hour at 4°C, washed off, then stained with a secondary anti–rabbit Cy3–conjugated antibody for 1 hour at 4°C (1:500 in PBS). Cells were visualized using an LSM NT confocal laser scanning microscope (Leica, Heidelberg, Germany). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of fluorescein isothiocyanate–conjugated deoxyuridine-triphosphate technique was performed as described previously.4,14

Statistics. Results from at least three independent experiments are expressed as the mean ± SEM (n = number of independent experiments). Results were analyzed using the Student t test; a P value of less than .05 was considered statistically significant.

Results

Transfection and Functional Characterization of the CD95–YFP and EGFR–CFP Constructs. Huh7 hepatoma cells were chosen as an experimental model because of their very low endogenous CD95 expression23 compared with rat hepatocytes (Fig. 1). EGFR-CFP and CD95–YFP constructs were transfected into Huh7 cells with high efficacy, as demonstrated by Western blot analysis (see Fig. 1). Approximately 42% ± 6% (n = 3) of the cells expressed the transfected EGFR-CFP and CD95–YFP, respectively. Similar transfection rates were found for the different CD95–YFP mutants with tyrosine/phenylalanine exchanges in positions 91, 232, and/or 291 (see Fig. 1).

Fig. 1. CD95–YFP and EGFR–CFP transfection of Huh7 hepatoma cells. Huh7 cells were transfected with YFP, wild-type CD95–YFP, mutated CD95 F/Y, or EGFR–CFP construct. Expression of CD95 (anti-CD95), EGFR (anti-EGFR), and the respective fusion proteins (anti-GFP cross-reacts with CFP and YFP) were determined via Western blotting. Primary 24-hour cultured rat hepatocytes and untransfected Huh7 cells served as controls. In all cells investigated, expression of EGFR (∼170 kd) was detectable, while Huh7 cells did not express endogenous CD95 (∼48 kd).23 In cells transfected with a CD95–YFP construct, the respective fusion protein was detectable at approximately 74 kd by using either an anti-CD95 or an anti-GFP antibody. Furthermore, in cells transfected with the EGFR–CFP construct, anti-EGFR and anti-GFP antibodies detected the fusion protein at approximately 196 kd. YFP, yellow fluorescent protein; EGFR, epidermal growth factor receptor; CFP, cyan fluorescent protein; W.b., Western blot.

Whereas untransfected or only YFP-transfected Huh7 cells were resistant against CD95L-induced apoptosis, cells transfected with wild-type CD95–YFP underwent apoptosis upon stimulation with CD95L (Fig. 2A). Twelve hours after addition of CD95L (100 ng/mL) the number of TUNEL-positive, CD95–YFP transfected Huh7 cells was 42% ± 5% (n = 3) compared with only 3.0% ± 1.3% (n = 3) of cells that were transfected with YFP only. In unstimulated cells, CD95–YFP was localized in the cytosol but was targeted within 2 hours to the plasma membrane upon stimulation with CD95L (Fig. 2B). These findings indicate that the wild-type CD95–YFP construct was functionally active in Huh7 cells. Furthermore, the transfected EGFR–CFP construct was functionally active. As shown in Fig. 3, addition of EGF–induced tyrosine phosphorylation not only of the endogenous EGFR, but also of the transfected EGFR–CFP construct.
Hyperosmotic Activation of the EGFR and CD95 in Huh7 Cells. As shown recently via Western blotting in rat hepatocytes, hyperosmotic exposure leads to a rapid ligand-independent but Yes-dependent activation of the EGFR, followed by EGFR–CD95 association, CD95 tyrosine phosphorylation, membrane translocation, and DISC formation.4,13 These results were also observed in Huh7 cells, which were transfected with the EGFR-CFP or CD95-YFP construct. In EGFR-CFP-transfected cells, the transfected EGFR-CFP construct was tyrosine-phosphorylated 1 minute after hyperosmotic exposure, indicating EGFR-CFP activation (see Fig. 3). This was also found for the endogenous EGFR in untransfected Huh7 cells (see Fig. 3). Similar to rat hepatocytes,13 hyperosmotic activation of both the endogenous EGFR and the transfected EGFR-CFP construct in Huh7 cells was sensitive to inhibition by cyclic adenosine monophosphate (cAMP) in an H89-sensitive manner but was insensitive to JNK inhibition (see Fig. 3). Inhibition of EGFR tyrosine kinase activity by AG1478 did not abolish overall EGFR tyrosine phosphorylation (see Fig. 3), but affected site-specific tyrosine phosphorylation, as described recently for bile salt–induced, Yes-dependent EGFR activation in hepatocytes.15 As shown in Supplementary Fig. 1, hyperosmolarity induced phosphorylation of EGFR-Y845, a known target of Src kinases24 and EGFR-Y1173, a autophosphorylation site; this is indicative of EGFR kinase activation.25 Whereas hyperosmotic-induced EGFR-Y845 phosphorylation was maximal within 1 minute, the maximum of EGFR-Y1173 phosphorylation was somewhat delayed (see Supplementary Fig. 1).

Fig. 2. CD95L-induced apoptosis in CD95-YFP transfected Huh7 cells. (A) Huh7 cells were transfected with CD95-YFP (a) or YFP only (g). Untransfected Huh7 cells served as controls (c). After 12-hour exposure to CD95L, the number of apoptotic cells was determined via TUNEL. Data represent the percentage of control, CD95-YFP, or YFP-transfected cells with positive TUNEL staining (n = 3). Transfection of CD95-YFP transfers sensitivity to CD95L-induced apoptosis. (B) Huh7 cells were transfected with CD95-YFP and exposed to normosmotic medium (305 mosmol/L; left panel) or CD95L (30 mosmol/L; right panel) for 2 hours. In control cells, CD95L is localized inside the cell, while CD95L triggers translocation of CD95-YFP to the plasma membrane. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; YFP, yellow fluorescent protein.

Fig. 3. Hyperosmolarity-induced EGFR activation in Huh7 cells. Untransfected and EGFR-CFP-transfected Huh7 cells were exposed for 1 minute to normosmotic medium (control, 305 mosmol/L), hyperosmotic medium (405 mosmol/L), or EGF (50 ng/mL, 305 mosmol/L). When indicated, cAMP (100 µmol/L), H89 (5 µmol/L), L-JNKI-1 (5 µmol/L), and AG1478 (5 µmol/L) were preincubated for 30 minutes and colchicine (5 µmol/L) and taxol (10 µmol/L) were preincubated for 60 minutes. Endogenous EGFR (upper panel) or transfected EGFR-CFP (lower panel) were immunoprecipitated, and EGFR tyrosine phosphorylation was detected via immunoblotting. Hyperosmolarity and EGF activated both the endogenous EGFR and the transfected EGFR-CFP within 1 minute. Hyperosmotic EGFR-EGFR-CFP activation was insensitive to inhibition of JNK or EGFR tyrosine kinase activity. Furthermore, disruption of microtubules by colchicine or taxol did not affect EGFR phosphorylation, whereas cAMP inhibited EGFR phosphorylation in a protein kinase A–dependent way. cAMP, cyclic adenosine monophosphate; JNK, c-Jun-N-terminal kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; P-Tyr, tyrosine phosphorylation; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; I.P., immunoprecipitation; W.B., Western blot.

Hyperosmolarity-induced EGFR phosphorylation at both sites was inhibited by the Src kinase inhibitor SU6656 (see Supplementary Fig. 1), whereas AG1478 had no effect on EGFR-Y845 phosphorylation but prevented EGFR-Y1173 phosphorylation. This suggests that EGFR-Y845 phosphorylation by the Src kinase Yes13,15 triggers activating autophosphorylation at Y1173. Little or no phosphorylation of EGFR-Y1045, which mediates recruitment of the adapter peptide c-Cbl for EGFR-internalization,20 was found in response to hyperosmolarity.
In CD95-YFP-transfected Huh7 cells, a JNK inhibitor–sensitive EGFR–CD95-YFP association and CD95-YFP–tyrosine phosphorylation was found 30 minutes after hyperosmotic exposure (Fig. 4). A JNK requirement for EGFR–CD95 association was shown recently in rat hepatocytes. CD95-YFP–tyrosine phosphorylation but not EGFR–CD95-YFP association was also inhibited by AG1478 and cAMP, as was reported recently for rat hepatocytes. All maneuvers preventing hyperosmotic CD95-YFP–tyrosine phosphorylation also prevented DISC formation, which was otherwise observed after 2 hours. As further shown in Fig. 4, addition of CD95L to CD95–YFP-transfected Huh7 cells also induced EGFR–CD95-YFP association, CD95-YFP–tyrosine phosphorylation, and DISC formation. Thus, hyperosmotic or CD95L-induced activation of the CD95 system in transfected Huh7 cells is not significantly different from that reported for rat hepatocytes.

In Vivo Demonstration of Hyperosmotic and CD95L-Induced EGFR-CFP–CD95-YFP Interactions and Membrane Translocation in Huh7 Cells Cotransfected With EGFR-CFP and CD95-YFP Constructs. The in vivo interaction of both fluorescent proteins in response to hyperosmolarity (405 mosmol/L) or CD95L (50 ng/mL) was studied by means of FRET. Such FRET signals are obtained only when EGFR-CFP and CD95-YFP associate and the CFP/YFP moieties come closer than 10 nm. As shown in Fig. 5A, no FRET signal was detectable in resting cells; however, hyperosmotic exposure generated an intracellular FRET signal within 30 minutes, indicating that an association of CD95-YFP with EGFR-CFP occurs in the cytosol. After 120 minutes, both EGFR-CFP and CD95-YFP were translocated to the plasma membrane, where the FRET signal and thus the EGFR–CD95 protein complex was still detectable (see Fig. 5A). No bleb formation was observed even after 6 hours of hyperosmotic exposure; this is in agreement with the previous finding that hyperosmolarity induces CD95 activation and DISC formation and sensitizes hepatocytes to CD95L-induced apoptosis but does not by itself execute cell death. The hyperosmotic EGFR targeting to the plasma membrane was also shown for the endogenous EGFR in Huh7 cells that were transfected with CD95-YFP (Fig. 6). However, in resting cells endogenous EGFR was localized in both the plasma membrane and the cytosol, and hyperosmotic exposure led to EGFR targeting to the plasma membrane within 2 hours, as it was observed for the transfected EGFR-CFP construct (compare Figs. 5A, 6). These hyperosmolarity effects strongly differed from those induced by EGF, which, in accord with the literature, results in EGFR internalization (see Fig. 6).

As shown by FRET, CD95L (50 ng/mL) also triggered an EGFR-CFP–CD95-YFP association in the cytosol within 30 minutes and triggered trafficking of the EGFR-CFP–CD95-YFP complex to the plasma membrane within 2 hours (Fig. 5B). However, in contrast to hyperosmotic exposure, stimulation with CD95L resulted after 6 hours in membrane bleb formation, indicating apoptosis (see Fig. 5B). Interestingly, FRET signals were also obtained in the bleb membranes, indicating that the...
EGFR–CD95 complex was contained in membrane blebs. These findings demonstrate that the hyperosmotic or CD95L-induced EGFR–CD95 complex forms in the cytosol and is subsequently targeted to the plasma membrane. The EGFR–CD95 association is apparently fairly stable, because it is observed even at time points after DISC formation (compare Figs. 4, 5). However, membrane targeting of the EGFR–CD95 complex and DISC formation may not inevitably result in cell death and may have the potential of reversibility, because hyperosmolarity alone does not execute apoptosis and leads to CD95 reinternalization after several hours. This reversibility is also reflected by the time-dependent decrease of cells with a membrane-associated FRET signal, which was 17.4% ± 2.4%, 10.5% ± 2.4%, and 6.3% ± 1.0% after 2, 4, and 6 hours of hyperosmotic exposure, respectively. These data indicate that the CD95–EGFR complex may have potential for reinternalization once it is targeted to the plasma membrane.

Hyperosmolarity (Fig. 7) and CD95L (Supplementary Fig. 2) induced no FRET signal in the presence of the JNK-inhibitory peptide L-JNKI-1, indicating that a JNK signal, which is triggered by hyperosmolarity, is required for CD95–EGFR association and membrane translocation. Neither AG1478 (see Fig. 7), an inhibitor of EGFR tyrosine kinase activity, nor cAMP (not shown) had any effect on hyperosmotic EGFR–CD95 association in the cytosol; however, membrane translocation of the protein complex was prevented. Because AG1478 prevents hyperosmotic (see Fig. 4) and CD95L-induced CD95 tyrosine

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Fig. 5. FRET analysis of hyperosmotic and CD95L-induced EGFR-CFP–CD95-YFP association. Huh7 cells were cotransfected with EGFR-CFP and CD95-YFP, then exposed to (A) hyperosmotic medium (405 mosmol/L) or (B) CD95L (50 ng/mL) for the periods indicated. FRET pictures were normalized with respect to FRET efficiencies (scale indicates FRET efficiency from blue [0] to red [255]). (A) Hyperosmolarity induced an intracellular EGFR-CFP–CD95-YFP association within 30 minutes as indicated by the FRET signal. After 120 minutes, translocation of the EGFR-CFP–CD95-YFP complex to the plasma membrane was observed. (B) CD95L induced an intracellular EGFR-CFP–CD95-YFP association within 30 minutes and translocation of the EGFR-CFP–CD95-YFP complex to the plasma membrane within 120 minutes. Within 6 hours, apoptosis occurred as indicated by bleb formation, and the EGFR-CFP–CD95-YFP complex was still detectable in the membrane blebs. EGFR, epidermal growth factor receptor; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein.
phosphorylation due to inhibition of EGFR tyrosine kinase activity, these findings underline the important role of CD95 tyrosine phosphorylation for CD95 membrane translocation and DISC formation, in line with recent data.

Role of Microtubules in Hyperosmotic and CD95L-Induced CD95 Activation. Pretreatment of Huh7 cells for 60 minutes with colchicine or taxol—but not with the inactive lumicolchicine—led to a disruption of microtubules (Supplementary Fig. 3). As shown in Fig. 3, disruption of microtubules had no effect on hyperosmotic EGFR activation, EGFR–CD95-YFP association, or CD95-YFP–tyrosine phosphorylation but prevented DISC formation (i.e., recruitment of FADD and caspase 8 to CD95-YFP) (see Fig. 3). As shown using FRET, hyperosmotic EGFR-CFP–CD95-YFP association still occurred in the cytosol in colchicine-pretreated cells; however, trafficking of the protein complex to the plasma membrane was prevented (Fig. 8). As for control, lumicolchicine had no effect on hyperosmotic EGFR-CFP–CD95-YFP association or membrane translocation (see Fig. 8). Similar findings were obtained after disruption of microtubules by taxol (see Fig. 8); however, in taxol-treated cells, the hyperosmolality-induced intracellular FRET signal was weaker and more spotted (see Fig. 8). This result may relate to the different modes of microtubule destruction by taxol and colchicine, respectively. Whereas colchicine inhibits tubulin polymerization, taxol induces bundling of microtubules (see Supplementary Fig. 3), and the EGFR-CFP–CD95-YFP complex may still bind to microtubular fragments.

Inhibition of membrane translocation of the EGFR–CD95 complex by microtubule disruption was also observed when CD95L was used as an apoptotic stimulus (Supplementary Fig. 4) and under these conditions apoptosis was inhibited (Table 1). These data indicate that microtubules are involved in the transport of the EGFR–CD95 complex to the plasma membrane, but not in the hyperosmolality-induced formation of the EGFR–CD95 complex. Interestingly, disruption of microtubules had no effect on CD95 tyrosine phosphorylation but prevented DISC formation (see Fig. 4). This suggests that in

Fig. 6. EGFR distribution in Huh7 hepatoma cells. CD95-YFP–transfected Huh7 cells were immuno-cytochemically stained for EGFR as described in Experimental Procedures to visualize hyperosmotic or EGF-induced EGFR translocations. In unstimulated cells, endogenous EGFR is found in the plasma membrane and the cytosol. Whereas hyperosmotic exposure (405 mosmol/L, upper panels) induces EGFR translocation to the plasma membrane within 30 to 60 minutes, addition of EGF (50 ng/mL, lower panels) induces EGFR internalization. EGF, epidermal growth factor.

Fig. 7. FRET analysis of the hyperosmotic-induced EGFR-CFP–CD95-YFP interaction in the presence of AG1478 and the JNK inhibitor. Huh7 cells were cotransfected with EGFR-CFP and CD95-YFP. FRET pictures were normalized with respect to FRET efficiencies (see Fig. 5). Inhibition of EGFR tyrosine kinase activity by AG1478 (5 μmol/L, preincubated for 30 minutes) did not affect the hyperosmotic (405 mosmol/L) EGFR-CFP–CD95-YFP association but prevented the translocation of the EGFR-CFP–CD95-YFP complex to the plasma membrane, which is otherwise observed within 120 minutes (compare with Fig. 5A). Inhibition of JNK by L-JNKI-1 (5 μmol/L, preincubated for 30 minutes) abolished the hyperosmotic (405 mosmol/L) EGFR-CFP–CD95-YFP association and targeting of EGFR-CFP and CD95-YFP to the plasma membrane. Similar findings were obtained for CD95L-induced EGFR-CFP–CD95-YFP interaction (see Supplementary Fig. 2). EGFR, epidermal growth factor receptor; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein; JNK, c-Jun-N-terminal kinase.
addition to CD95 tyrosine phosphorylation, membrane targeting of the CD95–EGFR complex and/or intact microtubules are prerequisites for DISC formation.

**CD95 Mutagenesis: Role of CD95 Tyrosine Residues.** CD95 tyrosine phosphorylation was recently shown to be essential for CD95 membrane targeting and

**Table 1. CD95L-Induced Apoptosis in CD95mut-YFP-Transfected Huh7 Hepatoma Cells**

<table>
<thead>
<tr>
<th>Huh7 Hepatoma Cells</th>
<th>Cells With Positive TUNEL Staining (%)</th>
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<tr>
<td>YFP</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>CD95wt-YFP</td>
<td>42.1 ± 4.6*</td>
</tr>
<tr>
<td>+ Colchicine</td>
<td>4.6 ± 1.0*</td>
</tr>
<tr>
<td>+ Lumicolchicine</td>
<td>37.1 ± 3.0†</td>
</tr>
<tr>
<td>+ Taxol</td>
<td>12.2 ± 1.6*</td>
</tr>
<tr>
<td>CD95Y91F-YFP</td>
<td>40.3 ± 3.0†</td>
</tr>
<tr>
<td>CD95Y232F-YFP</td>
<td>33.5 ± 6.3†</td>
</tr>
<tr>
<td>CD95Y291F-YFP</td>
<td>34.1 ± 5.3†</td>
</tr>
<tr>
<td>CD95Y91,232F-YFP</td>
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<tr>
<td>CD95Y91,291F-YFP</td>
<td>33.2 ± 5.1†</td>
</tr>
<tr>
<td>CD95Y232,291F-YFP</td>
<td>6.7 ± 0.9*</td>
</tr>
<tr>
<td>CD95Y91,232,291F-YFP</td>
<td>5.2 ± 1.0*</td>
</tr>
</tbody>
</table>

**NOTE.** Huh7 cells were transfected with YFP only, the wild-type CD95-YFP fusion protein (CD95wt-YFP), or CD95-YFP constructs with single tyrosine—phenylalanine exchange in positions 91, 232, and 291 and combinations thereof. When indicated, cells were preincubated for 60 minutes with colchicine (5 μmol/L) or taxol (10 μmol/L). Lumicolchicine (5 μmol/L, 60 minutes), a colchicine analog which does not affect microtubules, served as control. Cells were exposed for 12 hours to CD95L (100 ng/mL), and the number of apoptotic cells was detected using TUNEL and determined as the percentage of cells with positive TUNEL staining successfully transfected with CD95wt-YFP. Transfection of the CD95Y91F-YFP receptor significantly increased the number of apoptotic Huh7 cells after 12 hours of CD95L exposure compared with transfection of YFP alone (see also Fig. 2A). Apoptosis was significantly inhibited after pretreatment with colchicine and taxol, which suggests that microtubules are involved in CD95L-induced apoptosis. Single tyrosine—phenylalanine exchanges in positions 91, 232, or 291 of CD95mut-YFP or CD95Y91,232F-YFP and CD95Y91,291F-YFP did not significantly affect CD95L-induced apoptosis. However, mutations of tyrosine residues within the death domain (CD95Y232,291F-YFP, CD95Y91,232,291F-YFP) showed resistance to CD95L-induced apoptosis. *P < .05. †P value not significant.

**Fig. 8.** Disruption of microtubules has no effect on hyperosmotic-induced CD95–EGFR association but inhibits translocation of the EGFR-CFP–CD95-YFP complex to the plasma membrane. Huh7 cells were cotransfected with EGFR-CFP and CD95-YFP. FRET pictures were normalized with respect to FRET efficiencies (see Fig. 5). If indicated, cells were pretreated for 60 minutes with colchicine (5 μmol/L), taxol (10 μmol/L), or lumicolchicine (5 μmol/L, control). Cells were exposed to hyperosmolarity (405 mosmol/L) for the periods indicated. Disruption of microtubules did not prevent CD95–EGFR association, but it did inhibit translocation of the EGFR–CD95 complex to the plasma membrane. EGFR, epidermal growth factor receptor; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein.
EGFR-CFP and CD95mut-YFP indicated that all CD95-YFP mutants associated with EGFR-CFP in the cytosol within 30 minutes in response to hyperosmolarity or CD95L (not shown; for the CD95Y91F mutant, see Fig. 10), as did the wild-type CD95-YFP (see Fig. 5A). However, when CD95 Y232F/Y291F (see Fig. 10) or CD95 Y91F/Y232F/Y291F mutated receptors (not shown) were transfected, no CD95mut-YFP membrane translocation was observed after 2 hours of hyperosmotic exposure (see Figs. 9, 10), as it otherwise occurred with the wild-type CD95-YFP (see Fig. 5A). These mutants also failed to target to the plasma membrane in response to CD95L (see Fig. 10). As shown in coimmunoprecipitation studies with CD95mut-YFP transfected cells (Fig. 11), tyrosine phosphorylation was strongly diminished with the CD95Y232F,Y291F mutant and was fully abolished with the triple mutant CD95Y91F/Y232F/Y291F. Both mutants also failed to associate with FADD and caspase 8 in response to hyperosmolarity (see Fig. 11) and CD95L (Supplementary Fig. 5). However, their association with the EGFR was apparently not impaired, as shown by both coimmunoprecipitation (see Fig. 11) in CD95mut-YFP single-transfected cells and FRET experiments in cells that were cotransfected with EGFR-CFP and CD95 Y232F/Y291F-YFP (see Fig. 10). Similar findings were obtained when CD95L was added as a proapoptotic stimulus (see Fig. 10, Supplementary Fig. 5).

**Significance of CD95 Mutations for Apoptosis Induction.** As shown recently, hyperosmotic CD95 activation in rat hepatocytes sensitizes these cells to CD95L-induced apoptosis, but it does not execute apoptosis by itself. Therefore, CD95L was used to investigate the
role of CD95 tyrosine mutations for apoptosis induction.

As shown in Table 1, cells transfected with the CD95Y232F/Y291F-YFP or CD95Y91F/Y232F/Y291F-YFP mutants but not with the CD95 Y91F-, CD95 Y232F-, CD95 Y291F-, CD95 Y91F/Y232F-YFP; CD95 Y91F/Y291F-YFP mutant; or wild-type CD95-YFP were resistant to CD95L-induced apoptosis.

**Discussion**

Hyperosmotic hepatocyte shrinkage was recently shown to activate CD95 and to sensitize hepatocytes to apoptosis. This process involves a Yes-dependent activation of the EGFR and its association with the CD95 followed by an EGFR-catalyzed CD95 tyrosine phosphorylation. The present study employed the FRET technique to characterize these events in the living cell. Hyperosmotic EGFR–CD95 association was dependent upon JNK activation, in line with recent data, and the identification of the EGFR as a target of JNK-induced serine/threonine phosphorylation. CAMP prevented hyperosmotic EGFR activation but not EGFR–CD95 association in a H89-sensitive way, which suggests involvement of protein kinase A. One consequence of EGFR–CD95 interaction is a rapid CD95 tyrosine phosphorylation, which is probably achieved through EGFR tyrosine kinase activity in view of the AG1478 sensitivity of the process. As shown in the present FRET study, this EGFR–CD95 association occurs already in the cytosol. The protein complex is thereafter targeted to the plasma membrane, where DISC formation occurs. Interestingly, the EGFR–CD95 association in response to hyperosmolarity or CD95L appears to be fairly tight, and the kinetics of EGFR movements in response to hyperosmolarity differ strongly from those induced by EGF. Whereas EGF induces a rapid EGFR internalization, which may be accompanied by still ongoing mitogenic signaling, hyperosmotic or CD95L-induced EGFR activation leads to a long-lasting EGFR translocation to the plasma membrane after complex formation with CD95. These differences may be relevant for the understanding of the dual roles played by the EGFR in the control of cell proliferation and survival on the one hand and of cell death on the other. Multiple protein kinase systems, such as JNK, Yes, and protein kinases A and C are involved in this differential regulation. EGFR activation by EGF, the proapoptotic taurolithocholate-3-sulfate, or hyperosmolarity triggers EGFR tyrosine phosphorylation at Y845 and Y1173, whereas Y1045 is phosphorylated in response to EGF only. This latter phosphorylation site is known to mediate recruitment of the adapter protein Cbl, which is required for EGFR internalization, and the lack of Y1045 phosphorylation in response to proapoptotic stimuli may explain the persistent membrane localization of the EGFR. Colchicine was recently shown to protect mice from the lethal effect of an agonistic CD95 (Fas) antibody and to reduce surface expression of CD95. This effect may reside in the microtubule dependence of membrane translocation of the EGFR–CD95 complex. Interestingly, colchicine pretreatment did not prevent hyperosmotic or CD95L-induced EGFR–CD95 complex formation, suggesting that intact microtubules are not required for EGFR–CD95 association in the cytosol. Colchicine was also without effect on CD95 tyrosine phosphorylation, indicating that CD95 tyrosine phosphorylation by the EGFR is a cytosolic process that precedes targeting of the EGFR–CD95 complex to the plasma membrane. However, despite CD95 tyrosine phosphorylation, no DISC.
formation occurred after disruption of microtubules. This finding suggests that DISC formation either requires intact microtubules or is a membrane-associated process that does not occur in the cytosol. The finding that microtubules are required for CD95 membrane translocation, DISC formation, and apoptosis induction may explain some beneficial effects of colchicine in the treatment of cholestatic liver disease.42-44

The molecular mechanisms underlying the microtubule-dependent transport of the EGFR–CD95 complex to the plasma membrane are unknown; however, CD95 tyrosine phosphorylation but not EGFR phosphorylation is required. This is evidenced by the findings that inhibition of EGFR tyrosine kinase activity by AG1478 prevents membrane targeting of the EGFR–CD95 protein complex, whereas CD95 mutants lacking tyrosines 232 and 291 still associate with the activated EGFR in the cytosol, but the EGFR–CD95mut complex fails to undergo membrane targeting and DISC formation.

Furthermore, inhibition of hyperosmotic EGFR activation by cAMP,13 which allows for EGFR–CD95 association, but no longer for CD95 tyrosine phosphorylation, prevents membrane translocation of the EGFR–CD95 complex and DISC formation. Thus both, intact microtubules and CD95 tyrosine phosphorylation are prerequisites for CD95 membrane targeting and DISC formation. The CD95 mutagenesis experiments also demonstrate an important role of the CD95 tyrosine residues 232 and 291, which are located in the CD95 death domain, whereas Tyr-91 appears not to be essential for this process. However, Tyr-91 is apparently also phosphorylated in response to hyperosmolarity, because CD95Y232F/Y291F mutants still exhibited some immunoreactivity to a phospho-tyrosine antibody. However, the functional role of this phosphorylation site remains unclear. It is also not clear whether tyrosine phosphorylation in the death domain is essential only for translocation of the EGFR–CD95 complex to the plasma membrane or whether it also mediates the recruitment of FADD and caspase 8 to CD95. Mutation of critical CD95 tyrosine residues also prevented CD95L-induced apoptosis, emphasizing the important role of CD95 tyrosine phosphorylation for apoptosis induction.

Acknowledgment: The authors are grateful to Prof. Dr. Arndt-Jovin (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany), for kindly providing the EGFR-GFP construct.

References


The liver has the capacity to regenerate after acute injury by hepatocyte cell division. However, in circumstances where hepatocyte proliferation is attenuated or blocked, the liver is repopulated after induction of cell-signaling pathways that distinguish the alternative pathways are unknown. This study shows that in a mouse model, hepatic expression of lymphotoxin-β (LTβ) and interferon gamma (IFNγ) transcripts is increased in response to the choline-deficient, ethionine-supplemented (CDE) diet, which induces oval cell–mediated liver regeneration. Oval cells express LTβ and IFNγ transcripts, contributing to the increased expression in the liver of mice fed the CDE diet. An attenuated oval cell response to such a diet was observed in LTβ receptor–, LTβ–, and IFNγ-gene targeted mice. Loss of LTβ and LTβ receptor signaling reduced the number of oval cells expressing A6 and muscle pyruvate kinase. The lack of IFNγ signaling reduced muscle pyruvate kinase+, but not A6+, oval cells. In contrast, partial hepatectomy suppressed LTβ and IFNγ transcripts. We also show that IFNγ induces STAT-3 phosphorylation in an oval cell line. In conclusion, LTβ, LTβ receptor, and IFNγ are involved in oval cell–mediated, but not hepatocyte-mediated, liver regeneration, and the absence of these pathways impairs the oval cell–dependent regenerative response. (HEPATOLOGY 2005;41: 327-335.)

Abbreviations: PHx, partial hepatectomy; CDE, choline-deficient, ethionine-supplemented; TNF, tumor necrosis factor; IFNγ, interferon gamma; LTβ, lymphotoxin-β; LTα, lymphotoxin-α; LTβR, LTβ receptor; KO, knock-out; WT, wild-type; mRNA, messenger RNA; MPK, muscle pyruvate kinase; AST, aspartate transaminase.
quiescent cells into the G1 phase of the cell cycle. Cyclins and associated kinases, together with changes in the expression of inhibitory factors and activators, regulate cell cycle progression through the G1 and S phases.\(^1\)\(^-\)\(^3\)\(^-\)\(^5\) Cytokines such as tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) and interleukin 6 have important roles in hepatocyte-mediated liver regeneration,\(^1\)\(^6\)\(^-\)\(^7\) whereas interferon gamma (IFN\(\gamma\)) has been identified as an inhibitor of liver regeneration after PHx, an effect that may be the result of inhibition of Kupffer cell activation.\(^8\)

It has been proposed that the liver stem cell population resides in the terminal bile ducts—the Canals of Hering.\(^9\)\(^-\)\(^11\) Hepatocyte growth factor may promote the differentiation of oval cells, which express the hepatocyte growth factor-receptor c-Met, into hepatocytes.\(^12\)\(^,\)\(^13\) We previously showed that TNF\(\alpha\) signaling is necessary for an optimal oval cell–mediated regenerative response to a CDE diet,\(^14\) whereas IFN\(\gamma\) signaling has been implicated in the oval cell–mediated regenerative response to 2-acetylaminofluorene coupled with PHx in the rat.\(^15\)

Thus, TNF\(\alpha\) plays a positive role in both hepatocyte-mediated and oval cell–mediated liver regeneration; and although IFN\(\gamma\) inhibits hepatocyte-mediated regeneration, its effect on oval cells is unknown.

We recently reported expression of lymphotoxin-\(\beta\) (LT\(\beta\)) in oval cells and small portal hepatocytes during oval cell–mediated liver regeneration induced by chronic hepatitis C\(^1\),\(^6\) suggesting that this cytokine may be involved in the regenerative process. The LT\(\beta\) ligand is a membrane-bound member of the TNF ligand superfAMILY.\(^17\) It functions as a heterotrimer, comprising, in its most abundant form, two LT\(\beta\) subunits and one lymphotoxin-\(\alpha\) (LT\(\alpha\)) subunit (\(i.e.,\) LT\(\alpha_i\beta_j\)).\(^18\)\(^,\)\(^19\) The LT\(\beta\) ligand is expressed on the surface of activated T and B lymphocytes and NK cells\(^18\),\(^20\),\(^21\) and signals via the lymphotoxin-\(\beta\) receptor (LT\(\beta R\)),\(^18\)\(^,\)\(^19\),\(^22\) a protein ubiquitously expressed by nonlymphoid cells\(^23\)\(^-\)\(^25\) that also transduces a signal generated by a second ligand, LIGHT.\(^26\)

In this study, investigation of cytokine expression in whole liver and in a purified oval cell fraction identified TNF\(\alpha\), LT\(\beta\), and IFN\(\gamma\) transcripts. LT\(\beta\) and IFN\(\gamma\) transcripts were upregulated in mouse liver regenerating via oval cells (CDE diet), but were suppressed in liver regenerating via hepatocytes (PHx). Oval cell induction in LT\(\beta R\) knock-out (KO), LT\(\beta\) KO, and IFN\(\gamma\) KO mice was impaired, confirming a role for LT\(\beta\) and IFN\(\gamma\) in oval cell–mediated liver regeneration. Interferon gamma and LT\(\beta\) transcript abundance are tightly coordinated in the liver of CDE-treated mice but not PHx mice, suggesting their expression may be regulated by factors derived from a common cellular source, a common signal during chronic liver injury, or both. The induction of STAT-3 phosphorylation by IFN\(\gamma\) in a liver progenitor cell line suggests it may affect oval cells directly in vivo.

### Materials and Methods

**Animals.** Specific pathogen-free wild-type (WT) C57BL/6 mice, IFN\(\gamma\) KO mice (Jackson Laboratory, Bar Harbor, ME), LT\(\beta R\) KO mice (Prof. Klaus Pfeffer, The Technical University of Munich, Germany), and LT\(\beta\) KO mice (The Centenary Institute, Sydney, Australia) were housed under specific pathogen-free conditions. The animals experienced a 12-hour light-and-dark cycle and were fed sterile chow and fluid ad libitum. Animal care was provided in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, as specified by the National Health and Medical Research Council of Australia.

**PHx and Sham Procedures.** Six-week-old male C57BL/6 mice underwent one-third PHx or Sham procedure. Control mice had no operation. Before PHx, mice were anesthetized using 2.5% 2,2,2 tribromoethanol (Aldrich, Castle Hill, Australia) (0.015 mL/g body weight); the depth of anesthesia was monitored using the leg withdrawal reflex. The left lobe of the liver was externalized through a midabdominal incision and was ligated near the hilus, and the lobe was excised. Remnant liver was repositioned in the abdominal cavity, and the peritoneum and skin were sutured. Sham operations were carried out as described; however, as soon as the left lobe had been externalized, it was placed back in the abdomen before suturing. Control, sham-operated, and PHx groups were sacrificed at 4, 8, 24, and 48 hours after surgery.

**The CDE Diet.** Male mice at 4 to 5 weeks of age were housed in microisolators and fed choline-deficient pelleted mouse chow (Teklad, Indianapolis, IN) and ethionine dissolved in drinking water at 0.165% (wt/vol; Sigma, St. Louis, MO) ad libitum. Control mice were fed standard laboratory rodent chow and water ad libitum. Mice receiving control and CDE diets were sacrificed after 1, 2, and 3 weeks.

**Sacrifice, Perfusion of Intact Mouse Liver, and Collection of Samples.** At the designated times after PHx or administration of the CDE diet, mice were anesthetized using Avertin, blood samples were collected, the liver was perfused in situ via the hepatic portal vein with sterile phosphate-buffered saline at a flow rate of 3 mL/min, and tissue was collected for immunohistochemical procedures and RNA isolation. Tissues for immunohistochemistry were fixed in Carnoy’s solution and were either paraffin embedded or embedded in Cryomatrix (Shandon, Pittsburgh, PA), were snap frozen in liquid nitrogen,
and were stored at −80°C. Tissue for RNA isolation was snap frozen and stored at −80°C. Blood samples were allowed to clot at room temperature, and the serum was stored frozen at −20°C until analyzed.

Oval Cell Isolation. Collagenase digestion of intact liver and fractionation by centrifugal elutriation of the isolated cells were performed as described previously.14 This was followed by affinity purification of the oval cell–containing fraction using a MiniMACS column containing an anti-CD45 antibody (Miltenyi Biotec, Auburn, CA) following the manufacturer’s instructions. The cells in the CD45− column fraction were tested for oval cell markers by immunocytochemistry and were determined to contain oval cells at approximately 95% purity. The CD45+ fraction was considered to contain inflammatory cells.

RNase Protection Assay. RNA was isolated from whole liver and the cell fractions using TRIzol (Invitrogen, Mt. Waverley, Victoria, Australia) following the manufacturer’s instructions. An RNase protection assay was carried out on 20–μg aliquots of total RNA using the RiboQuant Multiprobe RNase Protection Assay System (BD Pharmingen, San Diego, CA) and the mCK3b probe set, following the manufacturer’s instructions. Protected probe fragments were electrophoresed in a 5% polyacrylamide gel, and transcript abundance was visualized by phosphorimaging and was quantified using the ImageGauge Software (Fuji Photofilm Co. Ltd., Tokyo, Japan). Interferon gamma, LTα, and LTβ messenger RNA (mRNA) abundance was normalized against GAPDH for each experimental group, the value for control animals was set at 100%, and the levels obtained for PHx and CDE-treated groups were expressed relative to this.

The levels of LTβ, IFNγ, and LTα transcripts in the liver of control mice sacrificed at different time points in the PHx experiments produced data that were not statistically different; these data were therefore pooled. Similarly, the data generated in mice fed the control diet within the CDE experiments were also pooled.

Immunohistochemistry. Immunohistochemical staining of liver tissues was performed using the rat antianmouse A6 monoclonal antibody and polyclonal goat antirabbit muscle pyruvate kinase (MPK; Rockland, Gilbertsville, PA), using an indirect staining protocol using goat antirat immunoglobulin G-horseradish peroxidase (HRP) (Amersham Biosciences, Castle Hill, New South Wales, Australia) and donkey antigoat immunoglobulin G-HRP (Rockland) second antibodies, respectively.27 The number of positive cells for each of these markers was expressed as a percentage of total cells.

Serum Aspartate Aminotransferase. Serum aspartate transaminase (AST) concentration was determined using the GO-transaminase kit (Sigma) following the manufacturer’s instructions.

Cell Culture. PIL-2 cells were grown at 37°C in a Williams E medium (Gibco BRL, Carlsbad, CA) containing 5% fetal calf serum and supplements as listed: 2 mM glutamine, 10−7 M dexamethasone, 1× ITS+ (Collaborative Biomedical, Bedford, MA), 20 ng/mL epithelial growth factor (Collaborative Biomedical), 1.1% (vol/vol) Fungizone (Gibco BRL), and 1.1% (vol/vol) penicillin/streptomycin (Gibco BRL).

Analysis of STAT-3 Phosphorylation. PIL-2 cells were grown overnight in serum-free medium followed by a further 4 hours in fresh serum-free medium the following day. Cells then were stimulated for 10, 30, and 60 minutes with IFNγ (400 U/mL; Genzyme, Cambridge, MA). The supernatants were removed, and the cells were lysed by addition of 2× Laemmlı buffer (10% glycerol, 2% SDS, 2% beta-mercaptoethanol, 0.001% bromophenol blue). An aliquot (20 μL) was subjected to SDS-PAGE, blotted to a nitrocellulose membrane (Amersham Biosciences), and incubated with polyclonal anti-phospho-STAT-3 Tyr-705 antibody (Cell Signaling Technology, Beverly, CA); it was then reacted against a secondary antibody coupled to peroxidase (polyclonal horseradish peroxidase-coupled goat antirabbit antibody; BioRad, Hercules, CA) and was visualized using the Amersham Biosciences ECL chemiluminescence kit. Loading consistency was evaluated with anti-β-actin antibody (ab6276; abcam, Cambridge, United Kingdom).

Statistical Analyses. The data represent the mean average ± SEM. Statistical significance was assessed using ANOVA analysis facilitated by the SuperAnova software program (Abacus Concepts, Cary, NC).

Results

A CDE Diet Induces TNFα, IFNγ, LTα, and LTβ Transcripts in Liver and Purified Oval Cells. To investigate hepatic cytokine expression, an RNase protection assay was carried out on whole liver samples from control and CDE-treated mice and on isolated oval and inflammatory cell fractions. Compared with mice fed a control diet (Fig. 1A), steady-state mRNA levels of LTβ, TNFα, and IFNγ increased in whole liver in response to the CDE diet (Fig. 1B). Substantial expression of LTβ mRNA was observed in purified oval cells isolated from murine liver after 3 weeks on the CDE diet (Fig. 1C). LTβ was also expressed by inflammatory cells (Fig. 1D). IFNγ mRNA was detected in the oval cell fraction, but not in inflammatory cells. The inflammatory cell fraction highly expressed TNFα mRNA, whereas oval cells expressed this transcript at lower levels. Interleukin 6
mRNA was expressed by inflammatory cells and was weakly detected in the oval cell fraction. Low-level interleukin 6 expression observed in the oval cell fraction may be the result of the 5% contamination (maximum) by similar-sized inflammatory cells, and therefore cannot be definitively attributed to the oval cells. LTα transcripts appeared in CDE liver and were observed in both cell fractions after longer exposure of the RNase protection assay gel (data not shown).

**PHx Suppresses Hepatic LTβ IFNγ mRNA Levels after Surgery.** Rapid suppression of the level of LTβ transcript was seen in both the sham and PHx groups. Four hours after surgery, there was a reduction to 33% to 34% of the control value (P < .001 in both groups). Later, LTβ mRNA levels in the sham-operated group rose, reaching control values 24 hours after surgery (P < .05 at 8 hours). LTβ mRNA abundance in the PHx animals remained suppressed below the control value for the duration of the experiment (P < .001 at 4 and 8 hours, and P < .01 at 24 and 48 hours after surgery) and below the level of the sham group 24 and 48 hours after surgery (Fig. 2A; P < .05 at 24 and 48 hours).

LTα mRNA abundance was below the detection limit of the RNase protection assay in both the sham and PHx animals after surgery (data not shown).

IFNγ mRNA abundance was not appreciably altered in sham-operated mice at any time point except at 24 hours (P < .05). The PHx mice showed suppression of IFNγ transcript abundance relative to controls at 24 and 48 hours after surgery (P < .001 at 24 hours, and P < .01 at 48 hours) and relative to sham-operated mice at 8, 24, and 48 hours after surgery (Fig. 2B; P < .05 at 8 and 48 hours, and P < .01 at 24 hours).

**The CDE Diet Increases LTα, LTβ, and IFNγ Abundance.** LTβ, IFNγ, LTα mRNA transcripts all increased in response to the CDE diet (Fig. 3A-C, respectively). LTβ and IFNγ mRNA levels rose rapidly, both peaking at 2 weeks at levels 15-fold more than those of controls and remained elevated at five- to six-fold more than those of controls at 3 weeks (P < .001 at all time points). Similarly, hepatic LTα mRNA abundance
peaked after 2 weeks on the CDE diet to fourfold more than that of controls (P < .001) and remained 2.4-fold higher than that of controls at 3 weeks (P < .05).

**IFNγ KO, LTβ KO, LTβR KO Mice Show Impaired Oval Cell–Mediated Liver Regeneration.** The CDE diet produced a significant A6⁺ oval cell response in each of the WT (Fig. 4A), IFNγ KO (Fig. 4B), LTβ KO (Fig. 4C), and LTβR KO (Fig. 4D) mice. Whereas WT and IFNγ KO mice showed similar increases in A6⁺ cells at each time point tested, the response was attenuated in LTβR KO and LTβ KO mice in comparison with WT mice (Fig. 4A-D; Fig. 5). A6⁺ oval cells were reduced in LTβR KO mice to 29% and 70% of the WT mice at 2 and 3 weeks, respectively (P < .05 at each time point) and in LTβ KO mice to 43% and 62% of the WT mice response (P < .05 at 2 weeks).

Quantification of oval cells on the basis of MPK expression also showed a significant increase in response to the CDE diet in WT mice (Fig. 4E) and in all the transgenic mice. Using MPK as a marker, the magnitude of this response was attenuated in IFNγ KO (Fig. 4F), LTβR KO (Fig. 4G), and LTβR KO (Fig. 4H) strains (Fig. 6). A more than 50% reduction in the peak MPK⁺ oval cell number is evident in each of the transgenic strains compared with WT mice (P < .001 at 2 and 3 weeks, all transgenic strains), demonstrating a markedly impaired MPK⁺ oval cell response to the CDE diet in these mice.

![Fig. 4. Oval cells detected by immunohistochemistry using (A-D) A6 and (E-H) muscle pyruvate kinase (MPK) antibody in (A, E) wild-type, (B, F) interferon gamma knock-out (KO), (C, G) lymphotxin-β (LTβ) KO, and (D, H) LTβ receptor KO mice. Scale bars, (A-D) 500 μm and (E-H) 200 μm. The inserts are further magnified by (A) x5 and (E) x2.](image)

![Fig. 5. The oval cell response determined by A6 antigen is attenuated in lymphotxin-β receptor (LTβR) knock-out (KO) and lymphotxin-β (LTβ) KO, but not interferon gamma (IFNγ) KO mice. Results are presented as the percentage of A6⁺ cells in mice fed the control diet or the choline-deficient, ethionine-supplemented (CDE) diet for 1, 2, and 3 weeks (wk). The data represent the mean ± SEM; there are five to seven mice in CDE-fed IFNγ KO and LTβR KO groups and between 9 and 17 controls (animals sacrificed at each time point have been combined). There were three mice in each of the LTβ KO control and CDE-treated groups. Statistical significance with respect to wild type (WT) is represented as *P < .05.](image)

![Fig. 6. The oval cell response determined by muscle pyruvate kinase (MPK) is attenuated in interferon gamma (IFNγ) knock-out (KO), lymphotxin-β receptor (LTβR) KO, and lymphotxin-β (LTβ) KO mice compared with wild-type (WT) mice. Results are presented as the percentage of MPK⁺ oval cells in mice fed the control diet or the choline-deficient, ethionine-supplemented (CDE) diet for 1, 2, and 3 weeks (wk). The data represent the mean ± SEM; there were five to seven mice in the CDE-fed IFNγ and LTβR KO group and between 9 and 18 controls (animals sacrificed at each time point have been combined). There were three mice in each of the LTβ KO control and CDE-treated groups. Statistical significance with respect to WT is represented as ***P < .001.](image)
CDE Diet–Induced Liver Damage Is Independent of LTβ.
Serum AST, a marker of liver damage, was measured to determine the extent of liver damage engendered by the CDE diet. Serum AST rose in the WT and transgenic strains in response to the CDE diet. However, there was no consistent trend in serum AST levels in the LTβ/H9252 KO and LTβR KO strains that would indicate that the degree of liver damage caused by the CDE diet in these mice was different from WT mice (data not shown).

LTβ and IFNγ Abundance Is Correlated During Oval Cell–Mediated, But Not Hepatocyte-Mediated, Liver Regeneration.
The similar response patterns of LTβ and IFNγ transcripts in WT mice fed the CDE diet (Fig. 3A-B) prompted us to investigate if there was a correlation in the abundance of the two transcripts. Wild-type mice fed the CDE diet showed a high correlation in the steady-state levels of LTβ and IFNγ mRNA, each of which were induced by the CDE diet (Fig. 7C; r = 0.994). In contrast, there was a poor correlation between the expressions of these transcripts in mice undergoing PHx or sham operation in which the transcript abundance is suppressed (Fig. 7B; PHx, r = 0.039; Fig. 7A; sham, r = 0.496). There was no correlation between the transcript abundance of any two of the cytokines LTα, TNFα, and either IFNγ or LTβ.

IFNγ Induces STAT-3 Phosphorylation of PIL-2 Liver Progenitor Cells in Culture. The addition of IFNγ (400 U/mL) to cultures of the PIL-2 liver progenitor cell line28 rapidly induced the phosphorylation of STAT-3 (Fig. 8). A significant signal above the basal level in untreated cells was observed as early as 10 minutes after exposure to the cytokine.

Discussion
Understanding the mechanisms underlying oval cell activation, proliferation, and differentiation will lead to clinically useful methods to replace liver cell mass lost because of injury or disease, and is particularly important in situations where the liver environment may impede or preclude regeneration from existing or transplanted hepatocytes. The identification of factors and knowledge of their mechanism of action would form the basis of strategies to induce oval cells both in vivo and in vitro.

We previously confirmed a role for TNFα in oval cell induction in studies using TNF receptor I KO mice.14 TNFα is also crucial in liver regeneration after PHx, where it has been assigned the role of priming hepatocytes so they can respond to hepatocyte growth factor.6,29 The substantial, but incomplete, suppression of oval cell proliferation in TNF receptor I KO mice in response to a CDE diet indicates that factors other than TNFα must be involved in this process. Their absence may explain why TNFα priming of hepatocyte proliferation after PHx does not also lead to the induction of oval cells.6,29

This study established that in addition to TNFα, the cytokines IFNγ, LTβ, and LTα are elevated during the oval cell response. In contrast, after PHx, steady-state levels of both IFNγ and LTβ mRNA are suppressed and LTα is not detectable. In summary, opposite situations are observed for these cytokines in the two models of liver regeneration.

Fig. 7. The steady-state levels of lymphotoxin-β (LTβ) and interferon gamma (IFNγ) are correlated in choline-deficient, ethionine-supplemented (CDE) diet-fed mice, but not after partial hepatectomy (PHx) and sham operation in C57BL/6 wild-type mice. No correlation between LTβ and IFNγ messenger RNA (mRNA) levels in mice after (A) sham (n = 17) or (B) PHx (n = 18). (C) Correlation in CDE mice after 1, 2, and 3 weeks on the diet (n = 19).

Fig. 8. Interferon gamma (IFNγ) rapidly induces STAT-3 phosphorylation in the liver progenitor oval cell line PIL-2. Cells are exposed to vehicle, hence untreated (UT) or IFNγ (400 U/mL) for times indicated. Extracts were prepared and Western blot analysis was performed as described in Materials and Methods. β Actin was used as a loading control.
It is also possible that these cytokines are involved in paracrine signaling of oval cells, because we demonstrate that all three transcripts are abundant in purified oval cells, and it has been shown by Bisgaard et al.\textsuperscript{15} that IFNγ receptor subunits are expressed by oval cell ductular structures after their induction by 2-acetylaminofluorene coupled with PHx.

Interestingly, during oval cell induction in response to the CDE diet, but not during hepatocyte proliferation in response to PHx, the expression of LTβ and IFNγ transcripts are highly correlated. This tight correlation is consistent with these cytokines being predominantly coexpressed by a common cell type, such as the oval cell. Alternatively, LTβ and IFNγ may be coregulated during oval cell induction, either by one cytokine regulating the expression of the other, or by the action of a common regulator.

We demonstrate a role for LTβ/LTβR signaling and confirm a role for IFNγ signaling in oval cell induction by establishing that the oval cell response is attenuated in LTβ KO, LTβR KO, and IFNγ KO mice. The degree of liver damage engendered by the CDE diet, assessed by quantification of serum AST, is similar between the WT and the gene-targeted mice, excluding a substantial reduction in liver damage as an explanation for the attenuated oval cell response in the gene-targeted mice. However, we did observe a poorer condition of these mice compared with WT mice in terms of the appearance of their fur, reflecting a reduced ability to groom and general physical activity.

Interferon gamma suppresses hepatocyte proliferation after PHx\textsuperscript{8}; therefore, its reduction in PHx would be promitogenic. In contrast, IFNγ has previously been implicated in oval cell–mediated liver regeneration. Subtractive hybridization comparing transcripts from nonparenchymal cells after 2-acetylaminofluorene coupled with PHx and PHx-identified genes induce IFNγ, IFNγ receptor subunits 1 and 2, primary and secondary response genes, and IFNγ-induced cell adhesion molecules.\textsuperscript{15} Using a liver progenitor cell line, PIL-2,\textsuperscript{28} we showed IFNγ rapidly induces STAT-3 phosphorylation when it is added to cultures. This result suggests a direct effect of IFNγ in activating this signaling pathway that, in several in vitro studies, is shown to link cytokine signaling with cell proliferation.\textsuperscript{30,31} Collectively, these data provide compelling evidence that IFNγ signaling is required for an optimal oval cell response.

Within the liver, oval cells can differentiate into bile duct cells or hepatocytes.\textsuperscript{32–34} Interestingly, the loss of IFNγ signaling leads to a reduction in MPK\textsuperscript{+} oval cells, but not A6\textsuperscript{+} oval cells. In our hands, A6 staining is confined to bile ducts in normal liver and mainly ductal and periductal oval cells in CDE-treated liver. In contrast, MPK staining detects few periportal cells in normal liver; but in CDE-treated liver, it stains periportal oval cells and many that have migrated from zone 1. More importantly, MPK staining is present in small hepatocytes, many of which also express the liver-specific isoenzyme of pyruvate kinase.\textsuperscript{35} This finding suggests that A6 and MPK may distinguish oval cells that have differentiated along the bile duct and hepatocyte lineages, respectively. If this is correct, the finding that in IFNγ KO mice only MPK\textsuperscript{+} oval cells are reduced suggests this cytokine affects the generation of hepatocytes, rather than bile duct cells, from oval cells. Metaplastic differentiation of pancreatic ductal cells into an hepatocytic phenotype was observed when IFNγ was constitutively expressed in the pancreas,\textsuperscript{46} supporting a role for IFNγ specifically in hepatocyte differentiation, directing progenitor cells toward this phenotype. The presence of low numbers of MPK\textsuperscript{+} cells in IFNγ KO mice receiving a control diet is consistent with this view.

Loss of the LTβR and functional LTβ produced comparable reductions in both A6- and MPK-expressing oval cells, suggesting that this reduction is the result of disruption of LTβ-dependent LTβR signaling rather than LIGHT signaling through this receptor. We previously demonstrated LTβ expression in oval cells and small portal hepatocytes in patients with chronic hepatitis C,\textsuperscript{16} and in this study, we have confirmed a role for this cytokine in oval cell–mediated liver regeneration.

LTβ is expressed by activated lymphocytes,\textsuperscript{18,20,21} and its role in lymphogenesis has been extensively studied. In some tumor cells, LTβ signals apoptosis,\textsuperscript{37,38} or nonapoptotic growth inhibition,\textsuperscript{39} and in WI38 fibroblasts, cellular proliferation.\textsuperscript{39} In vivo, LTβ/LTβR signaling is required for the development of secondary lymph organs, including Peyer’s patches and lymph nodes\textsuperscript{40–51} and the cellular composition and organization of the spleen,\textsuperscript{40–42,48–51} and lamina propria.\textsuperscript{42,47} LTβ receptor-mediated signaling has been shown to regulate gene expression by activation of nuclear factor KB\textsuperscript{39,52–54} to stimulate chemokine secretion,\textsuperscript{25,53,55} and to upregulate the expression of adhesion molecules.\textsuperscript{39,45,48,55–58} The development of splenic follicular dendritic cell networks facilitating T/B-cell segregation and the establishment of germinal centers\textsuperscript{51} and the differentiation of NK cells from NK precursors\textsuperscript{59,60} are both dependent on LTβ/LTβR signaling. In addition, LTβR-dependent signaling between thymocytes and thymic medullary epithelial cells is required for normal differentiation and numbers of medullary epithelial cells within the thymus.\textsuperscript{61} In summary, the biological role of LTβ/LTβR signaling in the lymphoid system seems to involve cell proliferation, differentiation,
perhaps apoptosis, and the regulation of cell trafficking. LTβ/LTβR signaling in oval cell-mediated liver regeneration also may facilitate recruitment, cell migration, and differentiation of oval cells into hepatocytes and biliary epithelium. Comparison of the characteristic patterns of A6 antigen expression in CDE-treated WT and LTβR KO mice did not identify any gross defects in the migration of A6+ cells within the liver; however, a more subtle defect cannot be excluded.

In conclusion, this study provides direct evidence that both LTβ/LTβR and IFNγ are required for optimal oval cell-mediated, but not hepatocyte-mediated, liver regeneration. Importantly, it shows signaling by both cytokines is actually reduced before hepatocyte cell division, conditions that would favor hepatocyte proliferation, and this also may act to suppress oval cell induction after acute injury. Transcripts of LTβ and IFNγ are coordinately regulated during the oval cell response, which suggests that a common regulator, cell type, or both, is involved in mediating the induction process.

Acknowledgment: The A6 antibody was kindly supplied by Dr. Valentina Factor (National Cancer Institute, Bethesda, MD).

References


Cytochrome P450 CYP2E1, But Not Nicotinamide Adenine Dinucleotide Phosphate Oxidase, Is Required for Ethanol-Induced Oxidative DNA Damage in Rodent Liver


The occurrence of malignant tumors of the upper gastrointestinal tract and liver is, based largely on epidemiological evidence, causally related to the consumption of ethanol. It is widely recognized that oxidants play a key role in alcohol-induced liver injury; however, it is unclear how oxidants may be involved in DNA damage. We asked whether nicotinamide adenine dinucleotide phosphate oxidase, cytochrome P450 CYP2E1, or both are responsible for the production of DNA damage. The rodent Tsukamoto-French model of intragastric ethanol infusion was used. Wistar rats, Cyp2e1−/−, p47phox−/−, and hCyp2e1 transgenic mice were used. The abundance of oxidative DNA adducts, mutagenic apurinic/apyrimidinic sites, and expression of base excision DNA repair genes was determined. In rats and wild-type mice, ethanol treatment for 4 weeks led to an increase in oxidative DNA damage and induction of expression of the base excision DNA repair genes that are known to remove oxidative DNA lesions. No increase in either of the endpoints was observed in ethanol-treated Cyp2e1−/− mice, whereas the magnitude of response in p47phox−/− mice and transgenic hCyp2e1 was identical to that in wild types. The increase in expression of DNA repair genes was completely abolished by treatment with the P450 inhibitor 1-aminobenzotriazole. In conclusion, the data support the hypothesis that oxidative stress to DNA is induced in liver by ethanol. Furthermore, although it was shown that nicotinamide adenine dinucleotide phosphate oxidase-derived oxidants are critical for the development of ethanol-induced liver injury, CYP2E1 is required for the induction of oxidative stress to DNA, and thus may play a key role in ethanol-associated hepatocarcinogenesis. (HEPATOLOGY 2005;41:336-344.)

Exposure to environmental agents, certain lifestyles, and dietary factors can result in the development of chronic diseases such as cancer. The American Cancer Society estimates that in 2002, there were nearly 1.3 million new cancer cases and more than 555,000 new deaths resulting from cancer in the United States alone. Although hepatocellular carcinoma (HCC) is not the most common type of cancer in this country, it is one of the deadliest. Worldwide, HCC is one of the most common (the seventh most prevalent cancer in men and the ninth in women) and devastating malignant tumors. HCC frequently has a grave prognosis because it progresses rapidly, is poorly responsive to nonoperative treatment, and has a low rate of resectability. In the United States, the most recent epidemiological studies of risk factors associated with HCC suggest that heavy alcohol consumption contributes to most HCC cases. Indeed, ethanol consumed orally is classified by the International Agency for Research on Cancer as a "known

Abbreviations: HCC, hepatocellular carcinoma; NADPH, nicotinamide adenine dinucleotide phosphate; ABT, 1-aminobenzotriazole; AP, apurinic/apyrimidinic; Ogg1, 8-oxoguanine DNA glycosylase/lyase 1; BER, base excision DNA repair.

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Received August 18, 2004; accepted October 20, 2004.

Supported by the National Institutes of Health (grants ES11391, ES11660, DK56350, and ES10126). I.R. was a recipient of a Transition to Independent Position award (grant ES11660) from the National Institute of Environmental Health Sciences.

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Conflict of interest: Nothing to report.
human carcinogen.5 Interestingly, the precise mechanisms of the tumorigenic effects of ethanol remain largely unknown, and the “known carcinogen” categorization is based almost exclusively on epidemiological evidence.

It is widely recognized that reactive oxygen species play a key role in alcohol-induced liver injury. Oxidants are well-known DNA damaging molecules, and DNA damage and repair are thought to be the key cancer-relevant genome-associated pathways. Indeed, alcohol drinking has been postulated to cause formation of several DNA-damaging molecules such as reactive oxygen species, products of lipid peroxidation, and acetaldehyde.6,7 It was shown that ethanol exposure leads to alterations in the structural integrity of mitochondrial DNA8; leads to the accumulation of single-strand breaks in rat liver parenchymal cells, an effect that closely matched the timing of CYP2E1 induction and was inhibited by dietary antioxidants9; increases in activity and amounts of 3-methyladenine and 8-oxoguanine DNA glycosylates in rat liver10; leads to increases in lipid peroxidation-derived ethenol-DNA adducts11; and leads to depletion of mitochondrial DNA in mouse liver and other organs.12

Much direct and indirect experimental evidence for increased production of oxidants in livers of rats and mice treated with ethanol has been presented (reviewed in Arteel13). Although few question a key role for oxidants in general, debates are ongoing as to the origin of these oxidants.6 It was shown that early alcohol-induced liver injury caused by 4-week intragastric administration of an ethanol-containing diet (Tsukamoto-French protocol) is absolutely dependent on Kupffer cell NADPH oxidase-derived oxidants.14 At the same time, induction of CYP2E1 was proposed as one of the central pathways by which ethanol generates a state of oxidative stress in hepatocytes.15 Surprisingly, it was demonstrated that Cyp2e1-null mice are not resistant to early alcohol-induced liver injury.16 Thus, oxidative stress is likely to occur in at least two cellular compartments in the liver, Kupffer cells and parenchymal cells, and oxidative stress-induced liver injury involves coordination of molecular events within and between these compartments.

In this study, we asked whether NADPH oxidase or CYP2E1 is responsible for the production of oxidative DNA damage. Studies were conducted using genetically engineered mice and a well-established Tsukamoto-French model of ethanol-induced liver injury. Our data strongly support the hypothesis that administration of ethanol leads to the accumulation of oxidative stress to DNA in liver. Importantly, we report here that although NADPH oxidase-derived oxidants are known to be critical for the development of ethanol-induced liver injury, activation of CYP2E1 is required for the induction of oxidative DNA damage, which may play a key role in ethanol-associated carcinogenesis in liver.

**Materials and Methods**

**Animals and Treatments.** For the most part, the studies detailed herein were performed using archived liver tissue samples (stored at −80°C) from previously reported animal studies (Table 1),14,16-19 where rats or mice were treated with high-fat diet or ethanol intragastrically for 4 weeks. Some wild-type mice also were administered daily with 1-aminobenzotriazole (ABT; 100 mg/kg intragastically in saline).20 Additional ethanol treatments were performed in a transgenic hCyp2e1 mouse line and matching wild-type mice (SV129 ter substrain Kono et al.16)

<table>
<thead>
<tr>
<th>Rodent Strain</th>
<th>Reference to the Genetically Engineered Model</th>
<th>Genetic Change</th>
<th>Control Strain</th>
<th>Reference to the Alcohol Study</th>
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<td>Rats</td>
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<td>N/A</td>
<td>N/A</td>
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</tr>
<tr>
<td>Mice</td>
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<td>Mouse CYP2E1 null</td>
<td>SV/129ter substrain</td>
<td>Kono et al.16</td>
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<td>NADPH oxidase deficient</td>
<td>C57Bl/6J</td>
<td>Kono et al.14</td>
</tr>
<tr>
<td>p47phox-null</td>
<td>Unpublished</td>
<td>Human CYP2E1</td>
<td>SV/129X FVB</td>
<td>This study</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, not applicable.
humanized mice will be published elsewhere (Cheung et al., Drug Metab Dispos., in press).

Mice were treated with an ethanol or control high-fat diet. All animals used specifically for this study or in earlier experiments were housed in sterilized metabolic cages in special facilities with a 12-hour night-and-day cycle. Temperature and relative humidity were held at 22°C ± 2°C and 50% ± 5%, respectively. The University of North Carolina Division of Laboratory Animal Medicine maintains these animal facilities, and veterinarians were always available to ensure animal health. All animals were given humane care in compliance with National Institutes of Health and institutional guidelines, and studies were performed according to approved protocols. Before experiments, animals were maintained on standard lab chow diet and purified water ad libitum.

**Enteral Ethanol Feeding Model in Mice.** Surgical procedures were performed using modifications of the method developed by Tsukamoto and French for rats. Mice were allowed to recover for 1 week after surgery before receiving liquid diet, during which time they were fed chow diet. Subsequently, liquid diets containing ethanol or isocaloric maltose-dextrin controls were administered via the gastric cannula (see Thompson and Reitz for diet composition). The composition of the diet had a total of 21% of calories as protein, 44% of calories as carbohydrate (35% of calories are ethanol in the ethanol-treated group), and 35% as fat from corn oil. Diets were supplemented with methionine and choline as described by Morimoto et al. Mice had access to purified water and nonnutritive cellulose pellets as a source of fiber. Administration of ABT daily to C57BL/6J mice resulted in effective inhibition of a broad panel of cytochrome P450 isoforms.

**Isolation of DNA.** DNA was extracted by a procedure slightly modified from the method reported previously. To minimize formation of oxidative artifacts during isolation, 2,2,6,6-tetramethylpiperidinoxyl (20 mM final concentration) was added to all solutions, and all procedures were performed on ice. Briefly, frozen tissues were thawed, homogenized in phosphate-buffered saline, and centrifuged at 2,000g for 10 minutes. The nuclear pellets were incubated in lysis buffer (Applied Biosystems, Foster City, CA) overnight at 4°C with protease K (500 mg/mL; Applied Biosystems). DNA then was extracted twice with a mixture of phenol, chloroform, and water, followed by ethanol precipitation. The extracted DNA was incubated in phosphate-buffered saline (pH 7.4) with RNase A followed by DNA precipitation with cold ethanol. The DNA pellet then was resuspended in sterilized distilled water and stored at −80°C until assayed.

**Abasic Sites.** Abasic (apurinic/apyrimidinic [AP]) sites were measured based on a procedure reported by Nakamura and Swenberg. Briefly, 8 μg of DNA in 150 μL of phosphate-buffered saline was incubated with 1 mM aldehyde reactive probe at 37°C for 10 minutes. After precipitation using cold ethanol, DNA was suspended in Tris-ethylenediaminetetraacetic acid (TE) buffer. DNA (250 ng) in TE buffer was heat denatured and loaded on a nitrocellulose membrane (110 ng DNA/slot; Hybond-C Super, Amersham Pharmacia Biotech, Piscataway, NJ) and soaked with 5×SSC (saline sodium citrate) then baked in a vacuum oven for 30 minutes. The membrane was preincubated with 10 mL of Tris-HCl containing bovine serum albumin (0.25%) and Tween 20 (0.1%) for 15 minutes and then incubated in the same solution containing streptavidin-conjugated horseradish peroxidase (1:10,000 dilution; Biogenex, San Ramon, CA) at room temperature for 45 minutes. The enzymatic activity on the membrane was visualized by enhanced chemiluminescence reagents. The nitrocellulose filter was exposed to x-ray film, and the developed film was analyzed using a Kodak Image Station 440 (Rochester, NY). Quantitation was based on comparisons to internal standard DNA containing a known amount of AP sites.

**Detection of 8-Oxoguanine DNA Glycosylase/Lyase 1-Sensitive Sites in DNA (Oxidized Purines).** A modified AP site assay was used. First, DNA was incubated with methoxyamine to reduce aldehydic DNA lesions (primarily abasic sites) to approximately one lesion per 10⁶ nucleotides. After ethanol precipitation, 8 μg DNA was incubated with or without human 8-oxoguanine DNA glycosylase/lyase 1 (hOgg1) enzyme at 37°C. hOgg1 is a glycosylase/lyase and removes oxidized purine lesions from DNA and also introduces a 3’ nick to the damaged site, leaving a 3’-AP site that can be detected by a standard abasic site assay (see previous section). The total number of hOgg1-sensitive sites, oxidized purine lesions, was calculated from the number of AP sites detected after hOgg1 incubation minus the number of AP sites detected without added hOgg1.

**Ribonuclease Protection Assays.** Total RNA was isolated using RNeasy total RNA extraction method (QIA-GEN, Valencia, CA) and dissolved in RNase-free water. Samples were stored at −80°C until assayed for no longer than 2 months to minimize degradation. The quality of preparations was determined using an Agilent Bio-Analyzer (Agilent Technologies, Palo Alto, CA). Expression of base excision DNA repair (BER) enzymes was determined using an RNase protection assay with mouse or rat multiprobe RNA probe template sets (mBER-1, mBER-2, and rBER; BD PharMingen, San Diego, CA) as described in Rusyn et al. Riboprobes were synthesized in
the presence of $^{32}$PdUTP (uracil-5'-triphosphate) to yield labeled antisense RNA probes. The RNase protection assays were performed on 30 µg of individual total RNA samples using a RibOQuant multiprobe RNase protection assay kit (BD PharMingen). Protected fragments were separated on 5% polyacrylamide nucleic acid separation gels, dried, and exposed to a phosphor imaging screen. The intensity of protected bands was quantified using a phosphor image analyzer and was normalized to the intensity of housekeeping gene L32.

**Western Blotting for CYP2E1.** Mouse liver tissues were homogenized in three volumes of 10 mM Tris chloride buffer, pH 7.4, containing 150 mM potassium chloride, 1 mM ethylenediaminetetraacetic acid, and 0.25 M sucrose, and centrifuged at 10,000 ×g for 30 minutes. Nine micrograms of supernatant was immunoblotted after separation of proteins on a 9% polyacrylamide gel. Nitrocellulose sheets were blocked overnight in 3% (wt/vol) nonfat dry milk in Tris-buffered saline (10 mM Tris chloride, pH 7.4, with 150 mM NaCl) at 4°C. CYP2E1 was detected using sheep antirabbit CYP2E1 antisera (1:5,000; a kind gift of Dr. D. R. Koop, Oregon Health Sciences University), with rabbit antiserum horseradish peroxidase (1:5,000), using enhanced chemiluminescence detection. Band intensity was determined with a Bio-Rad GS-363 molecular imager (Bio-Rad, Richmond, CA) and analyzed using Kodak 1D image analysis software.

**Monitoring of the Catalytic Activity of CYP2E1 in Liver.** The production of 6-hydroxy-chlorzoxazone was used to monitor the catalytic activity of CYP2E1 by high pressure liquid chromatography using a modification of the procedure described previously. It should be noted that other P450 isozymes could catalyze the oxidation of chlorzoxazone, including CYP1A1. However, the rate of CYP1A1 for chlorzoxazone is approximately 10-fold lower at V_max compared with CYP2E1. Further, although the substrate concentrations used here saturate CYP2E1, substrate concentrations are near K_m for CYP1A1, so the rate difference between these enzymes is even greater. Thus, it is likely that chlorzoxazone hydroxylation reflects predominantly CYP2E1 activity under these conditions. Nevertheless, a minor contribution of CYP1A1 cannot be completely excluded.

**Microsome Preparation and Immunoblotting for hCYP2E1.** Pooled human liver microsomes and recombinant expressed human CYP2E1 were purchased from BD Biosciences Discovery Labware (Bedford, MA). Mouse liver microsome samples were prepared as described previously. Proteins were separated by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and were transferred onto nitrocellulose. Human CYP2E1 was detected using a mouse monoclonal antibody (MAB 1-156-3, specific to human CYP2E1 and not crossreactive with mouse CYP2E1), as described previously.

**Results**

Administration of ethanol to male Wistar rats for 4 weeks by use of the enteral (Tsukamoto-French protocol) leads to typical liver injury manifested by inflammation, deposition of fat droplets, and focal necrosis, changes that are dependent on production of reactive oxygen species. Although liver injury in this model is tightly associated with ethanol-induced increased reactive oxygen species production, little is known about the potential for oxidative DNA damage to occur under these conditions. Thus, we first investigated whether oxidative DNA damage can be detected in livers of rats treated with ethanol. The number of AP sites, DNA lesions that may result from sugar oxidation or loss of oxidized DNA bases through spontaneous depurination or base excision repair, was increased nearly two-fold after enteral ethanol treatment for 4 weeks (Fig. 1A). Similarly, the number of oxidized purines, as measured by the number of DNA sites sensitive to cleavage by Ogg1 also was increased significantly, approximately four-fold after ethanol treatment (Fig. 1B).

Recently, it has been demonstrated that expression of base excision DNA repair genes is a sensitive in vivo marker for oxidative stress-induced DNA damage. To assess what effect a subchronic treatment with ethanol has on the expression of oxidative lesion-removing DNA repair proteins, a multitemplate RNase protection assay technique was used (Fig. 2A). Ethanol treatment led to a significant increase in the expression of OGG1, apurinic/
apyrimidinic endonuclease I, polymerase (DNA directed)
β, poly(ADP-ribose) polymerase, and proliferating cell
nuclear antigen, compared with expression levels in chow-
fed or control high-fat diet–fed animals (Fig. 2B). Impor-
tantly, the expression of O6-methyl guanine
methyltransferase, a protein that participates in a single-
step repair of alkylguanine residues but has no role in
repair of oxidative DNA damage, was not affected. Col-
lectively, the data presented in Figs. 1 and 2 suggest that
early ethanol-induced liver injury coincides with oxida-
tive DNA damage and upregulation of DNA repair genes.

As we previously showed, oxidants from NADPH ox-
idase in Kupffer cells, but not from CYP2E1 in liver pa-
renchymal cells, are critical for the development of early
alcohol-induced liver injury.14,16 Specifically, it was found
that both liver pathological features resulting from etha-

nal (steatosis, inflammation, and necrosis) and formation
of α-hydroxyethyl radicals were identical in wild-type and
Cyp2e1-null mice. Interestingly, NADPH oxidase-defi-
cient mice that lack a critical regulatory subunit of this
potent oxidant-generating enzyme, p47phox, were pro-
tected against ethanol-induced liver injury and increased
formation of α-hydroxyethyl radicals. Here, we asked
what oxidant-generating enzyme is responsible for pro-
duction of DNA damage resulting from ethanol. Liver
tissues that were available from two published studies14,16
were used. Genomic DNA and whole liver RNA were
isolated, and AP sites and expression of base excision
DNA repair genes were assessed. Consistent with our data
from studies in the rat (Fig. 1A), 4 weeks of intragastric
ethanol administration through the Tsukamoto-French
protocol caused an increase in AP sites in genomic DNA
in wild-type mice (Fig. 3A, +/−/ bars). Surprisingly, no
increase in AP sites was observed in Cyp2e1-null mice
(Fig. 3A, −/−/ bars). In addition, an increase similar to
that in wild-type mice was observed in NADPH oxidase-
deficient p47phox-null animals (Fig. 3B, −/−/ bars).

Analysis of expression of base excision repair genes in
wild-type mice treated with ethanol for 4 weeks showed a
response similar to that of the rat (Fig. 2). Namely, etha-
nol treatment caused a significant (P < .05) increase in
expression of the genes that are known to remove oxida-
tive DNA lesions: OGG1, N-methylpurine DNA glyco-
sylate, uracil DNA glycosylate, thymine DNA

Fig. 2. Ethanol causes an increase in expression of base excision DNA
repair genes in rat liver. Expression of base excision DNA repair genes in
livers of naive Wistar rats (Untreated), or animals fed high-fat diet (CON)
or high-fat ethanol-containing diet (ETH) for 4 weeks via intragastric
infusion (Tsukamoto-French protocol) was analyzed by (A) the RNase
protection assay (representative data). (B) The intensity of protected
bands was quantified using phosphor imaging and was normalized to the
intensity of housekeeping genes. Data shown are results of densitometry
analysis of images (mean ± SEM from three to four animals per group).
*Significant difference (P < .05, Student t test) compared with the
untreated group. OGG1, 8-oxoguanine DNA glycosylase/lyase 1; PARP,
poly (ADP-ribose) polymerase; APE, apurinic/apyrimidinic endonuclease 1;
Pol β, polymerase β; MPG, N-methylpurine DNA glycosylase; Pol δ,
polymerase δ; MGMT, O6-methylguanine-DNA methyltransferase; PCNA,
proliferating cell nuclear antigen.

Fig. 3. Cytochrome P450 CYP2E1, but not nicotinamide adenine
dinucleotide phosphate (NADPH) oxidase, is critical for accumulation of
apurinic/apyrimidinic (AP) sites resulting from ethanol treatment. The
number of AP sites was determined in genomic DNA from livers of
wild-type (+/−/), Cyp2e1-null (A, −/−/), or p47phox-null (B, −/−/ mice
that were given a high-fat diet (CON) or a high-fat diet containing ethanol
(ETH) for 4 weeks via intragastric infusion (Tsukamoto-French protocol).
Data are reported as means ± SEM from three to four animals per group.
*Significant difference (P < .05, Student t test) compared with the
corresponding strain control group.
The expression of BER genes was assessed, an increase similar to that in wild-type mice that were coadministered with the P450 inhibitor ABT. No elevation of BER gene expression was observed in wild-type mice fed ethanol diet and treated with ABT (Fig. 5B).

To test whether ethanol-induced activation of human CYP2E1 also would be associated with upregulation of expression of BER, hCyp2e1 transgenic mice were treated with ethanol diet for 4 weeks using the Tsukamoto-French protocol. Administration of ethanol resulted in a more than three-fold significant elevation in the activity of CYP2E1 in these livers (not shown), as well as a robust induction of hCYP2E1 protein in liver microsomes from hCyp2e1 transgenics (Fig. 4). When changes in expression of BER genes were assessed, an increase similar to that in wild-type mice and rats was observed in ethanol-fed hCyp2e1 transgenics compared with control-fed animals (Fig. 5A). Furthermore, the expression of BER genes was compared in livers of control- or ethanol-fed mice with that in wild-type mice that were coadministered with the P450 inhibitor ABT. No elevation of BER gene expression was observed in wild-type mice fed ethanol diet and treated with ABT (Fig. 5B).

**Table 2. Expression of Base Excision DNA Repair Genes in Livers of Cyp2e1-Null (−/−), p47phox-Null (−/−), or Corresponding Wild-Type (+/+) Mice That Were Given a High-Fat Diet or a High-Fat Diet Containing Ethanol for 4 Weeks via Intragastric Infusion (Tsukamoto-French Protocol) Analyzed by the RNase Protection Assay**

<table>
<thead>
<tr>
<th>DNA Repair Gene</th>
<th>Cyp2e1 (+/+</th>
<th>Cyp2e1 (−/−)</th>
<th>p47phox (+/+</th>
<th>p47phox (−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Ethanol</td>
<td>Control</td>
<td>Ethanol</td>
</tr>
<tr>
<td>OGG1 (8-oxoguanine DNA glycosylase)</td>
<td>4.4 ± 1.2</td>
<td>8.0 ± 0.7*</td>
<td>5.1 ± 0.2</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>UNG (uracil DNA glycosylase)</td>
<td>3.6 ± 0.4</td>
<td>14.3 ± 3.4*</td>
<td>4.8 ± 0.7</td>
<td>6.8 ± 1.2</td>
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<tr>
<td>MNG (N-methylpurine DNA glycosylase)</td>
<td>7.1 ± 1.6</td>
<td>11.4 ± 1.5*</td>
<td>7.6 ± 0.6</td>
<td>7.0 ± 1.4</td>
</tr>
<tr>
<td>T5D (thymine DNA glycosylase)</td>
<td>8.6 ± 0.8</td>
<td>12.2 ± 1.3*</td>
<td>7.5 ± 0.7</td>
<td>7.6 ± 0.8</td>
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<tr>
<td>NTH1 (endonuclease III homologue 1)</td>
<td>4.1 ± 0.6</td>
<td>5.1 ± 1.7</td>
<td>4.1 ± 0.4</td>
<td>3.0 ± 0.7</td>
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<tr>
<td>APE (apurinic/apyrimidinic endonuclease 1)</td>
<td>17.7 ± 2.2</td>
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<td>16.5 ± 1.5</td>
<td>11.5 ± 1.2</td>
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<tr>
<td>MGMT (O2-methylguanine-DNA methyltransferase)</td>
<td>10.2 ± 1.2</td>
<td>12.3 ± 1.6</td>
<td>10.9 ± 0.9</td>
<td>9.9 ± 0.9</td>
</tr>
</tbody>
</table>

**NOTE.** The intensity of protected bands was quantified using phosphor imaging and was normalized to the intensity of housekeeping genes. Data shown are results of densitometry analysis of images (mean values ± SEM from three to four animals per group). Abbreviations: Control, high-fat diet; Ethanol, ethanol-containing diet.

*Statistical difference (P < .05) from the corresponding control group by ANOVA analysis.
Discussion

This study shows that subchronic administration of ethanol to either rats or mice using a Tsukamoto-French enteral feeding protocol leads to accumulation of oxidative DNA damage. Here, we addressed the association between ethanol exposure and oxidative stress to DNA by drawing a parallel between (1) the measurements of oxidative DNA damage (i.e., oxidized purines and AP sites) that in the past may have been plagued by formation of additional damage during DNA isolation; and (2) assessment of changes in a biological response to oxidative DNA damage, namely, expression of base excision repair genes, a measurement that can not be enhanced artificially.

It should be noted that the cell’s response to DNA damage by upregulating repair pathways is not a completely innocuous process and may lead to accumulation of additional mutations. It has been suggested that accumulation of AP sites, overexpression of certain DNA repair genes, or both may result in enhancement of mutagenesis, as was observed in yeast and Escherichia coli. Importantly, it was shown recently that adaptive imbalance in base excision repair enzymes generates microsatellite instability in chronic inflammation in patients with ulcerative colitis, establishing a critical link between studies in single-cell model organisms and human disease. Thus, ethanol-induced upregulation of DNA repair genes also may contribute to long-term effects, such as cancer.

Much was learned about the molecular mechanisms of liver damage by ethanol in the last decade. Cell types proposed to be involved in the initiation and progression of the disease include not only hepatocytes, but also inflammatory cells (e.g., Kupffer cells, lymphocytes, and neutrophils) and activated stellate cells. One widely accepted hypothesis states that early alcohol-induced liver injury involves an increase in gut permeability to normal bacterial flora-derived endotoxin and significant elevation in circulating levels of endotoxin, leading to activation of hepatic resident macrophages, or Kupffer cells. Oxidant production by NADPH oxidase leads to activation of oxidant-sensitive signaling pathways (e.g., transcription factor nuclear factor-κB) in Kupffer cells that increases the formation of proinflammatory and cytotoxic cytokines, such as tumor necrosis factor-α. The cytokine- and adhesion molecule-mediated recruitment and activation of neutrophils and other immune cells exaggerates the damage to hepatocytes, leading to progression of organ damage. Although the role of Kupffer cell–derived oxidants in early alcohol-induced liver injury is widely accepted, the exact involvement of hepatocyte-derived sources of oxidants, such as CYP2E1, remains controversial.

CYP2E1 is known to be a major microsomal source of hydrogen peroxide and lipid peroxidation in liver (reviewed in Robertson et al). Based on the data presented here, we suggest a potential central role for hepatocyte CYP2E1 as a source of DNA damaging oxidants resulting from ethanol exposure. The strongest evidence for this argument comes from our findings that Cyp2e1-null or ABT-treated mice are protected from ethanol-induced DNA damage. Although the results from the ABT treatment experiments do not prove unequivocally the role of CYP2E1, in combination with the data from Cyp2e1-nulls, it is evident that induction of CYP2E1 by ethanol plays a major role in oxidative DNA damage in this model. Importantly, ethanol treatment–induced oxidative DNA damage correlates with an increase in expression and activity of CYP2E1. At the same time, liver injury still develops in Cyp2e1-null or ABT-treated mice. This suggests that the liver injury phenotype is mediated exclusively by endotoxin-induced activation of Kupffer cells and other innate immunity mechanisms, whereas P450 enzymes are extraneous to ethanol-induced organ damage.

Observations reported here strongly support an apparent complexity of the liver effects of ethanol: the site where oxidants are produced (Kupffer vs. parenchymal cells) and the outcome (liver injury vs. DNA damage). In fact, the usefulness of “generalization” of oxidative stress to the whole liver was questioned recently in a model of chemical-induced chronic oxidative stress in liver. Specifically, it was found that Kupffer and parenchymal cell oxidant-generating enzymes are involved in a separate way. Although Kupffer cells are activated rapidly by peroxisome proliferators and produce substantial amounts of oxidants and inflammatory cytokines, these events have no effect on genomic DNA damage in hepatocytes, where induction of “leaky” peroxisomal oxidases causes oxidative DNA lesions. These observations underscore the importance of careful consideration of molecular and cellular sources of oxidants in the studies on the mechanism of action of hepatotoxins.

Based on the data presented here, one can speculate that ethanol-induced steatohepatitis and oxidative stress to DNA seem to be unrelated molecular events, at least in this model of early alcohol-induced liver injury. It may seem counterintuitive to hypothesize that ethanol-associated activation of macrophages, recruitment of neutrophils, and increased production of inflammatory mediators in liver, all processes that involve production of oxidants, would not yield a measurable effect on oxidative DNA damage. There are several potential explanations
for the apparent divergence. First, reasonable doubt exists regarding reports that document erroneously high values for measured oxidative DNA damage in many previous studies with ethanol. Notably, our data are in agreement with consensus values established by the European Standards Committee on Oxidative DNA Damage. Second, careful examination of the evidence that seems to support the notion that macrophage-derived oxidants can reach genomic DNA in hepatocytes casts doubt on the conclusions. The coculture experiments in which activation of macrophages by endotoxin or cytokines led to hepatocellular DNA damage used an erroneously high proportion of macrophages to hepatocytes. Thus, further investigation is necessary to prove unequivocally or to discount the role of inflammatory cell-derived oxidants in DNA damage in whole liver.

It has been suggested that the development of both alcoholic and nonalcoholic steatohepatitis in Cyp2e1-null mice at least in part is the result of a compensatory induction of CYP4A isozymes and the oxidative stress that ensues. However, the link between CYP4A family members and oxidative stress in liver has been questioned by a recent report that demonstrated that induction of CYP4A10 and CYP4A14 is not associated with an increase in lipid peroxidation in liver. This observation is corroborated by the results from this study because, even though CYP4A enzymes are induced in Cyp2e1-null mice treated with ethanol, this compensatory mechanism does not lead to increased oxidative DNA damage.

In addition to evaluating the role of mouse CYP2E1 in oxidative DNA damage by using a knockout line, we also show that “humanized” mice that carry a knockout line of the gene in place of the mouse sequence (hCyp2e1) have a similar response. This important result demonstrates both qualitatively and quantitatively that the mechanism and extent of the ethanol-induced oxidative DNA damage is similar in mice and humans. It also strengthens the usefulness of the mouse as a model organism in studies of ethanol toxicity in liver.

In conclusion, our study demonstrates that ethanol exposure leads to formation of oxidative DNA lesions and results in adaptive changes in DNA repair gene expression. However, other important cellular signaling pathways most likely are also modulated by ethanol in response to induction of DNA damage. Thus, in addition to establishing the cellular and molecular mechanisms of ethanol-induced oxidative DNA damage in liver, additional studies are required to decipher the role of oxidative stress to DNA and its repair in the mechanisms of alcohol-associated hepatocellular carcinoma, as well as to determine what gene networks are modulated by ethanol in response to induction of DNA damage.

Acknowledgment: The authors thank Drs. James A. Swenберg and Jun Nakamura (University of North Carolina, Chapel Hill) for providing reagents for AP site and oxidized purines assays.

References

44. (ESCODD) European Standards Committee on Oxidative DNA Damage. Comparative analysis of baseline 8-oxo-7,8-dihydroguanine in mammalian cell DNA, by different methods in different laboratories: an approach to consensus. Carcinogenesis 2002;23:2129–2133.
Serum Bilirubin Levels and Mortality After Myeloablative Allogeneic Hematopoietic Cell Transplantation

Ted A. Gooley, Pankaj Rajvanshi, H. Gary Schoch, and George B. McDonald

Many patients who undergo hematopoietic cell transplantation experience liver injury. We examined the association of serum bilirubin levels with nonrelapse mortality by day +200, testing the hypothesis that the duration of jaundice up to a given point in time provides more prognostic information than either the maximum bilirubin value or the value at that point in time. We studied 1,419 consecutive patients transplanted from allogeneic donors. Total serum bilirubin values up to day +100, death, or relapse were retrieved—along with nonrelapse mortality by day +200 as an outcome measure—using Cox regression models with each bilirubin measure modeled as a time-dependent covariate. The bilirubin value at a particular point in time provided the best fit to the model for mortality. With bilirubin at a point in time modeled as an 8th-degree polynomial, an increase in bilirubin from 1 to 3 mg/dL is associated with a mortality hazard ratio of 6.42. An increase from 4 to 6 mg/dL yields a hazard ratio of 2.05, and an increase from 10 to 12 mg/dL yields a hazard ratio of 1.17. Among patients who were deeply jaundiced, survival was related to the absence of multiorgan failure and to higher platelet counts. In conclusion, the value of total serum bilirubin at a particular point in time after transplant carries more informative prognostic information than does the maximum or average value up to that point in time. The increase in mortality for a given increase in bilirubin value is larger when the starting value is lower.

During the process of undergoing hematopoietic cell transplantation, many patients experience hepatobiliary complications, including: sinusoidal obstruction syndrome (veno-occlusive disease); several cholestatic disorders (e.g., graft-versus-host disease [GVHD], cyclosporine cholestasis, cholangitis lenta, hepatotoxic drugs); hepatocellular injury caused by viruses, GVHD, drugs, and ischemia; iron overload; biliary obstruction; and infiltration of the liver by fungi or tumor cells.

We analyzed a cohort of 1,419 consecutive patients who received allogeneic transplants to assess the relationship of the level of jaundice to mortality after transplant. We made no effort to make precise diagnoses, but rather used total serum bilirubin as an index of the severity of underlying liver damage from all cumulative causes. Previous studies have clearly shown an association of jaundice with poor outcome in the setting of hematopoietic cell transplantation. However, these reports have generally considered only a single measure of bilirubin, and this measure has been modeled in a relatively restrictive manner using either dichotomous or linear variables. Because clinical experience had suggested that both the degree of jaundice and its duration were associated with increased mortality, we studied the relation of three bilirubin parameters—maximum value by a specified point in time, average value up to that point in time, and daily value at that time—to the hazard of day +200 nonrelapse mortality. We have not modeled any factors other than those associated with bilirubin, because consideration of nonbilirubin vari-
ables could confound the bilirubin effect that is of interest. We also analyzed patients who were deeply jaundiced to see if we could identify clinical and laboratory factors that predicted survival despite extreme hyperbilirubinemia.

**Patients and Methods**

**Hematopoietic Cell Transplantation**

All patients undergoing allogeneic transplantation received a myeloablative regimen followed by infusion of donor cells. The day of infusion was day 0, by convention. Graft recipients received prophylaxis against acute GVHD with immunosuppressive drugs—usually cyclosporine or tacrolimus plus methotrexate. Prophylaxis for infections included acyclovir, trimethoprim/sulfamethoxazole, oral fluconazole, and ganciclovir. Serum samples were collected for determination of total serum bilirubin at regular intervals through day +100. This retrospective analysis was performed under a protocol approved by our institutional review office.

**Patient Selection**

All recipients of allogeneic hematopoietic cells from 1993 through 1997 were evaluated.

**End Point and Bilirubin Parameter Definitions**

**Nonrelapse Mortality.** Day +200 nonrelapse mortality (NRM) was taken as the primary end point of this study. Failure to meet this end point was defined as any death within 200 days posttransplant that was not preceded by a relapse of the original malignancy.

**Total Serum Bilirubin Parameters.** Maximum serum bilirubin was defined as the highest observed level by a given point in time. The average bilirubin level was the arithmetic mean of the bilirubin values up to the day in question. The actual bilirubin level was the level on the day in question. For each of these bilirubin summary measures, all values from day of transplant to day +100 following transplant were recorded, along with the date of relapse of underlying malignancy or date of death. On days that no measurement was taken, the last measured bilirubin value was imputed.

**Statistical Methods**

To assess the association of each bilirubin measure with day +200 NRM, Cox regression models were fit with each bilirubin parameter as an explanatory variable. Patients who relapsed before day +200 were censored at time of relapse in the regression models. To assess the fit of each model, the Akaike Information Criterion (AIC) was calculated for the three models, where a smaller AIC implies a better fit to the data. The AIC was defined as $[-2\log L + 3p]$, where $\log L$ represents the log likelihood and $p$ represents the number of parameters contained in the regression model. The motivation behind this statistic is that if the only difference between two models is that one contains unnecessary covariates, the values of $-2\log L$ for the two models will not be very different (the value of the log likelihood will always decrease as additional covariates are added to the model), and as a result the value of the AIC will increase when unnecessary terms are added to the model (because the addition of $3p$ to the log likelihood will exceed the decrease in the log likelihood). The choice of the parameter $3$ in the AIC corresponds roughly to using a 5% significance level in comparing two nested models that differ by 1 to 3 parameters. Each bilirubin parameter was modeled as a time-dependent covariate. In addition to comparing the AICs for models containing only one of the bilirubin measures, models additionally containing a particular measure were compared to the model not containing this measure using the likelihood ratio test. These comparisons allow one to assess the additional contribution of each bilirubin parameter after other parameters have already been considered. In addition to assessing the association of bilirubin measures with outcome, many other clinical measurements were recorded among patients who were deeply jaundiced (total serum bilirubin $> 10$ mg/dL) at days +20 and +50. The association of these clinical variables with day +200 NRM was assessed in an effort to identify parameters associated with survival despite hyperbilirubinemia.

**Results**

**Description of the Study Cohort.** During the study period, 1,419 patients received an allogeneic transplant; their demographics are given in Table 1. Among these
patients, 419 (30%) died before day +200 without a prior relapse of disease. Fourteen of these deaths occurred between day 0 and day +10; the remaining 405 occurred between days +10 and +200.

### Table 2. Frequency of NRM at Day +200 After Allogeneic Hematopoietic Cell Transplant as a Function of Total Serum Bilirubin Values at 10-Day Intervals (N = 1,419)

<table>
<thead>
<tr>
<th>Total Serum Bilirubin (mg/dL)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
<th>Day 40</th>
<th>Day 50</th>
<th>Day 60</th>
<th>Day 70</th>
<th>Day 80</th>
<th>Day 90</th>
<th>Day 100</th>
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<td>38/415</td>
<td>39/472</td>
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<td>(8%)</td>
<td>(8%)</td>
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<td>(5%)</td>
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<td>(6%)</td>
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<td>10–13</td>
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<td>8/9</td>
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</table>

**NOTE.** Boldface data indicate cumulative summaries.

*Values represent the number of patients who died of nonrelapse causes before day +200, divided by the number of patients who were alive without relapse at the time noted (that is, days 10 through 100 after transplant), thus giving the percentage of patients at risk who later died from nonrelapse causes.

Modeling Bilirubin Parameters as Time-Dependent Covariates With Regard to Day +200 NRM. Table 2 displays the proportion of patients who died before day +200 without a prior relapse among those who

### Table 3. Total Serum Bilirubin Parameters as nth-Degree Polynomials and Their Fit to Day +200 NRM as Determined by the AIC

<table>
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<tr>
<th>Summary Measure</th>
<th>n</th>
<th>AIC</th>
<th>P Value</th>
</tr>
</thead>
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<tr>
<td>Daily total serum bilirubin</td>
<td>5</td>
<td>4,737</td>
<td></td>
</tr>
<tr>
<td>Daily total serum bilirubin*</td>
<td>6</td>
<td>4,691</td>
<td>&lt;.00001, 5th-degree vs. 8th-degree</td>
</tr>
<tr>
<td>Daily total serum bilirubin</td>
<td>9</td>
<td>4,699</td>
<td>.52, 8th-degree vs. 9th-degree</td>
</tr>
<tr>
<td>Maximum total serum bilirubin*</td>
<td>5</td>
<td>5,007</td>
<td></td>
</tr>
<tr>
<td>Maximum total serum bilirubin</td>
<td>6</td>
<td>5,008</td>
<td>.17, 5th-degree vs. 6th-degree</td>
</tr>
<tr>
<td>Average total serum bilirubin*</td>
<td>5</td>
<td>5,114</td>
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</tr>
<tr>
<td>Average total serum bilirubin</td>
<td>6</td>
<td>5,117</td>
<td>.79, 5th-degree vs. 6th-degree</td>
</tr>
</tbody>
</table>

*Best model for each summary measure.
were alive without relapse on days 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 as a function of the total serum bilirubin on these specific days. There is a substantial increase in NRM as the bilirubin level is increased, and a relatively large increase in mortality as the bilirubin level reaches above 4 mg/dL. Each of the three bilirubin measures was statistically significantly associated with the hazard of death $H_{200}$, and the association of each appeared to be monotone non-decreasing (data not shown). That is, increases in each of the parameters led to an increase in the hazard of NRM, although this increase levels off as the value of the parameter increases. In addition, these associations did not appear to depend on time after transplant (data not shown). Table 3 shows the results of modeling each measure as an nth-degree polynomial, where $n$ was chosen to be the highest value that led to the lowest AIC for a particular measure. A linear model for a particular bilirubin parameter assumes that the association for a particular increase in the measure is the same for all increases of this magnitude, regardless of the initial value for the measure. Use of a polynomial function in modeling allows nonlinear features to be captured; this is the motivation for the use of an nth-degree polynomial.

Figure 1 shows hazard ratios for relative increases in total serum bilirubin values of 1, 2, and 3 mg/dL when the daily bilirubin value is modeled as a continuous 8th-degree polynomial. For example, Fig. 1A shows that under this model, as the bilirubin value increases from 1 mg/dL to 2 mg/dL, there is an accompanying increase in the hazard of death $H_{200}$ of 190% (corresponding to a hazard ratio of 2.90). Note that for a specified starting bilirubin value, the hazard ratios increase when the relative increases in bilirubin get larger. For example, if a starting bilirubin value of 2 mg/dL is isolated on Fig. 1A (reflecting a change from 2 mg/dL to 3 mg/dL), Fig. 1B (a change from 2 mg/dL to 4 mg/dL), and Fig. 1C (a change from 2 mg/dL to 5 mg/dL), the corresponding hazard ratios increase from 2.21 to 3.95 to 6.01, respectively. For a specified magnitude of increase (e.g., an increase of 2 mg/dL [Fig. 1B]), the hazard ratios decrease as the starting bilirubin level increases. In other words, the hazard of NRM is increased less and less (although still increased, as demonstrated by a hazard ratio greater than 1.0 throughout) for specified rises in bilirubin as the starting level gets larger. For example, an increase in bilirubin from 1 mg/dL to 3 mg/dL is associated with a hazard ratio of 6.42, while increases in bilirubin from 4 mg/dL to 6 mg/dL and from 10 mg/dL to 12 mg/dL are associated with hazard ratios of 2.05 and 1.17, respectively.

Table 4. Results From Likelihood Ratio Test Comparing Nested Models

<table>
<thead>
<tr>
<th>Models Compared</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>5th-degree average + nth-degree daily vs.</td>
<td>&lt; .00001</td>
</tr>
<tr>
<td>5th-degree average</td>
<td></td>
</tr>
<tr>
<td>5th-degree maximum + nth-degree daily vs.</td>
<td>&lt; .00001</td>
</tr>
<tr>
<td>5th-degree maximum</td>
<td></td>
</tr>
<tr>
<td>8th-degree daily + nth-degree maximum vs.</td>
<td>.87 (n = 1), .65 (n = 5)</td>
</tr>
<tr>
<td>8th-degree daily</td>
<td></td>
</tr>
<tr>
<td>8th-degree daily + nth-degree average vs.</td>
<td>.05 (n = 1), .08 (n = 2)</td>
</tr>
<tr>
<td>8th-degree daily</td>
<td></td>
</tr>
</tbody>
</table>
Although this modeling indicates that the daily bilirubin measure fits the data better than the other two measures, these other measures may still add important information to the model that contains only the daily value. Table 4 summarizes the results from comparing various models via the likelihood ratio test. These results suggest that what matters most for predicting subsequent outcome is the actual bilirubin value at a particular point in time, and the route through which this value was reached is less important.

The association of daily bilirubin values with day/H11001/H1200 NRM appeared to be relatively independent of the average bilirubin value and the maximum value. If a linear term for the average value is added to the 8th-degree polynomial for daily value and an interaction between the linear terms is fit, the interaction term was not significant \((P = .28)\). The same holds if a linear term for the maximum value is added \((P = .41)\). Moreover, the association of the daily bilirubin value with outcome was not demonstrably different according to donor type, source of stem cells, severity of disease, or age at transplant (data not shown).

**Patients With Extreme Hyperbilirubinemia \( (>10 \text{ mg/dL})\).** Among the 1,419 patients studied, there were 292 (21%) whose total serum bilirubin exceeded 10 mg/dL before day +100. The NRM rate by day +200 among these patients was 230 (79%) of 292, compared with 189 (17%) of 1,127 among patients whose bilirubin never exceeded 10 mg/dL. The distribution of the day on which the serum bilirubin level first exceeded 10 mg/dL for these patients is shown in Fig. 2. This threshold was reached before day +20 in 156 patients (53%), between days +20 and +40 in 75 patients (26%), and after day +40 in 61 patients (21%). Sinusoidal obstruction syndrome (formerly known as venoocclusive disease of the liver) was the cause for most of the cases of extreme hyperbilirubinemia that occurred before day +20, and acute GVHD was the most common cause after day +40.

Although most patients who were deeply jaundiced died from nonrelapse causes before day +200, there were some who survived. We examined clinical parameters during a 10-day window from day +10 to +20 among patients who were deeply jaundiced at day +20 in an effort to identify a cohort of individuals that was at decreased risk of dying from nonrelapse causes by day +200. Table 5 displays the clinical factors at or before day +20 that were statistically significantly different among 14 survivors compared with 48 patients.
who died. Patients who survived had a lower frequency of multiorgan failure and were more likely to be platelet transfusion–independent. There were no statistically significant differences in a large number of other clinical factors between survivors and nonsurvivors, including bilirubin parameters, the underlying hematological malignancy, conditioning regimen, HLA match, GVHD prophylaxis, engraftment, documented infection, presence of GVHD, hematocrit, or any other laboratory test (data not shown). Analysis of 41 patients whose total bilirubin level was greater than 10 mg/dL at day +50 yielded similar results (data not shown); there were only 9 survivors from this cohort.

Discussion

Total serum bilirubin has previously been shown to be associated with increased mortality following hematopoietic cell transplant. Clinical experience has suggested that the totality of liver injury as measured by the duration of jaundice up to a point in time would prove to be a better indicator of subsequent outcome than only the bilirubin value at that particular point or the maximum value achieved by that time. We formally tested this hypothesis by using Cox regression models to represent each of these summary measures as a time-dependent covariate. In addition, each summary measure was modeled in a flexible manner, allowing for nonlinear associations between the measure and outcome.

Each of the summary measures that we examined was positively correlated with the hazard of day +200 NRM in the time-dependent models. The total serum bilirubin value at a particular point in time was more important in predicting subsequent outcome than either the average value or maximum value up to that point in time. Moreover, once the bilirubin level at a particular point in time is known, additional knowledge of either the maximum or the average bilirubin value from transplant up to that point in time did not lead to large improvements in the model. Thus, the route by which a patient reaches a specific serum bilirubin level is far less important than what the level is at that time. Relatively small increases in bilirubin are associated with a relatively large increase in mortality when the starting bilirubin is relatively low. Although these latter findings may be intuitively apparent, quantification of these associations provide clinicians with guidelines for determining when additional therapies should be attempted, and for defining futility of further treatment.

In many situations, development of jaundice in very ill patients with liver disease is well recognized as a sign of a poor prognosis. The question of how hyperbilirubinemia confers an adverse prognosis after hematopoietic cell transplant cannot be completely addressed by these data. The simplest explanation is that total serum bilirubin is a marker for several serious conditions that are known to have an adverse outcome, such as sinusoidal obstruction syndrome, GVHD, sepsis syndrome, and renal failure. An alternative but not mutually exclusive explanation is that liver dysfunction per se leads to morbidity. There is compelling evidence in both animals and man that liver dysfunction causes neurological, renal, cardiovascular, and pulmonary dysfunction. In the setting of hematopoietic cell transplant, pulmonary and renal dysfunction are statistically more frequent among patients with liver injury and commonly follow development of jaundice by days to weeks. Our data show a relatively constant relation between levels of jaundice and mortality across time, even though the causes of jaundice differ over time, suggesting that the cause of jaundice is not as important in prognosis as its degree.

These findings have several implications for the care of patients undergoing allogeneic transplantation. Because treatment of transplant patients who become deeply jaundiced can be futile, emphasis should be on early recognition and treatment of liver injury and on modifying the transplant process to prevent liver injury. New methods for preparing patients for infusion of stem cells should result in less regimen-related liver damage than with the standard regimens used in this cohort of patients. For example, myeloablative preparative regimens that contain busulfan are now dosing this drug to a metabolic end point or giving an intravenous formulation of busulfan. A study of the pharmacokinetics of cyclophosphamide has shown that its metabolism is highly variable and that liver toxicity is related to increased exposure to toxins of cyclophosphamide metabolism as well as the dose of irradiation. Liver toxicity might be reduced by dosing cyclophosphamide according to its metabolism or by replacing cyclophosphamide with another drug that has minimal liver toxicity (e.g., fludarabine), and by limiting the dose of total body irradiation. There is minimal regimen-related liver toxicity from nonmyeloablative conditioning regimens (e.g., fludarabine plus total body irradiation 200 cGy). Prophylactic use of oral ursodiol at 10 to 15 mg/kg/d can reduce the frequency of liver injury in patients undergoing transplantation, presumably by preventing hepatocyte damage that results from prolonged cholestasis. Prophylaxis with ursodiol may also confer a survival benefit.

An aggressive approach to diagnosis and treatment should be undertaken when the total serum bilirubin level rises above normal. Early treatment of sinusoidal obstruction syndrome may alter the prognosis of this disease. Patients with evidence of cholestatic liver
injury who are not receiving ursodiol should be started on this drug.39 When the differential diagnosis of liver disease includes infections that can be treated—such as hepatitis B virus, varicella zoster virus, herpes simplex virus, adenovirus, cytomegalovirus, or a fungal or mycobacterial process—transvenous liver biopsy should be performed to ascertain the cause.43,44 In acute GVHD, evidence of T-cell–mediated apoptosis of small bile ducts and ductopenia are diagnostic features that should lead to treatment.45,46 Some drugs in common use in the transplant setting are potential liver toxins (e.g., trimethoprim-sulfamethoxazole, itraconazole, voriconazole, and fluconazole).1 Cyclosporine and tacrolimus may elevate serum methoxazole, itraconazole, voriconazole, and fluconazole.1

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References


MELD Accurately Predicts Mortality in Patients With Alcoholic Hepatitis


Assessing severity of disease in patients with alcoholic hepatitis (AH) is useful for predicting mortality, guiding treatment decisions, and stratifying patients for therapeutic trials. The traditional disease-specific prognostic model used for this purpose is the Maddrey discriminant function (DF). The model for end-stage liver disease (MELD) is a more recently developed scoring system that has been validated as an independent predictor of patient survival in candidates for liver transplantation. The aim of the present study was to examine the ability of MELD to predict mortality in patients with AH. A retrospective cohort study of 73 patients diagnosed with AH between 1995 and 2001 was performed at the Mayo Clinic in Rochester, Minnesota. MELD was the only independent predictor of mortality in patients with AH. MELD was comparable to DF in predicting 30-day mortality (c-statistic and 95% CI: 0.83 [0.71-0.96] and 0.74 [0.62-0.87] for MELD and DF, respectively, not significant) and 90-day mortality (c-statistic and 95% CI: 0.86 [0.77-0.96] and 0.83 [0.74-0.92] for MELD and DF, respectively, not significant). A MELD score of 21 had a sensitivity of 75% and a specificity of 75% in predicting 90-day mortality in AH. In conclusion, MELD is useful for predicting 30-day and 90-day mortality in patients with AH and maintains some practical and statistical advantages over DF in predicting mortality rate in these patients. MELD is a useful clinical tool for gauging mortality and guiding treatment decisions in patients with AH, particularly those complicated by ascites and/or encephalopathy.

Alcoholic hepatitis (AH) is an acute, inflammatory syndrome associated with significant morbidity and mortality that occurs in a subset of patients that consume excessive amounts of alcohol.1 Mild forms of AH improve with conservative management, whereas more severe AH is associated with substantial mortality.2 Pharmacological therapies including corticosteroids and pentoxifylline have been proposed for patients with more severe disease.3-6 Consequently, the a priori identification of subsets of patients with significant disease who will not improve with conservative therapy is an area of active investigation.

The Maddrey discriminant function (DF) (DF = 4.6 × [prothrombin time (PT) in seconds − control PT] + serum bilirubin in mg/dL) was introduced in 1978 as a tool for predicting risk for mortality in AH and thereby identifying a subset of patients that may benefit from intervention with corticosteroids.7 Based on these analyses, corticosteroid treatment is advocated by many clinicians for patients with a DF score of more than 32, because these patients appear to have a mortality rate exceeding 50% in the absence of pharmacological intervention.4,8 This criterion was also used in a recent clinical trial evaluating the potential clinical efficacy of pentoxifylline in AH.5 However, use of a DF score of more than 32 for intervention has some drawbacks: (1) DF uses the PT, a variable that is poorly standardized across different laboratories; (2) initial validation of DF relationship to mortality is based on patient cohorts from several decades past; and (3) patients with a DF score of more than 32 may still have a notable risk of death of up to 17%.9-11 In addition to the DF, other studies have identified clinical
variables, most notably hepatic encephalopathy, as a risk factor for mortality in AH as well. However, these models rely heavily on subjective parameters, which render them less optimal.\(^3\)\(^{12}\)

The model for end-stage liver disease (MELD) is a survival model based on a composite of three laboratory variables: serum creatinine, serum bilirubin, and international normalized ratio (INR) for PT. The model was originally derived from a cohort of 231 patients to assess the short-term prognosis of patients with cirrhosis undergoing elective transjugular intrahepatic portosystemic shunt at four centers in the United States.\(^13\) This model was subsequently validated as an independent predictor of survival in several independent cohorts of patients with cirrhosis.\(^13\)^\(^{16}\) Because of the ability of MELD to accurately stratify patients according to mortality risk, it has now replaced the Child-Turcotte-Pugh score to prioritize and rank organ allocation of cadaveric livers for transplantation on the United Network for Organ Sharing liver waiting list.\(^14\)^\(^{16}\) The variables in MELD include PT and bilirubin, which are also included in the DF score; however, the bilirubin and PT are weighted appropriately based on extensive validation studies and are expressed as logarithm values to avoid extreme values, unduly influencing the results. Moreover, MELD—but not DF—includes serum creatinine as a variable. Elevated serum creatinine has been shown to be associated with poor outcome in patients with AH.\(^17\) Because of the demonstrated capacity of MELD to discriminate patients with cirrhosis—and in conjunction with the aforementioned limitations in current disease-specific models used to predict mortality in patients with AH—the aim of the current study was to develop, characterize, and validate a MELD-based strategy to predict mortality in AH.

**Patients and Methods**

**Patient Information.** This was a Mayo Clinic Institutional Review Board–approved retrospective cohort study of patients with a diagnosis of AH (ICD-9 code 571.1) who were seen at the Mayo Clinic Rochester inpatient or outpatient facilities between January 1, 1995, and December 31, 2001. In all potential cases, inpatient and outpatient records were reviewed and demographic, clinical, and laboratory data were extracted. The presence of acute AH was confirmed via clinical and laboratory criteria that included the following: (1) alcohol consumption within 2 months and exceeding 40 g/d for male and 20 g/d for female patients; (2) an aspartate/alanine aminotransferase ratio above 1.5 with an aspartate aminotransferase level above 45 U/L; (3) a total bilirubin level above 2 mg/dL; and (4) absence of an alternative primary cause of liver disease based on clinical history and serological studies (11 patients with AH had underlying viral hepatitis but were not excluded because the clinical basis of the admission/visit was due to AH). These threshold levels allowed for the inclusion of patients with both mild and severe AH. A total of 182 patients were diagnosed with AH based on ICD coding. After reviewing the medical record, 98 of these patients met the entry criteria of the study as outlined above. Of these 98 patients, 73 had all the requisite laboratory data (PT, INR, serum creatinine, and serum total bilirubin within 24 hours of presentation) and comprised the final patient sample. The first available laboratory tests within 24 hours of presentation were used to calculate baseline MELD and DF scores. Laboratory data to calculate MELD on day 7 following admission were available in 27 patients. The change in MELD—day 7 MELD — MELD on admission—was defined as \( \Delta \text{MELD} \). For patients with more than one episode of AH in the time period, only the initial episode was included. Presence or absence of ascites and encephalopathy were based on physical examination findings described in the chart by the primary care physician. Survival was verified with hospital record and social security death index.

**Statistical Analyses.** The statistical end point was death due to any cause within 90 days of the hospital admission. Univariate logistic regression was used to screen the variables reported in Table 1 for associations with 90-day mortality. Variables that were statistically significant formed a pool of potential independent predictors that are reported in Table 2. These predictors were entered into a backward elimination variable selection procedure (logistic regression); the criteria for retaining predictors was a \( P \) value less than .05. This variable selection process was repeated using forward variable selection procedure. Concordance (range, 0.0-1.0) is equivalent to the area under the receiver operating characteristic curve and quantifies the prognostic validities of variables. The concordance (c) statistics of MELD and DF were compared using Delong’s test.\(^18\) Thirty-day c-statistics of MELD and DF were also calculated for comparison, because this was the time point for which the DF score was originally derived.\(^7\) The statistical program used was SAS version 8.0 (Cary, NC). MELD was calculated using the following formula: \( \text{MELD} = 9.57 \times \log_2(\text{Cr mg/dL}) + 3.78 \times \log_2(\text{bili mg/dL}) + 11.20 \times \log_2(\text{INR}) + 6.43 \).\(^{16}\) The probability of 90-day mortality in AH was calibrated using the data from logistic regression (\( P = e^{(-4.3 + 0.16 \times \text{MELD})} / [1 + e^{(-4.3 + 0.16 \times \text{MELD})}] \)).

**Results**

**Patient Demographics and Variables Associated With Mortality on Univariate and Multivariate Analysis.** Demographic, clinical, and laboratory variables from the initial patient presentation retrieved from
the patient record and analyzed with univariate logistic regression are outlined in Table 1. The patient cohort consisted of 73 patients with a median age of 47 years (range, 24-65), with 16 deaths occurring in this group by 90 days. Thirty-three patients had cirrhosis based on histology or imaging. All biopsies were consistent with alcoholic hepatitis. Eleven patients had liver-relevant diagnoses concomitant to AH, including hepatitis C virus, hepatitis B virus, and recent acetaminophen consumption. These patients were not excluded because the reason for their current illness, based on the hospital record and the retrospective chart review, was solely due to alcoholic hepatitis. The vast majority of the patient cohort did not receive active pharmacotherapy, consistent with standard practice at our institution; however, 12 patients did receive active therapies including corticosteroids and etanercept in the context of a clinical trial.

Table 1. Demographic, Clinical, and Laboratory Variables Analyzed With Univariate Logistic Regression

<table>
<thead>
<tr>
<th>Demographic Data</th>
<th>History of Present Illness</th>
<th>Past Medical History</th>
<th>Physical Examination</th>
<th>Laboratory Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (47 ± 10)</td>
<td>Amount of alcohol consumption (139.8 ± 141.1 g/d)</td>
<td>Hypertension (23.3%)</td>
<td>Fever (13.4% ≥38°C)</td>
<td>Total bilirubin (12.1 ± 12.0 mg/dL)</td>
</tr>
<tr>
<td>Sex (31.5% female)</td>
<td>Fatigue (37.0%)</td>
<td>Coronary artery disease (8.2%)</td>
<td>Encephalopathy (23.3%)</td>
<td>Creatinine (1.1 ± 1.0 mg/dL)</td>
</tr>
<tr>
<td>BMI (28.1 ± 6.0)</td>
<td>Anorexia (27.4%)</td>
<td>Renal insufficiency (8.2%)</td>
<td>Ascites (57.5%)</td>
<td>ALT (68.6 ± 61.9 U/L)</td>
</tr>
<tr>
<td></td>
<td>Nausea/vomiting (34.3%)</td>
<td>Diabetes mellitus (2.7%)</td>
<td>Hepatomegaly (71.2%)</td>
<td>AST (244.3 ± 218.7 U/L)</td>
</tr>
<tr>
<td></td>
<td>Abdominal pain (27.4%)</td>
<td>Concomitant viral hepatitis (15.0%)</td>
<td>Spleenomegaly (26.0%)</td>
<td>Albumin (6.6 ± 11.6 g/dL)</td>
</tr>
<tr>
<td></td>
<td>Weight loss (28.8%)</td>
<td></td>
<td>Spider angioma (50.7%)</td>
<td>Platelet count (161.0 ± 97.2 × 10^9/L)</td>
</tr>
<tr>
<td></td>
<td>Diarrhea (13.7%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Gastrointestinal bleeding (21.9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Concurrent acetaminophen use &gt;1 g/d (4.1%)</td>
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</table>

NOTE. Values reported are percentage or mean ± standard deviation of patients with the finding.
Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WBC, white blood cell.

Table 2. Risk Factors Associated With 90-Day Mortality

<table>
<thead>
<tr>
<th>Univariate Logistic Regression</th>
<th>Multivariate Logistic Regression (MELD + Risk Factor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odds Ratio (95% CI)</td>
<td>Odds Ratio (95% CI)</td>
</tr>
<tr>
<td>MELD score 1.17 (1.08–1.26)</td>
<td>1.01 (0.98–1.05)</td>
</tr>
<tr>
<td>DF 1.04 (1.02–1.07)</td>
<td>2.39 (0.26–21.96)</td>
</tr>
<tr>
<td>History of renal insufficiency 9.17 (1.50–55.93)</td>
<td>2.39 (0.26–21.96)</td>
</tr>
<tr>
<td>Gastrointestinal bleeding 4.15 (1.23–14.02)</td>
<td>2.27 (0.52–9.96)</td>
</tr>
<tr>
<td>Edema 6.00 (1.70–21.11)</td>
<td>3.00 (0.72–12.39)</td>
</tr>
<tr>
<td>Encephalopathy 7.87 (2.28–27.18)</td>
<td>2.67 (0.61–11.68)</td>
</tr>
<tr>
<td>Asci 7.25 (1.51–34.85)</td>
<td>3.63 (0.65–20.20)</td>
</tr>
<tr>
<td>Total bilirubin 1.08 (1.03–1.14)</td>
<td></td>
</tr>
<tr>
<td>Creatinine 2.00 (1.10–3.64)</td>
<td></td>
</tr>
<tr>
<td>INR 13.36 (2.72–65.52)</td>
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</tr>
</tbody>
</table>

* c-Statistic associated with MELD alone. The P value of MELD remained <.05 when MELD was combined with any risk factor in multivariate analysis.
accuracy. Table 2 shows that when the six remaining variables were added to MELD one at a time using forward selection, all six variables lost significance, and the combined (c) statistics did not significantly improve compared with MELD alone.

Validation and Calibration of MELD as an Independent Predictor of Mortality. Because MELD emerged as the only independent predictor of mortality in the multivariate analysis, we next sought to further validate and calibrate MELD to predict mortality in AH. First, the probability of 90-day mortality in AH was calibrated using the data from logistic regression (\( P = \frac{e^{-4.3 + 0.16 \times \text{MELD}}}{1 + e^{-4.3 + 0.16 \times \text{MELD}}} \)). Figure 1 depicts the plotted curve demonstrating the estimated mortality for a given MELD score using this data. Next, receiver operating characteristic curves were generated to compare MELD with DF for 30-day mortality, the time point for which the DF was originally derived, as well as to examine the prognostic use of MELD for predicting an extended 90-day mortality. For 30-day mortality (Fig. 2A), the c-statistic was 0.83 (95% CI: 0.71-0.96) for MELD and 0.74 (95% CI: 0.62-0.87) for DF, with the optimal cut points of 22 and 41 for MELD and DF, respectively. For 30-day mortality using the optimal cut point, MELD had a sensitivity of 0.75 and a specificity of 0.75, while DF had a sensitivity of 0.75 and a specificity of 0.69. For 90-day mortality (Fig. 2B), the c-statistic was 0.86 (95% CI: 0.77-0.96) for MELD and 0.83 (95% CI: 0.74-0.92) for DF, with the optimal cut points of 21 and 37 for MELD and DF, respectively. For 90-day mortality using the optimal cut point, MELD had a sensitivity of 0.75 and a specificity of 0.75, while DF had a sensitivity of 0.88 and a specificity of 0.65. The differences in c-statistic between MELD and DF were not statistically significant for 30-day or 90-day mortality. Furthermore, the addition of other variables on physical examination and biochemical testing that emerged from the univariate analysis did not significantly increase the c-statistic achieved by MELD alone in predicting mortality (Table 2).

These analyses suggest an equivalency of MELD to DF rather than a substantive advantage of one of the parameters. To further address the question of whether MELD scores may diverge from DF in some select patients, we plotted MELD and DF scores in patients that survived and patients that died at 30 days (Fig. 3). Visual inspection of this plot demonstrates that while DF largely correlates with MELD at lower values, at higher values, many patients have disproportionally higher MELD score com-
pared to DF. Among these patients, deaths appear to track more closely with MELD rather than DF.

**Ascites and Encephalopathy as Predictors of Mortality.** Because the physical examination findings of ascites and encephalopathy were significantly associated with mortality in the univariate analysis and are routinely assessed in patients with AH, these two variables were further examined as predictors of 90-day mortality. Table 3 shows the 90-day mortality of patients who evidenced presence and absence of ascites and/or encephalopathy. Notably, patients that lacked both ascites and encephalopathy had 100% survival at 90 days. However, survival was highly variable in the majority of patients that evidenced the presence of one or both of the physical examination findings of ascites and encephalopathy, highlighting the need for prognostic models such as MELD in this patient population.

### Discussion

This study was undertaken to examine the accuracy of MELD in predicting mortality in patients with AH and thereby optimize strategies to prognosticate patients. The traditional disease-specific formula used to predict 30-day mortality in patients with AH is the DF.7 In this study, the c-statistics calculated to compare prognostic validity of MELD and DF in AH were comparable for 30-day as well as 90-day mortality. Interestingly, in our multivariate logistic regression, MELD, not DF, emerged as the only independent predictor of 90-day mortality. This may be due to the incorporation of serum creatinine in MELD. Serum creatinine correlates with survival in a number of disease states, including AH.10,17 Although a recent study demonstrated that temporal changes in serum bilirubin during the course of AH are effective in predicting subsequent patient mortality,20 in our study the baseline MELD was the best independent predictor of subsequent mortality, while interval changes in MELD (ΔMELD)21 did not emerge as a predictor of mortality in the univariate analysis. Indeed, no other variables were significantly associated with mortality after correction for MELD; furthermore, no other variables augmented the capacity of MELD to predict mortality. The optimal cut point of 21 was obtained for MELD in the 90-day receiver operating characteristic mortality analyses, with a c-statistic that was similar to the DF. A series published by Sheth et al.22 recently provided initial evidence that MELD may be useful in estimating 30-day prognosis in patients with AH; a more recent analysis of MELD conducted by Said et al.23 across a broad spectrum of liver disease, which included a cohort of patients with AH, also supports this concept. The current study, performed in a large characterized cohort, establishes the ability of MELD to rank patients with AH by risk of death and provides evidence that MELD accurately predicts mortality up to 90 days. Although prospective validation would be required to definitively prove or disprove that MELD exceeds DF in predicting mortality in AH, some practical points favoring the use of MELD in this setting should be considered.

In the current era, with laboratories using INR as a standardized analysis of coagulation status rather than PT expressed in seconds, there are tangible benefits in using predictive formulas that use INR, such as MELD. The PT expressed as INR is comparable across all laboratories, because the calculation accounts for the sensitivity of the thromboplastin reagent used in the test. In contrast, PT expressed in seconds is highly dependent on the sensitivity of the thromboplastin used. Therefore, the same patient may have markedly variable values for PT expressed in seconds from laboratory to laboratory if varying sensitivities of thromboplastin are used.21 For instance, a patient with a PT of 12.6 seconds with a thromboplastin sensitivity of 3 will have a PT of 14 seconds with a thromboplastin sensitivity of 2, and a PT of 20 seconds if thromboplastin of sensitivity 1 is used. Furthermore, DF was developed several decades ago7 compared with MELD, which has been prospectively and retrospectively validated in heterogeneous cohorts of patients derived from the current era.13-16 With the increasing use of MELD in many applications, most prominently in the replacement of the Child-Turcotte-Pugh score for allocation of liver allografts,15 many convenient approaches to calculate MELD have arisen, including the maintenance of the formula on personal handheld computers—or, alternatively, the use of a common Web site calculator. Our analysis suggests a use for this calculation to estimate mortality in AH patients, and a website is available to estimate MELD-based mortality in patients with AH (http://www.mayoclinic.org/gi-rst/mayomodel7.html).

An important consideration in the use of prognostic models in patients with AH is to allow for the determination of which patients should undergo therapy with biologically active medications versus which patients should be managed supportively with the anticipation of spontaneous improvement. In this regard, a DF score of more

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<thead>
<tr>
<th>Encephalopathy</th>
<th>Ascites</th>
<th>90-Day Mortality/ Total Number (%)</th>
<th>Mean ± SD (Range of MELD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>7/9 (78)</td>
<td>29 ± 9 (17-38)</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>7/33 (21)</td>
<td>17 ± 8 (6-36)</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>2/8 (25)</td>
<td>21 ± 8 (7-33)</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>0/23 (0)</td>
<td>10 ± 4 (0-18)</td>
</tr>
</tbody>
</table>
than 32 predicts a mortality exceeding 50% and has traditionally been used as an indication for corticosteroid therapy. This criterion has been frequently used as an inclusion criterion in new treatment trials of patients with AH, as well. However, a significant minority of patients (10%-17%) with a DF score below 32 may still die from AH. Because the rationale for the cut point of 50% mortality as a trigger for treatment was based on the risk-benefit ratio specific to corticosteroids, if an effective therapy was available with a lower adverse event profile than corticosteroids, the therapeutic intervention could be initiated at a cut point of mortality lower than 50%. In this regard, the present study identifies a MELD score of 21 as having the highest sensitivity and specificity to predict mortality with an estimated 90-day mortality of 20% for patients with this score. Thus, patients with AH and a MELD score of more than 21 could be considered for entry into studies addressing the use of potential therapeutic agents, although the specific MELD cut point used may depend on the degree of treatment-related mortality.

Interestingly, our analyses suggest that physical examination signs of encephalopathy and ascites may be useful as a quick bedside screening test for mortality in AH. According to our patient sample, in the absence of encephalopathy or ascites, 90-day mortality approaches 0, thereby reducing the use of detailed prognostic calculations to predict mortality. Although the absence of encephalopathy and ascites were useful screening tests in this context, many patients with AH do have one or both of these findings. Furthermore, the clinical diagnoses of encephalopathy and ascites can be subjective, as evidenced by large interobserver variability. Indeed, these limitations in accurately detecting the presence or absence of these physical examination parameters was a major reason that MELD was adopted as a nonbiased approach to prioritize and rank organ allocation of cadaveric livers for transplantation on the United Network for Organ Sharing liver waiting list.

In summary, MELD is useful for predicting 30-day and 90-day mortality in patients with AH and maintains some practical and statistical advantages over DF in predicting mortality rate in these patients. These data suggest that in patients with AH—particularly those complicated by ascites, encephalopathy, or both—MELD is a useful clinical tool with which to gauge mortality and guide treatment decisions.

References
Incidence, Natural History, and Risk Factors for Biliary Sludge and Stones During Pregnancy

Cynthia W. Ko,1 Shirley A. A. Beresford,2 Scott J. Schulte,3 Alvin M. Matsumoto,1,4 and Sum P. Lee1

Gallstones are strongly associated with higher parity in women. This study prospectively assessed the incidence, natural history, and risk factors for biliary sludge and stones during pregnancy and the postpartum in 3,254 women at an army medical center. Women with a prior cholecystectomy or with stones at their first study ultrasound were excluded. Gallbladder ultrasound and subject questionnaires were obtained in each trimester and at 4 to 6 weeks postpartum. Serum glucose, lipids, insulin, leptin, estradiol, and progesterone were measured at 26 to 28 weeks’ gestation. A nested case-control study was done to examine the effects of serum leptin and insulin on incident gallbladder disease. At least two study ultrasounds were available for 3,254 women. Sludge or stones had been found on at least one study ultrasound in 5.1% by the second trimester, 7.9% by the third trimester, and 10.2% by 4 to 6 weeks postpartum. Regression of sludge and stones was common, such that overall 4.2% had new sludge or stones on the postpartum ultrasound. Twenty-eight women (0.8%) underwent cholecystectomy within the first year postpartum. Prepregnancy body mass index was a strong predictor of incident gallbladder disease (P < .001). Serum leptin was independently associated with gallbladder disease (odds ratio per 1 ng/dL increase, 1.05; 95% CI, 1.01, 1.11), even after adjusting for body mass index. In conclusion, incident gallbladder sludge and stones are common in pregnancy and the postpartum, and cholecystectomy is frequently done within the first year postpartum. Prepregnancy obesity and serum leptin are strong risk factors for pregnancy-associated gallbladder disease. (HEPATOLOGY 2005;41:359-365.)

In the United States, gallstone disease is the most common and costly of all digestive diseases, requiring more than 700,000 cholecystectomies annually.1–4 Both the frequency and number of pregnancies are major risk factors for cholesterol gallstones.5–9 European studies have suggested that new biliary sludge (a precursor to gallstones) and gallstones may form in as many as 31% and 2% of pregnant women, respectively.10–12 Gallbladder disease is the most common non-obstetrical cause of maternal hospitalization in the first year postpartum.13

This study prospectively evaluated the incidence and natural history of pregnancy-related gallbladder sludge and stones in the United States and closely examined potential demographic, medical, and behavioral risk factors for their development. Pregnancy may constitute a defined period of metabolic stress in which subclinical tendencies are transiently revealed. For example, pregnant women with gestational diabetes or high blood pressure are at risk of later developing diabetes mellitus or hypertension.14–18 A similar phenomenon may happen with gallbladder sludge and stones. With careful interpretation, our results also may help clarify risk factors for development of gallbladder disease in the general population.

Patients and Methods

Consecutive women attending obstetrics orientation class were approached. Women were excluded because of age younger than 18 years, language comprehension, plans to move away within 3 months, or being more than 20 weeks pregnant. Of 8,929 women approached, 4,897 (55%) were eligible and interested. We excluded an additional 208 women with stones on entry ultrasound and 33 women with a prior cholecystectomy. If sludge was seen on the entry ultrasound, women were followed to see
whether sludge progressed to stones. All women gave written informed consent, and the study was approved by the Institutional Review Boards of the participating institutions.

Women were scheduled for an entry ultrasound during their first trimester of pregnancy (9-12 weeks) if possible. Otherwise, their second trimester (16-22 weeks) ultrasound was their entry examination. Additional study ultrasounds were scheduled during the early third trimester (26-32 weeks) and at 4 to 6 weeks postpartum, regardless of the entry ultrasound findings. Serum obtained at 26 to 28 weeks’ gestation was tested for fasting glucose and lipid values. Additional serum samples were frozen at −70°C. At entry, subjects completed a questionnaire covering their medical, obstetrical, family, and social histories. Prepregnancy body mass index was calculated from self-reported height and weight immediately before pregnancy. Weight gain during pregnancy was calculated as the difference between predelivery weight and prepregnancy weight. Additional questionnaires were completed in the early third trimester and at 4 to 6 weeks postpartum. Women were invited back at 1 year postpartum for a final study ultrasound and questionnaire. Follow-up data for the first postpartum year were extracted from medical records for all subjects.

Gallbladder ultrasonography was performed with a standard imaging protocol using a 3.5- to 7.0-MHz rotatory sector scanning transducer (ATL Inc., Bothell, WA, or Acuson Corp., Mountain View, CA). All study ultrasounds were performed by sonographers with special training in gallbladder ultrasound and with women fasting or having drunk only sips of water. Findings were recorded by the sonographers, and images and findings were reviewed by one of two designated study radiologists with expertise in gallbladder ultrasound. Sludge was defined as the presence of low-level echoes that shift with position changes and without postacoustic shadowing. Stones were defined as high-amplitude echoes greater than 2 mm in diameter with postacoustic shadowing. In 10,887 scans, there were discrepancies between the radiologist’s and the sonographer’s readings in 70 regarding the diagnosis of sludge (kappa = .93) and in 25 regarding the diagnosis of stones (kappa = .98). In case of discrepancy, the radiologist’s reading was accepted as correct.

Table 1. Number of Women With Ultrasound Examinations at Scheduled Time Points

<table>
<thead>
<tr>
<th></th>
<th>No Third Trimester or Postpartum</th>
<th>Third Trimester Only</th>
<th>4–6 Weeks Postpartum Only</th>
<th>Both Third Trimester and 4–6 Weeks Postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td>First trimester, no second trimester (n = 48)</td>
<td>0</td>
<td>26</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Second trimester, no first trimester (n = 1,842)</td>
<td>0</td>
<td>684</td>
<td>0</td>
<td>1,158</td>
</tr>
<tr>
<td>First and second trimester (n = 1,364)</td>
<td>138</td>
<td>349</td>
<td>16</td>
<td>861</td>
</tr>
</tbody>
</table>

Estradiol and leptin levels were measured by radioimmunoassay (ICN Biomedicals, Inc., Costa Mesa, CA, and Linco, St. Charles, MO). Progesterone levels were measured by enzyme-linked immunoassay (Systems Labs, Inc., Webster, TX). Total immunoreactive insulin was measured by a double-antibody radioimmunoassay developed at the Diabetes Endocrinology Research Center at the University of Washington. All assays were performed on stored serum samples obtained at 26 to 28 weeks’ gestation.

The cumulative incidence and natural history of gallbladder sludge and stones were determined from the serial gallbladder ultrasounds. Women with new sludge, new stones, or progression of sludge on entry ultrasound to stones were considered to have incident gallbladder disease. The cumulative incidence of sludge or stones was determined, with the denominator representing the total number of study subjects who had at least two ultrasound examinations by that time. We analyzed risk factors for incident gallbladder disease in women with sludge or stones seen on the early postpartum ultrasound. Differences in the risk of developing sludge or stones in various subgroups were compared using t tests or chi-square tests, as appropriate (Stata 8.0, Stata Corp., College Station, TX). Logistic regression models were developed to examine independent risk factors for sludge or stones. Risk factors examined included demographic factors, medical history, body weight, reproductive factors, lipid levels, and behavioral factors including coffee intake, smoking, and alcohol consumption. Two-sided P values less than .05 were considered statistically significant.

Results

Of 4,897 eligible and interested women, 3,254 (66%) with at least two interpretable study ultrasounds were included in this analysis. The number of women with study ultrasound examinations at scheduled times is shown in Table 1.

By the second trimester, 3.2% and 1.9% of women had formed new sludge or stones, respectively, and 2% had progression of baseline sludge to stones. By the third trimester, the cumulative incidence of new sludge and stones was 4.5% and 1.8% of women, respectively, with
progression of sludge to stones in 1.5%. By the first postpartum examination, 5.1% of women had new sludge, 2.8% had new stones, and 2.3% had progression of baseline sludge to stones. Therefore, by 4 to 6 weeks postpartum, the cumulative incidence of new sludge, new stones, or progression of baseline sludge to stones was 10.2%. Results were similar when we restricted the analysis to only women who had the first-trimester ultrasound (data not shown). Development and regression of sludge and stones was common (Tables 2 and 3). Overall, 4.2% of women had new sludge or stones that persisted to the early postpartum ultrasound.

Fifty-four women had an ultrasound at 1 year postpartum. Of these, 16 had no prior sludge or stones, 27 had prior sludge, and 10 had prior stones. This ultrasound was normal in 26 of 27 women with prior sludge, but in only 2 of 10 women with prior stones. The remaining 8 women with prior stones had sludge, persistent stones, or a combination of both. New sludge was seen in 1 of the 16 women without prior sludge or stones.

Sludge and stones were asymptomatic in most women. Four women (1.2%) with sludge or stones had symptoms that they attributed to their gallbladder. Four other women (0.1%) without sludge or stones on any ultrasound reported symptoms that they attributed to their gallbladder. Twenty-eight women (0.8%) underwent cholecystectomy for symptomatic gallbladder sludge or stones during the first year postpartum; 5 of these had surgery for severe biliary colic within 6 weeks postpartum. No woman had complications of cholecystitis, such as cholecystitis, pancreatitis, or cholangitis.

Risk factors for sludge and stones persisting to the early postpartum ultrasound were similar, and results are presented for a combined endpoint of sludge and stones. On univariable analysis, Hispanic women were significantly more likely to develop gallbladder disease than non-Hispanic women, with other racial differences also seen ($P < .001$) (Table 4). Age, prior hormonal contraception, coffee consumption, and smoking at any time during pregnancy were unrelated to the risk of gallbladder disease. Consumption of alcoholic beverages at any time during pregnancy was associated with a slightly decreased risk of gallbladder disease. The risk of incident gallbladder disease also varied with parity.

Being overweight (body mass index 25-29.9 kg/m²) or obese (body mass index $\geq$ 30 kg/m²) immediately pre-pregnancy was significantly associated with incident gallbladder disease. For example, 2.7% of women with normal prepregnancy body mass index developed incident gallbladder disease, compared with 11.7% of obese women. Weight gain during pregnancy was inversely associated with the risk of incident gallbladder disease.

Because cholesterol gallstones have previously been linked with diabetes and altered lipid metabolism, we examined fasting serum glucose and lipid levels (Table 4). Higher triglyceride levels were associated with an increased risk of sludge or stones, whereas high-density lipoprotein (HDL) cholesterol was inversely related. Total cholesterol, low-density lipoprotein cholesterol, and glucose were not significantly associated with gallbladder disease.

We developed a multivariable model to identify independent risk factors for incident gallbladder disease.

### Table 2. Incidence and Regression of Biliary Sludge and Stones in Women With 3 Ultrasounds During Pregnancy

<table>
<thead>
<tr>
<th>First Trimester</th>
<th>Ultrasound Finding</th>
<th>N</th>
<th>Second Trimester</th>
<th>Ultrasound Finding</th>
<th>N</th>
<th>Third Trimester</th>
<th>Ultrasound Finding</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 1,206)</td>
<td>Normal</td>
<td>1,146</td>
<td>Normal</td>
<td>1,067</td>
<td>57</td>
<td>Stones +/− sludge</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sludge</td>
<td>39</td>
<td></td>
<td></td>
<td>36</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stones +/− sludge</td>
<td>21</td>
<td></td>
<td></td>
<td>8</td>
<td>0</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Sludge (n = 4)</td>
<td>Normal</td>
<td>2</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sludge</td>
<td>0</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stones +/− sludge</td>
<td>2</td>
<td></td>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Incidence and Regression of Biliary Sludge and Stones in the Early Postpartum

<table>
<thead>
<tr>
<th>Entry Ultrasound</th>
<th>Final Ultrasound During Pregnancy (Second or Third Trimester)</th>
<th>N</th>
<th>Postpartum Ultrasound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ultrasound Finding</td>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>Normal (n = 3,206)</td>
<td>Normal</td>
<td>3,042</td>
<td>1,870</td>
</tr>
<tr>
<td></td>
<td>Sludge</td>
<td>122</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Stones +/− sludge</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td>Sludge (n = 48)</td>
<td>Normal</td>
<td>42</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Sludge</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Stones +/− sludge</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>
Prepregnancy obesity was strongly associated with the risk of incident gallbladder disease (odds ratio, 4.45; 95% CI, 2.59, 7.64) (Table 5). Parity and HDL cholesterol were also inversely associated with incident gallbladder disease.

To examine physiological mechanisms for incident gallbladder disease, we analyzed serum estradiol, progesterone, leptin, and insulin levels measured at 26 to 28 weeks’ gestation in a nested case-control study of 50 case and 101 randomly chosen control subjects. Cases and controls had similar serum levels of estradiol (45,803 ± 1,659 vs. 42,808 ± 1,461 pmol/L, \( P = .29 \)), progesterone (1,221 ± 413 vs. 1,148 ± 305 nmol/L, \( P = .29 \)), and insulin (153.4 ± 75.1 vs. 158.4 ± 124.3 pmol/L, \( P = .77 \)). Leptin levels correlated with body mass index (\( r = \)).

### Table 4. Potential Risk Factors for Development of Gallbladder Sludge or Stones

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>No Sludge or Stones</th>
<th>New Sludge, New Stones, or Progression of Sludge to Stones</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, n (%)</td>
<td>335 (92.0)</td>
<td>29 (8.0)</td>
<td>.42</td>
</tr>
<tr>
<td>&lt;20</td>
<td>1,169 (90.3)</td>
<td>125 (9.7)</td>
<td></td>
</tr>
<tr>
<td>20-4</td>
<td>878 (88.3)</td>
<td>116 (11.7)</td>
<td></td>
</tr>
<tr>
<td>25-9</td>
<td>439 (90.0)</td>
<td>39 (10.0)</td>
<td></td>
</tr>
<tr>
<td>30-4</td>
<td>118 (90.1)</td>
<td>13 (9.9)</td>
<td></td>
</tr>
<tr>
<td>≥40</td>
<td>13 (86.7)</td>
<td>2 (13.3)</td>
<td></td>
</tr>
<tr>
<td>Race, n (%)</td>
<td>2,022 (89.9)</td>
<td>27 (10.1)</td>
<td>.005</td>
</tr>
<tr>
<td>White</td>
<td>377 (91.7)</td>
<td>34 (8.3)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>221 (93.2)</td>
<td>16 (6.8)</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>41 (89.1)</td>
<td>5 (10.9)</td>
<td></td>
</tr>
<tr>
<td>Other/mixed</td>
<td>26 (89.7)</td>
<td>3 (10.3)</td>
<td></td>
</tr>
<tr>
<td>Hispanic origin, n (%)</td>
<td>307 (84.1)</td>
<td>58 (15.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Yes</td>
<td>2,633 (90.5)</td>
<td>276 (9.5)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>580 (90.3)</td>
<td>62 (9.7)</td>
<td>.48</td>
</tr>
<tr>
<td>Any smoking during pregnancy, n (%)</td>
<td>2,235 (89.4)</td>
<td>265 (10.6)</td>
<td>.02</td>
</tr>
<tr>
<td>Yes</td>
<td>817 (91.6)</td>
<td>75 (8.4)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1,998 (88.8)</td>
<td>252 (11.2)</td>
<td></td>
</tr>
<tr>
<td>Any alcohol consumption during pregnancy, n (%)</td>
<td>1,258 (89.7)</td>
<td>144 (10.3)</td>
<td>.82</td>
</tr>
<tr>
<td>Yes</td>
<td>1,557 (89.5)</td>
<td>183 (10.5)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.84 ± 0.07</td>
<td>0.84 ± 0.07</td>
<td>.53</td>
</tr>
<tr>
<td>Waist-hip ratio at entry</td>
<td>0.84 ± 0.07</td>
<td>0.84 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Body mass index prepregnancy, n (%)</td>
<td>1.288 (97.3)</td>
<td>36 (2.7)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>&lt;25</td>
<td>443 (95.5)</td>
<td>21 (4.5)</td>
<td></td>
</tr>
<tr>
<td>25-29.9</td>
<td>218 (88.3)</td>
<td>29 (11.7)</td>
<td></td>
</tr>
<tr>
<td>≥30</td>
<td>1,556 (93.1)</td>
<td>116 (6.9)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Highest previous body mass index, n (%)</td>
<td>856 (88.7)</td>
<td>109 (11.3)</td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>478 (82.4)</td>
<td>102 (17.6)</td>
<td>.002</td>
</tr>
<tr>
<td>25-29.9</td>
<td>528 (93.1)</td>
<td>39 (6.9)</td>
<td></td>
</tr>
<tr>
<td>≥30</td>
<td>561 (96.9)</td>
<td>18 (3.1)</td>
<td></td>
</tr>
<tr>
<td>Weight gain during pregnancy, quartiles, n (%)</td>
<td>552 (96.5)</td>
<td>20 (3.5)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>335 (97.4)</td>
<td>9 (2.6)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/L, mean ± SD</td>
<td>6.20 ± 1.12</td>
<td>6.12 ± 1.26</td>
<td>.58</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L, mean ± SD</td>
<td>4.11 ± 0.45</td>
<td>4.18 ± 0.55</td>
<td>.20</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L, mean ± SD</td>
<td>1.70 ± 0.43</td>
<td>1.62 ± 0.42</td>
<td>.02</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L, mean ± SD</td>
<td>3.39 ± 1.06</td>
<td>3.34 ± 1.07</td>
<td>.39</td>
</tr>
<tr>
<td>Triglycerides, mmol/L, mean ± SD</td>
<td>2.38 ± 0.90</td>
<td>2.59 ± 0.81</td>
<td>.04</td>
</tr>
</tbody>
</table>

Abbreviation: LDL, low-density lipoprotein.
Table 5. Multivariable Analysis of Risk Factors for Incident Gallbladder Disease

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Odds Ratio*</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepregnancy body mass index, kg/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>1.00</td>
<td>&lt; .001</td>
<td></td>
</tr>
<tr>
<td>25–29.9</td>
<td>1.63</td>
<td>0.92, 2.88</td>
<td></td>
</tr>
<tr>
<td>≥30</td>
<td>4.45</td>
<td>2.59, 7.64</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td>.01</td>
</tr>
<tr>
<td>0</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.38</td>
<td>0.21, 0.72</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td>0.71</td>
<td>0.43, 1.19</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol (per 1 mmol/dL increase)</td>
<td>0.54</td>
<td>0.30, 0.96</td>
<td>.04</td>
</tr>
</tbody>
</table>

*Adjusted for age, race, and ethnicity.

Table 6. Body Mass Index, Leptin, and the Risk of Incident Gallbladder Disease

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Odds Ratio*</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepregnancy body mass index, kg/m²</td>
<td></td>
<td></td>
<td>.43</td>
</tr>
<tr>
<td>&lt;25</td>
<td>Referent</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>25–29.9</td>
<td>0.50</td>
<td>0.17, 1.52</td>
<td></td>
</tr>
<tr>
<td>≥30</td>
<td>1.11</td>
<td>0.22, 4.29</td>
<td></td>
</tr>
<tr>
<td>Serum leptin, per 1 ng/dL increase</td>
<td>1.05</td>
<td>1.01, 1.11</td>
<td>.02</td>
</tr>
<tr>
<td>HDL cholesterol, per 1 mmol/L increase</td>
<td>0.24</td>
<td>0.09, 0.61</td>
<td>.003</td>
</tr>
</tbody>
</table>

*Also adjusted for weight gain during pregnancy, parity, and serum insulin.

Discussion

When gallstones form, changes in bile composition lead to precipitation of bile solutes. These microprecipitates aggregate and grow and are known as biliary sludge ("microlithiasis"). When they are more than 2 mm in size, they produce higher amplitude echoes with post-acoustic shadowing, and are diagnosed as stones. Sludge can spontaneously disappear and reform over time, or it can evolve to become gallstones. Not all cases of biliary sludge will evolve to gallstones. Conversely, sludge is a necessary precursor to gallstone formation. Changes in bile composition and gallbladder stasis during pregnancy may contribute to sludge and stone formation.19-21

In this study, we examined the incidence, natural history, and risk factors for gallbladder sludge and stones during pregnancy. Over a 7- to 8-month period from the first trimester to the early postpartum, the cumulative incidence of sludge was 5%, whereas an additional 5% developed incident stones or progressed from baseline sludge to stones. Overall, 4.2% had new sludge or stones that were seen on the early postpartum ultrasound. Prior cross-sectional studies of nonpregnant women in the United States have found that 6.5% of women ages 20 to 29 years and 10.2% of women ages 30 to 39 years have prevalent gallstones or a prior cholecystectomy.2 Because regression of stones is relatively uncommon, we thus believe that pregnancy is a high-risk period for gallstone formation. In addition, our results likely underestimate the true incidence of sludge or stones. Transient sludge or stones, not present at the time of any study ultrasound, would not have been detected. Also, the sensitivity of ultrasound for microscopic sludge is only 50% to 60%. Therefore, some women may have had sludge that was not detected by a study ultrasound. Although the clinical significance of transient sludge and stones is not clear, these entities may cause biliary symptoms or complications. In the postpartum period after gallbladder motility is restored, sludge and stones may pass from the gallbladder, potentially causing biliary colic or other complications. Alternatively, postpartum changes in bile composition may favor regression of sludge or stones.22

Most women with gallbladder disease in this study were asymptomatic, but 0.8% underwent cholecystectomy in the first postpartum year. Because more than 4 million women give birth annually,23 we can estimate that more than 32,000 young, otherwise healthy women will require postpartum cholecystectomy each year. Thus, pregnancy-associated gallbladder disease is a significant cause of morbidity in young, otherwise healthy women.

Our results differ from those of Maringhini et al.,11 who found incident sludge in 31% and stones in 2% of pregnant women. Differences in the sonographic definition of sludge and stones may account for part of this discrepancy. Also, our population was slightly younger and less overweight. In contrast to our study, Maringhini et al. found that sludge was inversely related to body weight. However, they did not account for weight gain or loss during pregnancy, which may confound the relationship between prepregnancy weight and sludge. Women who weighed more prepregnancy in our study gained less weight during pregnancy, likely explaining the inverse association between weight gain during pregnancy and gallbladder disease.
The independent risk factors for incident sludge or stones were prepregnancy body mass index, HDL cholesterol, and parity. The association of prepregnancy body mass index is consistent with prior data showing an increasing prevalence of gallstones with increasing weight in the general population. Our data further demonstrate that the risk of developing new gallbladder disease is strongly associated with being obese, but only moderately associated with being overweight. As the prevalence of overweight and obesity in young women increases, pregnancy-associated gallbladder disease may become an even greater problem.

Similarly, the inverse association of parity with incident gallbladder disease was unexpected. However, this is likely attributable to our selection criteria. Women of higher parity with an underlying tendency to form stones may have already done so in previous pregnancies. These women would have been excluded by our criteria. Women of higher parity and without an underlying tendency toward stone formation would still be eligible for inclusion and would be less likely to form stones during the study pregnancy. As an approximation, our data indicate that approximately 5.1% of women develop gallbladder disease after one pregnancy, 7.6% after two pregnancies, and 12.3% after 3 or more pregnancies. These data are generally similar to those found in other population-based studies.

HDL cholesterol was inversely related to sludge and stone formation, similar to findings with prevalent stones. HDL promotes reverse cholesterol transport by facilitating cholesterol uptake into the liver. Preformed cholesterol in HDL is a major source of biliary cholesterol. Therefore, a direct mechanistic correlation seems unlikely. A simplistic explanation may be that low HDL levels are associated with reduced capacity to solubilize the total body cholesterol pool. The excess cholesterol may be distributed to the biliary system, where it precipitates as sludge or stones. Alternatively, low HDL co-exists with obesity, insulin resistance, and hypertriglyceridemia. The harmful effect of low HDL may be mediated through its association with obesity or insulin resistance.

The only potentially modifiable risk factor for incident gallbladder disease was body mass index. Possible mediators of the association between obesity and gallbladder disease include the hormones insulin and leptin, both of which are associated with body mass index. Insulin levels were associated with prevalent gallbladder disease in prior studies, whereas leptin levels were not. Leptin may mediate secretion of cholesterol into bile by the liver, potentially promoting cholesterol lithogenesis in obese patients. We found leptin levels were increased relative to those in nonpregnant women, consistent with known changes in leptin levels during pregnancy. Although serum leptin level may be a proxy measure for leptin resistance commonly found in obesity, our data show that it is independently associated with incident gallbladder disease. Our data suggest leptin may mediate the association between obesity and gallbladder disease. Further studies to confirm this finding are needed.

In conclusion, more than 4% of pregnant women have incident gallbladder sludge or stones persisting to the early postpartum. Although most women will remain asymptomatic, 0.8% will require cholecystectomy within the first year postpartum. Therefore, gallbladder disease is a significant cause of morbidity for young, otherwise healthy women. Being overweight or obese prepregnancy is a significant, but potentially modifiable, risk factor for developing gallbladder disease during pregnancy. Further interventions aimed at controlling body weight may be beneficial in reducing the burden of this disease in young women.

Acknowledgment: The authors acknowledge contributions to the study by Andrea K. Herron, Lori J. Green, Dianne Walkup, Lorna Imbruglio, Nancy Allison, and Brett Gates.

References


Outcome in Adulthood of Biliary Atresia: a Study of 63 Patients Who Survived for Over 20 Years With Their Native Liver

Panayotis Lykavieris,1 Christophe Chardot,2 Maroun Sokhn,1 Frédéric Gauthier,2 Jacques Valayer,2 and Olivier Bernard1

To define the long-term prognosis of children undergoing the Kasai operation for biliary atresia, a retrospective study was undertaken comprising 271 patients operated between 1968 and 1983. Twenty years after surgery, 63 (23%) were alive with their native liver. Serum bilirubin was normal in 21 of these patients, 12 also had normal serum aminotransferase and γ-glutamyltransferase activities, all but 2 had signs of cirrhosis, 44 had signs of portal hypertension, 19 had late bacterial cholangitis, and 6 had gallstones. Seven female patients gave birth to 9 children, and 3 male patients fathered 6 children. After age 20, 2 patients died of liver failure and 14 underwent or are awaiting liver transplantation. Twenty-year survival with native liver was significantly better in children with biliary atresia restricted to the hepatic ducts or with cysts at the porta hepatis. In conclusion, in the long term, less than 18% of infants with biliary atresia who are treated with corrective surgery may avoid liver transplantation, but even these patients require assiduous lifelong care. (HEPATOLOGY 2005;41:366-371.)

See Editorial on Page 231

Congenital biliary atresia is characterized by complete obstruction of all or part of the extrahepatic bile duct and is always associated with abnormalities of the intrahepatic bile ducts.1,2 Its cause is unknown, and its incidence ranges from 1 in 3,400 to 1 in 20,000 births.3 It results in death if left untreated. The Kasai operation or its variants constitute the first step in the surgical treatment of infants with biliary atresia4; liver transplantation is performed secondarily when bile flow is not restored or when complications of biliary cirrhosis occur. Many authors have reported the short-term results and prognostic factors of the Kasai operation, but few data are available regarding its results in adulthood. The present study was therefore undertaken to evaluate the liver status and general condition of patients with biliary atresia who underwent the Kasai operation and survived with their native liver for at least 20 years.

Patients and Methods

From 1968 to 1983, 271 infants were investigated and operated on for biliary atresia by the same medical and surgical teams. In all cases the diagnosis of biliary atresia was confirmed by examination during surgery and by histological study of the liver and biliary remnants.5 In 14 children, biliary atresia was associated with malformations described in the polysplenia syndrome. In 208 children, atresia involved the entire extrahepatic biliary tree, and all of these patients underwent hepatic portoenterostomy. In 43 children, the gallbladder, cystic duct, and common bile duct were patent, and all of these patients underwent hepatic portocholecystostomy (which had to be changed to hepatic portoenterostomy in 3 patients). In 19 children, a biliary cavity was communicating with dystrophic intrahepatic ducts at the porta hepatitis, and all of these patients underwent cystojejunostomy. Lastly, 1 child with biliary atresia limited to the common bile duct underwent cholecystojejunostomy.

Of the 271 children, 1 child who survived with her native liver was lost to follow-up at age 13 years, 175 died (mostly before liver transplantation was commonly practiced in France), 32 underwent liver transplantation before the age of 20 years, and the remaining 63, who survived with their native liver for at least 20 years, form the basis of this report. The indications for liver transplantation before the age of 10 years were persistent jaundice and/or endstage liver disease in 17 children and hepatopulmonary syndrome in 1 child. The indications for liver
transplantation between the ages of 10 and 20 years were hepatopulmonary syndrome in 5 children, pulmonary hypertension in 2 children, and persistent or relapsing jaundice in 7 children.

Data were collected from patients’ hospital records at their latest checkup or at the time of transplantation for patients who underwent transplantation after the age of 20 years. These data concerned clinical evaluation of growth and liver and spleen sizes, biochemical study of liver function, and a search for ultrasonographic and endoscopic signs of portal hypertension and signs of hepatopulmonary syndrome, pulmonary hypertension, and liver cancer. Cirrhosis was considered to be present either when proven by liver histology (studied in 33 patients aged 5 months to 33 years; median, 7 years), or as shown on abdominal ultrasonography by a nodular liver with signs of portal hypertension, and/or when a hard, enlarged liver was associated with splenomegaly. In 44 patients, ursodeoxycholic acid was given at a dose of 600 mg/m²/d. Assessment of patients’ condition also included data regarding general health, professional status, and whether they had children.

The following survival rates were calculated: (1) survival with native liver, which starts at birth and ends at either death or liver transplantation; and (2) patient survival, which starts at birth and ends at death. Actual survival rates, as well as categorical data, were compared using the χ² test (with Yate’s correction if indicated) and the Fisher exact test. Survival rates were calculated using the Kaplan-Meier method and compared using the log-rank test. All significance tests were two-tailed. Differences were considered significant at a P value of less than .05. All analyses were performed with Statview software, version 5.0 (SAS Institute Inc., Cary, NC).

Results

Twenty-Year Survival After Kasai Operation

After the Kasai operation, 63 (23%) of the 271 children survived with their native liver for at least 20 years. They comprised 27 females and 36 males. Two patients died of liver failure at the ages of 24 and 30 years, respectively. Ten patients underwent liver transplantation at ages 20 to 28 years; the indications for this transplantation were endstage liver disease in 6 patients, persistent jaundice in 3 patients, and hepatopulmonary syndrome in 1 patient. Two patients died within 6 months of transplantation. Therefore, at the time of writing, 59 patients are still alive, including 51 with their native liver; the oldest patient is 35 years old (Fig. 1).

The relationship between the type of operation and 20-year survival was significantly better in children who underwent hepatic portocholecystostomy or cystojejunostomy (35% and 40%, respectively) than in children who underwent hepatic portoenterostomy (19%). Age at surgery did not differ significantly in these two groups. However, when only patients surviving with their native liver and displaying normal total serum bilirubin at the last follow-up were considered, there was no significant difference in outcome according to type of initial surgery.

The median age at the time of operation was 69 days (range, 27-163) and was 90 days or more in 12 patients (19%). The relationship between 20-year survival and age at operation is shown in Table 2. Twenty-year survival with native liver was significantly better in children who were operated on before the age of 90 days. However, there was no significant difference according to age at initial surgery in patients alive with a native liver and normal serum bilirubin at the last follow-up.

Only 1 of the 14 children with associated polysplenia syndrome is alive with a native liver after 20 years. Of the other 13, 6 died of end-stage liver disease before liver transplantation became available, 4 died of bacterial infection, 1 died of unexplained sudden death, and 2 underwent liver transplantation. Twenty-year survival with native liver was similar in children who were operated on during the early period of study (1968-1977) and the late period (1977-1983) (25 of 125 [20%] vs. 38 of 146 [26%]; P value not significant).

Characteristics of the 63 Patients Alive With Their Native Liver at 20 Years or More (Tables 3 and 4)

Growth. Adult height was equal to or above the mean in 49 patients. The tallest man measured 190 cm; the tallest woman, 176 cm. Two women had a height below −1.5 SD; 1 had had an intracranial hemorrhage and bacterial meningitis at the age of 5 years combined with growth hormone deficiency and primary amenorrhea, and 1 had mental retardation and ventricular dilatation. Except for these patients, all the girls had their first men-
strual periods between the ages of 12 and 15 years. All male patients had achieved pubertal maturation.

**Liver Condition.** Hard hepatomegaly and splenomegaly were present in 21 and 29 patients, respectively. Thirteen complained of pruritus. Total serum bilirubin levels were within normal limits (below 17 μmol/L) in 21 patients (including 33% of survivors); 12 of these patients also displayed normal serum γ-glutamyltransferase and transaminase activities. Total serum bilirubin levels were elevated (range, 18-656 μmol/L) in the 42 remaining patients; 26 of these (62%) had a normal serum bilirubin level at least once but experienced a relapse of jaundice later. The others never had normal serum bilirubin levels. All patients with elevated total serum bilirubin levels at or after 20 years also had elevated serum transaminase levels (range, 2-7 x N) and γ-glutamyltransferase levels (range, 1.5-30 x N). Prothrombin time was below 70% in 19 patients, all with elevated total serum bilirubin levels; however, in 7 of these patients, serum bilirubin levels were only slightly above normal (<40 μmol/L). Serum albumin concentrations were below 3.5 g/dL in 11 patients, all with elevated total serum bilirubin levels; in 2 of these patients the serum bilirubin concentration was only slightly above normal (<40 μmol/L). Histological, clinical, and/or ultrasonographic signs of cirrhosis were present in all but 2 patients. One large liver nodule (>6 cm) was seen on ultrasound in 5 patients and proven by histology to be a regenerative nodule; serum alpha-fetoprotein levels were normal in all patients studied. Two patients are hepatitis C virus carriers.

**Portal Hypertension.** Clinical (splenomegaly), ultrasonographic, or endoscopic signs of portal hypertension were present or had been detected previously in 44 of the 63 20-year survivors with their native liver. Gastrointestinal bleeding had occurred in 20 patients at ages 2 to 27 years (median, 8 years). Three patients bled after age 20 years. Thirteen children underwent a successful surgical portosystemic shunt; none of them had further bleeding at follow-ups ranging from 4 to 22 years (mean, 15 years), but 1 patient presented with a transient episode of encephalopathy at age 22 years, 19 years after surgery. Four other patients underwent sclerosis or ligation of varices, and 2 received propranolol. Eleven of the remaining 17 patients in whom upper gastrointestinal endoscopy (performed at ages 16 to 24 years) disclosed esophageal varices are also being given propranolol. Another patient bled from a peptic ulcer related to *Helicobacter pylori* infection. One patient presented with hepatopulmonary syndrome at the age of 24 years.

**Biliary Lesions.** Nineteen patients, all of whom underwent hepatic portoenterostomy, cystojejunostomy, or cholecystojejunostomy, presented at ages 17 to 30 years.

### Table 1. Survival With Native Liver Among 271 Children Undergoing Surgery for Biliary Atresia According to Type of Surgery

<table>
<thead>
<tr>
<th>Type of Surgery</th>
<th>Number of children</th>
<th>Alive with native liver at 20 years</th>
<th>Alive with native liver and normal serum bilirubin at last follow-up</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic Portoenterostomy</td>
<td>208</td>
<td>40 (19%)</td>
<td>15 (7%)</td>
<td></td>
</tr>
<tr>
<td>Hepatic Portocholecystostomy</td>
<td>43</td>
<td>19 (35%)</td>
<td>3 (7%)</td>
<td></td>
</tr>
<tr>
<td>Others*</td>
<td>20</td>
<td>8 (40%)</td>
<td>3 (15%)</td>
<td>NS</td>
</tr>
<tr>
<td>Total</td>
<td>271</td>
<td>63 (23%)</td>
<td>21 (7.7%)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>P Values</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviation: NS, not significant.*

<table>
<thead>
<tr>
<th>Type of Surgery</th>
<th>Number of patients</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pruritus</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Total serum bilirubin concentration &gt;17 μmol/L</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Prothrombin time &lt;70%</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Portal hypertension</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Late bacterial cholangitis</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Gallstones</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Hepatopulmonary syndrome</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviation:** NS, not significant.

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with at least one episode of acute bacterial cholangitis, which responded to antibiotics. Six patients presented with gallstones during the follow-up period (Table 4): in all but one of these patients, the gallbladder had been used for surgery; in 2 patients the stones were asymptomatic, but the other 4 patients presented with jaundice and/or bacterial cholangitis.

**Quality of Life.** Complete information regarding quality of life was available for 52 patients. Thirty-eight can be considered to lead a normal life, because 21 are regularly employed and 17 are university students. Twenty are married or have a stable partnership. Seven female patients have given birth to 9 children, 2 of them small for their birth date. Three male patients have each fathered 2 healthy children. Six patients are suffering from depression, and 3 of them are heavy drinkers.

**Outcome at Last Follow-Up.** Of the 271 children operated on for biliary atresia between 1968 and 1983, 51 are currently alive with their native liver. However, 4 of these survivors are awaiting liver transplantation because of permanent jaundice and bilirubinemia above 150 \( \mu \text{mol/L} \); survival without the immediate need for liver transplantation can therefore be estimated at 17% of the cohort. All but 2 of the 51 survivors have cirrhosis; 21 (7%) have normal serum bilirubin levels; 8 (3%) have normal transaminase and \( \gamma \)-glutamyltransferase activities and no signs of portal hypertension; and 5 (2%) have normal transaminase and \( \gamma \)-glutamyltransferase activities, no signs of portal hypertension, and no late cholangitis.

**Discussion**

If untreated, children with biliary atresia rarely survive beyond the age of 3 years. Regular use of the Kasai operation since the late 1960s and of liver transplantation since the mid-1980s has significantly improved the prognosis; consequently, the successive use of these two types of surgery by experienced teams currently yields an overall short- and medium-term survival of approximately 90%. Liver transplantation is chiefly needed when the Kasai operation fails to restore a significant bile flow and jaundice persists; such children undergo liver transplantation in the first years of life. However, reports on the medium-range outcome of the Kasai operation after a significant improvement in bile flow indicate that liver transplantation nevertheless has to be performed later in some of these children for relapse of jaundice or the complications of portal hypertension. The results reported here in a group of 63 patients who were operated on for biliary atresia and reached adulthood indeed indicate that, except for a small minority of patients, the Kasai operation cannot be considered as a cure for the disease and that careful lifelong follow-up is necessary to detect the complications of biliary cirrhosis that require liver transplantation, even well into adulthood. This is chiefly because biliary atresia is not restricted to the extrahepatic bile duct; thus even children in whom the Kasai operation has been successful display abnormalities of the intrahepatic biliary tree, including stenoses, dilatation, and sometimes pseudocystic areas. The resulting image on cholangiograms is similar to that of sclerosing cholangitis. This has two main consequences: (1) ongoing cholestasis, which further aggravates (although at a variable pace) the cirrhosis already present in virtually all children at an early age; and (2) bile duct damage due to bacterial cholangitis in children whose bile ducts are directly in contact with the gut cavity, and cholelithiasis, the risk of which may be greater in children whose gallbladder was patent and used for anastomosis.

Even when biliary atresia is successfully treated via the Kasai operation, it is an ongoing disease that, as already indicated, requires careful management throughout life.

**Table 4. History of 6 Long-term Survivors With a Native Liver After Surgery for Biliary Atresia who Presented With Gallstones**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type of Surgery</th>
<th>Age at Appearance of Symptoms (yr)</th>
<th>Type of Symptoms</th>
<th>Site of Lithiasis</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cystojejunostomy</td>
<td>17–20</td>
<td>Relapsing bacterial cholangitis</td>
<td>Intrahepatic</td>
<td>Partial hepatectomy</td>
<td>Alive and well</td>
</tr>
<tr>
<td>2</td>
<td>Cholecystojejunostomy</td>
<td>21</td>
<td>Acute bacterial cholangitis</td>
<td>Biliodigestive anastomosis</td>
<td>Surgical removal of stone</td>
<td>Alive and well</td>
</tr>
<tr>
<td>3</td>
<td>Hepatic portocholecystostomy</td>
<td>9</td>
<td>Jaundice</td>
<td>Gallbladder</td>
<td>Cholecystoduodenostomy</td>
<td>Alive and well</td>
</tr>
<tr>
<td>4</td>
<td>Hepatic portocholecystostomy</td>
<td>17</td>
<td>Jaundice</td>
<td>Multiple intrahepatic</td>
<td>Patient refused transplantation</td>
<td>Death at age 23</td>
</tr>
<tr>
<td>5</td>
<td>Hepatic portocholecystostomy</td>
<td>17</td>
<td>Fortuitous finding</td>
<td>Gallbladder</td>
<td>None</td>
<td>Alive and well</td>
</tr>
<tr>
<td>6</td>
<td>Hepatic portocholecystostomy</td>
<td>20</td>
<td>Fortuitous finding</td>
<td>Gallbladder</td>
<td>None</td>
<td>Alive and well</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>Jaundice Liver failure</td>
<td>Gallbladder</td>
<td>Awaiting liver transplantation</td>
<td></td>
</tr>
</tbody>
</table>
This becomes clear when one compares the results of the present study of 63 children who had surgery for biliary atresia between 1968 and 1983 and survived for 20 years with their native liver to the results for 40 children who survived with their native liver for more than 10 years after surgery performed between 1968 and 1977. The rates of survival with native liver for the 20- and 10-year groups (23% vs. 32%), as well as the proportions with normal serum bilirubin levels (33% vs. 52%) and normal liver function tests (21% vs. 27%), were all less satisfactory in the 20-year population, but the proportion of children with portal hypertension (69% vs. 70%) was high in both groups. Early detection of the cardiopulmonary complications of portal hypertension is especially important: here, 6 of the 85 patients who were operated on between 1968 and 1983 and survived for over 10 years with their native liver developed hepatopulmonary syndrome between the ages of 10 and 24 years, while 2 developed pulmonary hypertension at ages 10 and 11 years, respectively. The risk of liver cancer also must be carefully explored.

There are few reports of the rate of survival with native liver for 20 years or more in children who have undergone surgery for biliary atresia. In one report by the Sendai group, survival with native liver was 40% (45 of 112 children who underwent surgery between 1969 and 1980). In another report by the Tokyo group, survival with native liver was 14% (16 of 115 children who underwent surgery between 1968 and 1985); here the proportion of survivors was intermediate (23%). There is little information in the previous reports to explain the differences in survival, although the longer experience of the Sendai group may have helped to improve their results. The results of this group also show that fewer than 10% of patients survive with their native liver after age 30 years (7 of 78 children who underwent surgery between 1951 and 1968).

Furthermore, little information has been published on the medical status of survivors into adulthood with a native liver after the Kasai operation. Two Sendai group reports respectively describe the condition of 21 and 30 patients aged 20 years or more. Although the proportion of patients with elevated serum bilirubin levels was smaller than in the present study, these two reports included examples of relapse of jaundice in adulthood and stressed the risks of portal hypertension and gallstones; moreover, the rate of cholangitis may have been higher than in the present series. Similar observations were reported by another Japanese group for 6 (15%) of 39 patients who survived for 20 years after the Kasai operation.

In a multicenter study, the anatomical pattern of the extrahepatic biliary remnant was shown to be linked to the medium-term results of the Kasai operation. Actuarial 10-year survival with native liver decreased from 83% in type I atresia (limited to the common bile duct) to 56% in type II atresia (atresia with a cyst in the liver hilum communicating with dystrophic intrahepatic ducts), 36% in type III atresia (biliary atresia with a patent gallbladder, cystic duct, and common bile duct), and 21% in type IV atresia (complete extrahepatic biliary atresia). The present single-center study shows that the prognostic value of the anatomical pattern persists in adulthood because 20-year survival with native liver was 40% in patients who underwent choledochocystostomy and cystojejunostomy (types I and II), 35% in those who underwent hepatic portocholecystostomy (type III), and 19% in those who underwent hepatic portoenterostomy (type IV). These results suggest that the severity of extrahepatic biliary lesions correlates with the severity of the extrahepatic lesions, and that the worst prognosis is associated with complete extrahepatic biliary atresia.

Many reports show that the positive medium-term results of the Kasai operation decrease when the age at surgery increases. The present study shows the same trend in long-term results. Similarly, the unfavorable prognostic value of the polysplenia syndrome, which was not found in some studies, seems confirmed by our long-term results; in the present series, only 1 patient with this syndrome survived with a native liver for more than 20 years.

In conclusion, although the Kasai operation undoubtedly improves survival and should therefore be maintained as a first-line treatment for infants with biliary atresia, the results reported here indicate that assiduous, lifelong care must be given to all long-term survivors to identify those who will require liver transplantation well into adulthood for relapse of jaundice or the complications of cirrhosis.

Acknowledgment: We are grateful to our colleagues who provided follow-up information on some patients—especially Drs. E. Fort, Willy Alo, Marianne Besnard, Laurent Schouler, Stefano Martellossi, and Pietro Vajo—and to Mathilde Dreyfus for English-language editing.

References


Racial and Ethnic Distribution of Nonalcoholic Fatty Liver in Persons With Newly Diagnosed Chronic Liver Disease

Shiobhan R. Weston,1 Wendy Leyden,3 Rose Murphy,3 Nathan M. Bass,1 Beth P. Bell,4 M. Michele Manos,2,3 and Norah A. Terrault1

We performed a cross-sectional study of newly diagnosed cases of nonalcoholic fatty liver disease (NAFLD) identified between December 1998 and December 2000 in the Chronic Liver Disease Surveillance Study. We compared the demographic and clinical features of NAFLD in a racially diverse representative U.S. population (Alameda County, CA). Diagnostic criteria for probable NAFLD were persistent unexplained elevation of serum aminotransferase levels, radiology (ultrasound or computed tomography scan) consistent with fatty liver, and/or two or more of the following: (i) body mass index of 28 kg/m² or more, (ii) type 2 diabetes, or (iii) hyperlipidemia, in the absence of significant alcohol use. Definite NAFLD cases required histological confirmation. Of the 742 persons with newly diagnosed chronic liver disease, 159 (21.4%) had definite or probable NAFLD. The majority were nonwhite: Hispanics (28%), Asians (18%), African Americans (3%), and other race(s) (6%). African Americans with NAFLD were significantly older than other racial or ethnic groups ($P < .001$), and in Asians, NAFLD was 3.5 times more common in males than in females ($P = .016$). Clinical correlates of NAFLD (obesity, hyperlipidemia, diabetes) were similar among racial and ethnic groups, except that body mass index was lower in Asians compared with other groups ($P < .001$). Compared with the base population (Kaiser Permanente members), Hispanics with NAFLD were overrepresented (28% vs. 10%) and whites were underrepresented (45% vs. 59%). In conclusion, these racial and gender variations may reflect differences in genetic susceptibility to visceral adiposity, including hepatic involvement, and may have implications for the evaluation of persons with the metabolic syndrome. Clinicians need to be aware of the variable presentations of NAFLD in different racial and ethnic groups. (HEPATOLOGY 2005;41:372-379.)

Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized to be among the most common causes of chronic serum aminotransferase elevation in the United States1 as well as in several other countries, including Japan, Australia, Europe, and the Middle East.2—6 Additionally, the histological lesion associated with more advanced disease, nonalcoholic steatohepatitis, has emerged as an important cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma.3,7,8 Clinical conditions commonly associated with a fatty liver include central obesity, type 2 diabetes mellitus, and dyslipidemia.1,9 As the prevalence and severity of these conditions continues to rise in the United States, the prevalence and disease burden of NAFLD are predicted to increase substantially.10 The prevalence rates for an overweight state, defined as a body mass index (BMI) of more than 25 kg/m², have risen to 64.5%11 in the United States and the age-adjusted prevalence of obesity, defined as a BMI of 30 kg/m² or more, is more than 30%. Based on current U.S. census figures, it is estimated that more than 30 million obese adults may have steatosis and that 8.6 million may have steatohepatitis.1 Prevalence rates for type 2 diabetes have similarly risen over the past decade.

Abbreviations: NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; KP, Kaiser Permanente.

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Received July 24, 2004; accepted November 9, 2004.

Supported by the Chronic Liver Disease Surveillance Study (DC U50 CCU915546) and the UCSF Liver Center Grant (P30 DK-26743). Dr. Weston was supported by a Schering-Plough Award (2002-2003).

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DOI 10.1002/hep.20554

Conflict of interest: Nothing to report.
Currently, 7.8% of the U.S. population has diabetes, representing a 49% increase from the period 1990 through 2000.12

NAFLD has been described in persons of all ages in the United States, but the populations studied have been predominantly white.2,4,13—16 In this study, we focused on the clinical presentation of NAFLD in different racial and ethnic groups in a geographically representative and racially diverse segment of the U.S. population.

Patients and Methods

The institutional review boards of each of the participating institutions approved the study.

Study Population

The study population was composed of adult members of the Kaiser Permanente Medical Care Program who participated in the Alameda County Chronic Liver Disease Surveillance Study. This site is one of three in the United States performing surveillance for chronic liver disease. In this cooperative study supported by the Centers for Disease Control and Prevention, new cases of chronic liver disease are identified and detailed demographic and clinical data are collected to establish the etiology, risk factors, and comorbidities associated with liver disease. The Kaiser Permanente (KP) membership is generally representative of the Alameda County population, except at the extremes of income.17 The proportion of KP members with an annual household income of less than $15,000 or more than $100,000 is lower than in the general Northern California population (6% vs. 10% with income of less than $15,000, and 18% vs. 24% with income of more than $100,000). Alameda County residents followed at one of four KP medical facilities (Pleasanton, Fremont, Hayward, and Oakland) serving Alameda county residents were eligible to participate. Additionally, study participants were required to be 18 years of age or older and to have continuous health plan membership for 18 months before initial identification for inclusion in the study.

A case of newly identified chronic liver disease was defined by specific laboratory, histological, or radiological criteria. A computer-based search was used to identify cases using predefined criteria. Laboratory criteria were consistently elevated liver blood tests (serum alanine aminotransferase and serum aspartate aminotransferase levels, alkaline phosphatase, or bilirubin), with nonliver causes excluded. The same liver test had to be elevated on at least two occasions at least 6 months apart, but not greater than 18 months apart, with the second test abnormality identified between December 1998 and December 2000. Radiological criteria required evidence of cirrhosis (nodular or shrunken liver) or portal hypertension (splenomegaly, varices, recanalized umbilical vein, or ascites). Histological criteria required a liver biopsy result consistent with chronic liver disease.18

Participants in the Chronic Liver Disease Surveillance Study were assigned the diagnosis of definite or probable NAFLD based on the following criteria. Definite NAFLD cases required histological confirmation. Probable NAFLD cases were defined as persistent unexplained elevation of serum aminotransferases with ultrasound or computed tomography scan results consistent with fatty liver and/or two or more of the following clinical conditions: (i) a BMI of 28 kg/m² or more, (ii) type 2 diabetes, or (iii) hyperlipidemia; and an absence of significant alcohol use, defined as a current daily alcohol consumption of 40 g/d or more in males and 20 g/d or more in females based on lifetime drinking history. Patients with serological or histological evidence of viral hepatitis B or C, autoimmune, metabolic, or other identifiable liver diseases were excluded based on available laboratory data. Serological tests for viral hepatitis (anti-hepatitis C virus, total anti–hepatitis B core antigen, and hepatitis B surface antigen) were performed on all study participants. Hepatitis C virus infection was confirmed by testing for hepatitis C virus RNA using qualitative and quantitative assays in those persons with positive anti-hepatitis C virus test results. Autoimmune markers (serum antinuclear antibody ± anti smooth muscle antibody) were available in 48% of participants, and metabolic markers (ferritin, total iron binding capacity, percent iron saturation, serum ceruloplasmin, and α₁-antitrypsin level) were available in 25%.

Study Methods

All participants had the following performed: (i) review of computerized medical records; (ii) in-person interview including self-reporting of race and ethnicity, comorbidities, and alcohol intake19; (iii) phlebotomy with testing of blood samples for viral hepatitis; (iv) height, weight, and BMI at the time of interview; and (v) standardized review of liver pathology, if available.

Review of Computerized Records. Review of computerized medical records was performed on all participants to confirm eligibility. Additionally, clinical data, pharmacy records, and physician-assigned diagnoses between the month in which the patient met the case definition (index month) and the 12 months of follow-up were reviewed. Laboratory variables included serum bilirubin, serum aspartate aminotransferase, serum alanine aminotransferase, alkaline phosphatase, serum albumin, prothrombin time, blood glucose, glycosylated hemoglobin, and triglycerides. Pharmacy record review focused on
use of corticosteroids, lipid-lowering agents, oral hypoglycemic agents, and insulin.

**In-Person Interview.** All patients were interviewed using a standardized questionnaire that included (i) demographic information such as age, sex, race, and ethnicity; (ii) a detailed history of all medications, including current use of corticosteroids or lipid-lowering agents as well as potential hepatotoxins; (iii) quantitation of current and lifetime alcohol intake; and (iv) comorbidities, including obesity, type 2 diabetes, dyslipidemia, ulcerative colitis, Crohn’s disease, and asthma within a 5-year period before interview. Race and ethnicity were determined by self-report.

**Histological Evaluation.** Liver biopsies obtained between July 1997 and December 2001 were retrieved and reviewed by a single pathologist. Liver biopsy results were evaluated for steatosis, ballooning of hepatocytes, inflammation, and fibrosis using the Brunt criteria.18

**Reference Population Used for Comparison of NAFLD Distribution by Race and Ethnicity.** To gain an understanding of differences in the racial and ethnic distribution of NAFLD in the study population versus the Kaiser Permanente base population, we used membership race and ethnicity information from a survey conducted in 1999. The survey, developed by the Division of Research at Northern California Kaiser Permanente, was mailed to a stratified random sample of 5,080 adult health plan members followed up at the Pleasanton, Fremont, Hayward, and Oakland KP facilities in Alameda County. Only Kaiser Permanente Medical Care Program members continuously enrolled within 3 months before administration of the survey were eligible. Respondent data were weighted so that the final sample used to create the profiles of sociodemographic characteristics reflected the actual age and sex distribution of the four medical center service populations in 1999 rather than that of the respondent sample. The overall survey response was 53%. Lower response rates were seen for younger adults (20-44 yr) compared with older age groups and for African Americans compared with Hispanics, Asians, and whites (NP Gordon. Characteristics of Adult Health Plan Members in the Northern California Region Membership, as Estimated from the 1999 Member Health Survey. Personal communication, December 31, 2003.).

**Statistical Methods**

Median and mean, and range and standard deviation were used for descriptive statistics, as appropriate. Categorical data were analyzed using the Fisher’s exact test and chi-square test. Continuous variables were tested with Student t test or a 1-way ANOVA. A P value of less than .05 was considered statistically significant.
Most participants in this study had probable NAFLD, because liver biopsies were uncommon in persons with a diagnosis of NAFLD. Only 19 participants (12% of the total NAFLD group) underwent liver biopsy. Among those participants with available histological results, 18 (94.7%) had features consistent with NAFLD (Fig. 1). Six had simple macrosteatosis, 12 had steatohepatitis, and the single remaining participant had nondiagnostic liver histological results. A total of 112 (70.0%) of the participants with probable and definite NAFLD had either abdominal ultrasound or computed tomography study results. Radiological findings consistent with fatty liver were present in 88% of these patients. Ultrasound was performed more frequently than computed tomography. In 90 of 103 (87.4%) participants who had undergone abdominal ultrasound and in 19 of 29 (65.5%) participants who had undergone a computed tomography scan of the abdomen, findings were consistent with fatty infiltration.

Factors Associated With Fatty Liver: Alcohol and Medication Use

Based on prescription records, only 20 (12.4%) participants had a history of being prescribed medications such as prednisone, amiodarone, methotrexate, or hepatotoxic substances associated with fatty liver. Forty-four (27.7%) participants were lifelong nondrinkers; 54 (34.0%) were current drinkers. The remainder (n = 61; 38.4%) were former drinkers who had not consumed alcohol within the 1 year before the interview. The median period of abstinence for former drinkers was 14 years. Among current drinkers, the average amount of alcohol consumed was 8.6 g/d (range, 0.9-31.3 g/d) for a mean of 13.6 years (range, 1-45 years). Current alcohol consumption was more common in males than in females; females were likely to be lifelong nondrinkers (Fig. 2). Of females who were current drinkers, the lifetime mean number of drinks consumed per day was 0.36, equivalent to 4.87 g/d of alcohol (range, 0.9-18.1 g/d), and the mean number of years of alcohol consumption was 15.3 years (range, 1-37 years). Currently drinking males consumed a lifetime average of 0.79 drinks per day or 10.57 g/d of alcohol (range, 2.27-31.3 g/d) over a mean period of 12.9 years (range, 1-45 years).

Race and Ethnicity and NAFLD

Of the 19 participants with definite NAFLD, 8 (44.4%) were white, 5 (27.8%) were Hispanic, 3 (16.7%) were Asian, 1 (5.7%) was American Indian, and 1 (5.7%) was of more than one race. In the combined definite and probable NAFLD population, 44.7% were white, 28.3% were Hispanic, 17.6% were Asian, 3.1% were African American, and 6.3% other or more than one race or ethnicity. Figure 3 depicts the distribution of race and ethnicity in the combined definite and probable NAFLD groups compared with the KP membership. The proportion of Hispanics in the NAFLD study population was higher than the KP membership, and the proportion of whites in the NAFLD study population was lower than the KP membership. Whites, who accounted for 59% of the KP membership, made up 45% of the NAFLD study population, and Hispanics, who accounted for 10% of the KP membership, made up 28% of the NAFLD study population. The proportion of Asians in the KP membership and the NAFLD study population was similar.
can Americans, who comprised 9% of the KP members, accounted for only 3% of the NAFLD study population, but the number of African-American participants with NAFLD was small.

The clinical and biochemical characteristics of participants with NAFLD by racial and ethnic group in shown in Table 1. The mean age of NAFLD participants was 50 years, with most NAFLD participants (61.5%) between the ages of 45 and 64 years. There were differences in the mean age of participants by racial and ethnic group (\( P < .001 \); Table 1). African Americans were 9 to 15 years older, on average, than whites, Hispanics, or Asians.

NAFLD was as frequent in males as females (52.2% vs. 47.8%) overall, but sex differences in the distribution of NAFLD were seen among Asians and African Americans (Fig. 4).

Further evaluation of participants of Asian race with NAFLD revealed that males were significantly younger than females (median age, 44.0 vs. 55.0 yr; \( P = .0085 \)). There were no significant differences in the proportion of Asian males (vs. females) with BMI of more than 28 kg/m² (\( P = .91 \)), diabetes mellitus (\( P = .11 \)), dyslipidemia (\( P = .14 \)), or current alcohol use (\( P = .93 \)). However, 68% of Asian males were previous drinkers, compared with 17% of Asian females (\( P < .02 \)).

### Discussion

NAFLD is rapidly emerging as one of the most important chronic liver diseases of the twenty-first century. Epidemiological studies in patients with other types of chronic liver disease have shown that race and ethnicity can be predictive of disease complications and response to treatment. For example, among patients with chronic

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**Table 1. Characteristics of NAFLD Participants by Race and Ethnicity**

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>White (n = 71)</th>
<th>Hispanic (n = 45)</th>
<th>African American (n = 5)</th>
<th>Asian (n = 28)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>31:40</td>
<td>23:22</td>
<td>0:5</td>
<td>22:6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Age (mean; yr)</td>
<td>52</td>
<td>46</td>
<td>61</td>
<td>46</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>BMI (mean; kg/m²)</td>
<td>34.0</td>
<td>34.2</td>
<td>36.6</td>
<td>26.8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>34 (48)</td>
<td>21 (47)</td>
<td>3 (60)</td>
<td>7 (25)</td>
<td>.16</td>
</tr>
<tr>
<td>Fastig glucose (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>143</td>
<td>164</td>
<td>141</td>
<td>133</td>
<td>.42</td>
</tr>
<tr>
<td>Range</td>
<td>72-302</td>
<td>87-294</td>
<td>90-181</td>
<td>83-238</td>
<td>.22</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>35 (49)</td>
<td>20 (44)</td>
<td>3 (60)</td>
<td>16 (57)</td>
<td>.72</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>266</td>
<td>265</td>
<td>210</td>
<td>300</td>
<td>.89</td>
</tr>
<tr>
<td>Range</td>
<td>75-845</td>
<td>113-1376</td>
<td>134-273</td>
<td>108-1351</td>
<td>.17</td>
</tr>
<tr>
<td>Current drinker, n (%)</td>
<td>29 (41)</td>
<td>16 (36)</td>
<td>0</td>
<td>7 (25)</td>
<td>.17</td>
</tr>
<tr>
<td>Alcohol use in current drinkers (g/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>8.1</td>
<td>10.1</td>
<td>–</td>
<td>5.4</td>
<td>.44</td>
</tr>
<tr>
<td>Range</td>
<td>0.9-31.3</td>
<td>1.36-30.8</td>
<td>–</td>
<td>0.9-14.1</td>
<td></td>
</tr>
<tr>
<td>ALT (IU/L)‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>53</td>
<td>60</td>
<td>39</td>
<td>61</td>
<td>.09</td>
</tr>
<tr>
<td>Range</td>
<td>15-143</td>
<td>19-147</td>
<td>38-42</td>
<td>24-97</td>
<td></td>
</tr>
<tr>
<td>AST (IU/L)‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>41</td>
<td>46</td>
<td>48</td>
<td>44</td>
<td>.92</td>
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<tr>
<td>Range</td>
<td>17-177</td>
<td>17-118</td>
<td>28-63</td>
<td>28-86</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index.

*Ten patients of Other Race or More than One Race excluded.

†Chi-square test for categorical variables and 1-way ANOVA for continuous variables.

‡Based on all available values in 12 months before entry into study.

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**Fig. 4. Gender distribution by race and ethnicity in persons with NAFLD.** The overall number of males and females with NAFLD was similar, but sex differences in the prevalence of NAFLD were evident in persons of Asian (\( P = .016 \) vs. other races combined) and African American race (\( P = .023 \) vs. other races combined). *Excludes participants of Other Race and More than One Race categories (n = 10; 6 males, 4 females). Males (n = 77), white; females (n = 72), black.
hepatitis C, African Americans have a lower response to antiviral treatment than whites. Asians and Alaskan Natives with chronic hepatitis B virus infection experience higher rates of hepatocellular carcinoma compared to whites. Thus, identification of racial-ethnic correlates in persons with NAFLD may lead to an improved understanding of the natural history and treatment of this disease. Additionally, sensitivity to differences in disease characteristics in different racial-ethnic groups will aid in the development of appropriate educational programs aimed at the early recognition and prevention of NAFLD within specific communities.

This study determines the distribution of NAFLD in a racially and ethnically defined population in the United States. Our study confirms the emerging importance of NAFLD as a cause of chronic liver disease, with one in every five cases of newly diagnosed chronic liver disease being definite or probable NAFLD. The above figures are in keeping with previous population estimates of NAFLD and of the metabolic syndrome in North America.

Among cases of newly diagnosed NAFLD, nonwhites comprised two thirds of the study population, with Hispanics making up the largest group (28%), followed by Asians (18%) and African Americans (3%). Pacific Islanders, Native Americans, and Native Hawaiians were also represented in the NAFLD study population, but at a low frequency. Because all NAFLD patients were enrolled in the KP healthcare plan, this study is not biased in terms of healthcare access. However, because the diagnosis of NAFLD was dependent on patients being seen by a physician and certain test results being obtained, it is possible that differences in healthcare-seeking behavior among persons of different racial and ethnic groups may affect rates of newly identified NAFLD in specific groups. Persons at the extremes of income (less than $15,000 or more than $100,000 per year) are underrepresented in the KP membership. This may influence the generalizability of our findings to other populations, but does affect the differential representation of NAFLD in the various racial-ethnic groups. The racial-ethnic distribution of our study population is not necessarily representative of that of all NAFLD patients in the United States and reflects, at least in part, the underlying racial-ethnic distribution of our base population (i.e., KP members in Alameda County).

Compared with the estimated racial-ethnic distribution of the base population (KP members) from Alameda County, the distribution of participants in the NAFLD study group showed a higher proportion of Hispanics (28% vs. 10% in the membership survey). The higher proportion of Hispanics in the NAFLD group suggests this racial-ethnic group is at higher risk for this condition. Differential rates of participation in the NAFLD study versus the KP membership survey would be unlikely to explain this substantial difference in proportions. Additionally, there are other lines of evidence suggesting Hispanics may be an ethnic group at higher risk for NAFLD. Other studies have found Hispanics have a higher BMI compared with several other ethnic groups and in women with similar BMI and socioeconomic status, Hispanic women have a greater amount of adiposity compared with white women. Although the overall representation of African Americans in this study population is too small to draw any firm conclusions, our results are consistent with those of Caldwell et al., who similarly reported a low prevalence of NAFLD in African Americans. In contrast, in the Third National Health and Nutritional Evaluation Survey (NHANES III) study of possible NAFLD cases (based on abnormal liver test results only), an increased prevalence of NAFLD among African-American males was found. Although differences in study methodology may account for the lack of consistency across studies, several studies suggest genetic or intrinsic physiological factors may underlie the differences in the prevalence of NAFLD in African Americans versus other racial groups.

We found that NAFLD patients who were of Asian race had a significantly lower BMI than all other racial groups. Although the World Health Organization currently defines overweight and obesity as a BMI of 25 kg/m² or more and 30 kg/m² or more, respectively, these definitions may vary by racial-ethnic group. Ko et al. recently proposed a lower cutoff value for BMI in persons of Asian race (BMI ≥ 23 kg/m² for overweight and BMI ≥ 27 kg/m² for obesity) based on the observation that Hong Kong Chinese have a higher percentage of body fat than whites for a given BMI. Our results support the recommendation for a lower cutoff for overweight and obesity in Asians. In another study, Asians were shown to have a higher amount of visceral adiposity and more subcutaneous fat than whites, highlighting the limitations of BMI as a single surrogate marker for obesity and obesity-related comorbidities in non-Caucasian racial-ethnic groups. Racial-ethnic differences in the relationship between BMI, percent body fat, and body-fat distribution among whites, Hispanics, and African Americans also have been reported and may contribute to the sex and ethnic variations in NAFLD prevalence. Although waist circumference was not measured in our study, persons with an increased waist circumference have been shown to be more likely to exhibit type 2 diabetes, dyslipidemia, and other components of metabolic X syndrome than persons with a normal waist circumference independent of race, BMI, age, income, socioeconomic class, or physical activity. Although waist-to-hip ratio
generally is thought to correlate with visceral adiposity, Conway et al. have shown that waist-to-hip ratio correlates with visceral adiposity in white women but not in African-American women, again highlighting the existence of racial variability in these anthropometric parameters.

One of the limitations of this study was the lack of liver biopsies in most patients. Additionally, although tests to exclude viral hepatitis were available for all participants, testing for autoimmune and metabolic liver diseases was incomplete in many patients. Thus, it is possible that a few participants with incomplete laboratory testing to exclude these less common chronic liver diseases (autoimmune hepatitis, hemochromatosis, Wilson’s disease, and α1-antitrypsin deficiency) were included in the study cohort. However, given the low prevalence of these conditions in the population, the effects of this bias are not likely of sufficient magnitude to change the results of our study.

This U.S. study finds sex differences in NAFLD among Asians. NAFLD was strikingly more common in males than females. It is possible that our findings may reflect differences in study participation or healthcare-seeking behavior by sex among Asian KP members. However, in support of sex differences in NAFLD among Asians, a recent study of 3,432 Japanese adults seen at a single hospital in Nagasaki in which ultrasound was used to detect fatty liver is not known. Previous studies in patients with NAFLD have used various cutoffs for alcohol consumption and insulin resistance are more or less pronounced in individuals of different races or ethnicities remains unclear, but the current study highlights the variability in risk factors by sex as well as race and ethnicity.

The exact amount of alcohol ingestion required to cause fatty liver is not known. Previous studies in patients with NAFLD have used various cutoffs for alcohol consumption ranging from 40 to 180 g/d as criteria to exclude alcohol-related liver disease. This study uses a validated instrument for quantification of lifetime and current alcohol consumption to ascertain alcohol use accurately. In this study, two thirds of patients were either lifelong nondrinkers or had been abstinent from alcohol for at least 12 months. In those who were current consumers of alcohol, the mean daily alcohol consumption was 8.6 g/d, which is below the maximum recommended daily alcohol intake for men (40 g/d) and women (20 g/d) and well below the level traditionally associated with alcohol-related liver injury.

In summary, we have shown that NAFLD affects individuals of diverse race and ethnicity within Alameda County, Northern California. Our findings highlight the need for increased awareness of NAFLD within minority racial-ethnic groups, the need for culturally sensitive educational programs aimed at the early recognition and prevention of NAFLD, as well as the need for ensuring adequate representation of different racial-ethnic groups in future clinical studies.

Acknowledgment: The authors thank Dr. Arthur Reingold for his ongoing support in the Chronic Liver Disease Surveillance Study and Dr. Raphael Merriman for his helpful comments during study planning.

References


Serum Alanine Aminotransferase in Skeletal Muscle Diseases

Rahul A. Nathwani, Shireen Pais, Telfer B. Reynolds, and Neil Kaplowitz

Although elevation of the levels of serum alanine aminotransferase (ALT) following liver injury is well known, confusion exists concerning skeletal muscle injury as the cause of this rise. We reviewed the records of 16 patients who had muscle necrosis without evidence of liver disease. The patients were divided into three groups: extreme exercise, polymyositis, and seizures. All patients exhibited markedly elevated creatine kinase and lactate dehydrogenase levels consistent with muscle injury. In acute cases, aspartate aminotransferase (AST) and ALT were both elevated, and the AST/ALT ratio was greater than 3, but this ratio approached 1 after a few days because of a faster decline in AST. In conclusion, this difference in half-life accounts for the comparable AST and ALT levels in our cases with chronic muscle injury. (HEPATOLOGY 2005;41:380-382.)

It is well known that by way of its presence in various cells such as hepatocytes, cardiac and skeletal myocytes, erythrocytes and brain cells, serum aspartate aminotransferase (AST) levels can be elevated in a wide spectrum of clinical disorders. In contrast, elevation in alanine aminotransferase (ALT) levels is widely viewed as a specific indicator of liver necrosis. However, skeletal muscle is known to contain isozymes of creatine kinase, lactate dehydrogenase (LDH), AST, and ALT, which may be released into the blood stream following muscle necrosis. Although reports of elevations in AST and ALT levels in patients with isolated skeletal muscle injury exist in the literature, there is still a pervasive lack of recognition of the correlation between skeletal muscle injury and ALT elevation, which has led to the unsubstantiated conclusion that serum aminotransferase elevations are due to liver injury or that liver injury is a secondary consequence of rhabdomyolysis. Therefore, we evaluated the pattern of serum aminotransferase elevation in patients with skeletal muscle injury due to various etiologies in the documented absence of liver disease.

Methods

The Liver Consult Service, under the supervision of Dr. Telfer Reynolds, retained records on all patients seen in consultation at the Los Angeles County—University Southern California Medical Center and filed these according to the final diagnosis.

We reviewed the records of patients seen from 1975 to 2002 who had a muscle disorder as the apparent sole cause of abnormal liver enzymes. Patients with identified liver disease, including viral hepatitis, biliary tract disease, use of potentially hepatotoxic drugs, and recent episodes of hypotension and heart failure were excluded. Some patients with fairly convincing predominant muscle injury also abused alcohol and/or cocaine, and because these agents can injure the liver we decided to exclude these subjects. Patient demographics, mode of presentation, hospital course, and peak elevations in liver tests and muscle enzymes were evaluated. All values are presented as the median with the range in parentheses.

Results

There were 16 patients (75% males, 44% Hispanic, 38% African American, 2% Asian) with a mean age of 39.8 ± 3.1 years. Four patients presented soon after extreme exercise—3 following vigorous squatting sessions (a practice of prison inmates) and 1 after a long-distance run. Another 6 patients had previously established diagnosis of polymyositis based on muscle biopsies and/or serum aldolase levels. The last 6 patients presented soon after a single grand mal seizure.
Peak simultaneous abnormalities in the serum biochemical profile are shown in Table 1. An extensive work-up for other abnormalities to account for the observed aminotransferase abnormalities was negative. The median AST/ALT ratio was between 3 and 5 in the seizure and extreme exercise groups and 1 in the polymyositis group. Interestingly, this ratio fell rapidly to 1.12 and 0.96 in patients in the seizure and extreme exercise groups, respectively, over a median of 3 days, even though these values were still elevated. Figure 1 shows the daily AST and ALT levels over 4 days following peak elevations in two patients in the seizure group in whom detailed follow-up was available. By day 4, the AST levels had sharply declined, whereas the ALT levels had changed minimally; as a result, the two reached similar levels. All patients in the extreme exercise and seizure groups had documented myoglobinuria, and 1 patient in the seizure group had renal failure requiring dialysis. Patients in all three groups were treated for their underlying disorder with a discomparable improvement in liver tests and muscle enzymes.

**Discussion**

Elevation in liver tests following extreme physical exertion has been reported previously. Some authors have attributed this to a reversible form of hepatic dysfunction believed to result from unclear mechanisms. Patients in our report had normal serum bilirubin levels and prothrombin times despite significant elevations in transaminase and LDH levels, an unlikely finding had the liver been the source of the enzyme abnormality. We also observed a similar picture in patients with cocaine or ethanol intoxication (delirium tremens), although liver damage secondary to the intoxicant itself cannot be reliably ruled out in these patients. Furthermore, cocaine can induce hyperpyrexia, hypotension, and/or arterial vasospasm, which can lead to an ischemic liver injury.

Interestingly, patients with polymyositis had roughly equal AST and ALT levels as noted by others, which may reflect the difference between the half-lives of the two enzymes. With acute rhabdomyolysis, the AST peak is initially higher than the ALT peak, although as the problem resolves, AST and ALT levels become comparable (reflecting the shorter half-life of AST as illustrated in Fig. 1). Thus, if a delay in testing were to occur, the patient with acute rhabdomyolysis may exhibit moderately increased aminotransferase levels and roughly similar AST and ALT levels. For the same reason, we assume that in patients with polymyositis, the more rapid turnover of muscle enzymes.7 With acute rhabdomyolysis, the AST peak is initially higher than the ALT peak, although as the problem resolves, AST and ALT levels become comparable (reflecting the shorter half-life of AST as illustrated in Fig. 1). Thus, if a delay in testing were to occur, the patient with acute rhabdomyolysis may exhibit moderately increased aminotransferase levels and roughly similar AST and ALT levels. For the same reason, we assume that in patients with polymyositis, the more rapid turnover of muscle enzymes.

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**Table 1. Peak Simultaneous Serum Biochemical Profile and Liver Tests in 16 Patients With Skeletal Muscle Disease**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>CPK (Normal: 25–525 IU/L)</th>
<th>LDH (Normal: 90–220 IU/L)</th>
<th>AST (Normal: 10–40 IU/L)</th>
<th>ALT (Normal: 0–1 mg/dL)</th>
<th>ALAST/ALT</th>
<th>Total Bilirubin (Normal: 0–1.1 mg/dL)</th>
<th>INR (Normal: 0.8–1.2)</th>
<th>Alkaline Phosphatase (Normal: 45–140 IU/L)</th>
<th>BUN (Normal: 4–20 mg/dL)</th>
<th>Cr (Normal: 0.6–1.2 mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extreme exercise</td>
<td>4</td>
<td>81,432</td>
<td>6,879</td>
<td>2,466</td>
<td>497</td>
<td>5.1</td>
<td>0.7</td>
<td>1</td>
<td>82</td>
<td>13</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(37,300–392,890)</td>
<td>(6,014–7,744)</td>
<td>(421–3,967)</td>
<td>(115–712)</td>
<td>(3.6–5.6)</td>
<td>(0.5–1.1)</td>
<td>(1.1–1.1)</td>
<td>(74–92)</td>
<td>(10–20)</td>
<td>(0.8–1.6)</td>
</tr>
<tr>
<td>Polymyositis</td>
<td>6</td>
<td>12,923</td>
<td>1,305</td>
<td>436</td>
<td>406</td>
<td>1.2</td>
<td>0.6</td>
<td>1.1</td>
<td>77</td>
<td>13.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(536–43,911)</td>
<td>(399–2,394)</td>
<td>(53–580)</td>
<td>(35–847)</td>
<td>(0.1–1.9)</td>
<td>(0.4–0.9)</td>
<td>(1.1–1.1)</td>
<td>(69–105)</td>
<td>(3.8–20)</td>
<td>(0.6–1.1)</td>
</tr>
<tr>
<td>Seizures</td>
<td>6</td>
<td>26,621</td>
<td>2,273</td>
<td>1,448</td>
<td>383</td>
<td>3.8</td>
<td>0.8</td>
<td>1</td>
<td>124</td>
<td>18.5</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1,019–50,029)</td>
<td>(306–4,840)</td>
<td>(235–10,000)</td>
<td>(152–670)</td>
<td>(1.5–14.9)</td>
<td>(0.3–1.9)</td>
<td>(0.9–1)</td>
<td>(66–219)</td>
<td>(12–120)</td>
<td>(0.9–9.3)</td>
</tr>
</tbody>
</table>

**NOTE.** Values are expressed as the median with the range in parentheses.

Abbreviations: CPK, creatine phosphokinase; INR, prothrombin time international normalized ratio; BUN, blood urea nitrogen; Cr, serum creatinine.

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**Fig. 1.** Serum AST and ALT levels in two representative patients (A and B) in the seizure group for 4 days following peak elevation. By day 4, both levels were still over four times the upper limit of normal and were comparable. AST, aspartate aminotransferase; ALT, alanine aminotransferase.
with an increased AST/ALT ratio should point to the correct diagnosis. However, creatine kinase, LDH, and AST levels may decline much more rapidly than ALT levels; if tests are delayed a few days, a misleading picture may emerge that suggests mild to moderate liver injury.

**Conclusion**

Elevated ALT levels in the absence of other evidence of liver disease should lead to consideration of muscle injury, which is confirmed by elevated creatine kinase and LDH levels.

**References**

Bioartificial Liver in Acute Liver Failure: Impostor or Simply Misunderstood?


Abstract

Objective: The HepatAssist liver support system is an extracorporeal porcine hepatocyte-based bioartificial liver (BAL). The safety and efficacy of the BAL were evaluated in a prospective, randomised, controlled, multicenter trial in patients with severe acute liver failure. Summary Background Data: In experimental animals with acute liver failure, we demonstrated beneficial effects of the BAL. Similarly, Phase I trials of the BAL in acute liver failure patients yielded promising results. Methods: A total of 171 patients (86 control and 85 BAL) were enrolled. Patients with fulminant/subfulminant hepatic failure and primary nonfunction following liver transplantation were included. Data were analyzed with and without accounting for the following confounding factors: liver transplantation, time to transplant, disease etiology, disease severity, and treatment site. Results: For the entire patient population, survival at 30 days was 71% for BAL versus 62% for control (P = 0.26). After exclusion of primary nonfunction patients, survival was 73% for BAL versus 59% for control (n = 147; P = 0.12). When survival was analyzed accounting for confounding factors, in the entire patient population, there was no difference between the 2 groups (risk ratio = 0.67; P = 0.13). However, survival in fulminant/subfulminant hepatic failure patients was significantly higher in the BAL compared with the control group (risk ratio = 0.56; P = 0.048). Conclusions: This is the first prospective, randomized, controlled trial of an extracorporeal liver support system, demonstrating safety and improved survival in patients with fulminant/subfulminant hepatic failure.

Comments

The comparison of the search for an effective liver support device to that of the endeavor to find the “holy grail” is clichéd and parochial, but it effectively articulates the importance of such a device to liver disease. The clinical burden of liver disease is increasing and is anticipated to continue to do so for at least another 10 to 15 years. The management of liver failure is becoming progressively more resource intensive and expensive, as futility is replaced by optimism for positive clinical outcomes in these settings. Liver transplantation is pivotal to the management of patients with both acute and chronic liver failure. However, only a minority of patients with liver failure get access to a liver transplant and then with limited control over the timing of the intervention and the clinical condition of the patient at the time of surgery. An effective liver support device would stabilize critically ill patients, optimize patients for liver transplantation, and allow some patients to escape the need for transplantation altogether.

Although enthusiasm for liver support devices has waxed and waned over the last 40 years, no device has established itself in clinical practice outside the realm of the enthusiasts. The initial enthusiasm generated by the early studies of charcoal hemoperfusion in the 1970s and 1980s was dashed by the apparent negativity of the pivotal trial.1,2 This immediately evoked concerns about trial design and interpretation and concerns “that the baby was being thrown out with the bathwater.”3,4 This was to become a recurring theme. The systems that have been or are currently being evaluated are classifiable as biological, nonbiological, or hybrid systems. The biological systems include bioartificial livers (BALs) utilizing cell-based therapies including porcine cells, human hepatoblastoma cells (C3A cell line), or human hepatocytes. The nonbiological systems include charcoal hemoperfusion, hemodiabsorption using powdered-activated charcoal, high volume plasmapheresis and albumin dialysis (including MARS). A systematic review of trials published using these devices up to September 2002 evaluated the outcome in 353 patients with acute liver failure and 130 patients with acute-on-chronic liver failure.5 It was concluded that these systems had no effect on mortality in randomized trials of patients with acute liver failure, but a 33% reduction in mortality was seen in patients with acute-on-chronic liver failure. No significant benefit in bridging patients to transplantation was identified. A significant improvement in encephalopathy was identified but this was considered a “soft” outcome measure prone to observer bias.

A challenge in the development of a liver support device is the reality that good trials are difficult to design and execute in acute liver failure, which has been the conventional testing ground for these devices. Acute liver failure is a heterogenous condition, with etiology, age, and the tempo of disease progression all significantly influencing the capacity for spontaneous regeneration. The latter is a clearly a prerequisite for studies that assess the ability of a device to bridge patients to transplant-free survival, but it
is probably also relevant to studies evaluating devices as bridges to transplantation. This has become the leading objective of studies over the last decade or so, and there is little evidence of confidence in testing the ability of current devices in bridging patients to transplant-free survival. The profile of patients with the best prospects of recovery is of young patients with rapidly progressive disease who have acetaminophen induced liver failure and hepatitis A or B. The patients least likely to reflect a benefit from liver support are older, have subacute liver failure, and have seronegative (or indeterminate) hepatitis or idiosyncratic drug reactions. There is also considerable heterogeneity with respect to the clinical complications that arise in these patients, particularly with regard to intracranial hypertension, SIRS/sepsis, hemodynamic instability, and renal failure, and these too need to be considered at the stage of trial design.

Modern trials of liver support devices in acute liver failure are “hostages to fortune” by virtue of the practice of diverting these patients to liver transplantation once a donor organ has been allocated. Reform of the United Network for Organ Sharing allocation system has advanced patients with acute liver failure in the United States, and now the majority of patients are receiving transplants within 48 hours of being wait-listed, a situation that is comparable to that of the United Kingdom. This results in an inadequate period of time available to appropriately assess the efficacy of a device in a substantial cohort of a study population.

Demetriou and his colleagues have recently reported the outcome of a controlled trial of a porcine hepatocyte–based BAL in 171 patients with acute liver failure or primary nonfunction after liver transplantation. With this device, separated plasma is initially pumped through a charcoal column before passing through a cartridge with hollow perforated fibers (pore size, 1.5 μm) that facilitate contact with the porcine hepatocytes. Twenty centers across the United States and Europe recruited 171 patients over a 3-year period, including 24 patients with primary nonfunction, 121 with fulminant hepatic failure, and 26 with subacute liver failure. Of these, 85 were randomized to BAL and 86 were controls. The primary end point was 30-day survival. The average number of treatments was 2.9 (range, 0-9), and the results were analyzed on an intention-to-treat basis.

The data and safety monitoring board terminated the trial after 171 patients had been recruited “because it determined that trial continuation, under the protocol in place and using this type of data analysis, was likely to be futile for the primary end point of 30-day survival.” The 30-day survival was 71% in the BAL group and 62% in the control group (P = .26). Subset analyses showed no difference in 30-day survival in the patients with primary nonfunction (75% vs. 58%; P = .667) or acute liver failure (73% vs. 59%; P = .117). A conclusion that can, and has been, drawn is that this is a negative study.

However, further analyses, although possibly post hoc, have identified a number of observations that deserve further consideration before the baby disappears with the bathwater. The first of these observations is that survival benefit was seen in the subgroup of 80 patients with acute liver failure with an identifiable etiology. This cohort had a 44% reduction in mortality at 30 days and a significant delay in the time to death. This observation could be a quirk of overanalysis, but equally it could be a reliable observation given that this group is very similar to the patient profile outlined above as most likely to have early liver regeneration, and hence it is very credible that these patients would show benefit from the device when others might not.

The study was clear in demonstrating the safety of the BAL device in a population where that could not be taken for granted. It also found no evidence of transmission of porcine endogenous retrovirus, an important theoretical risk that could limit the acceptance of this device. The device also appeared to reduce serum bilirubin levels, but the contribution of the different components of the device to this phenomenon remains uncertain. However, the study failed to shed light on one of the most intriguing issues—i.e., the ability of liver support devices to prevent or treat intracranial hypertension. An early study with BAL did show a reduction in intracranial pressure, but from levels that were not particularly high (mean, 17 mm Hg) and certainly not in the range that would lead to brainstem herniation or ischemic/hypoxic brain injury. The incidence of recognized intracranial hypertension in the latest study was low in both groups (11.6% vs. 7.1%), but these figures are almost certainly underestimates as systematic use of intracranial pressure monitoring was not utilized in this study (mainly reflecting the challenge of multicenter studies). This important issue remains to be resolved.

For the regulators and proponents of evidenced-based medicine, this trial probably represents a “full stop.” To the community of clinicians practicing hepatology and in need of an effective device it is probably a “semicolon.” However, to the clinical researcher with a commitment to developing an effective liver support device it probably represents only a “comma.” It is tempting to speculate that a different trial design and patient selection could have given a positive outcome and totally altered the climate for the development of liver support devices. Enthusiasts will hope that the positive messages of this trial, together with the lessons learned, will galvanize the search...
for the “holy grail.” The need for clinical trials persists, but these trials must set achievable targets for the device, use appropriate clinical end points, and be conducted in a patient population likely to demonstrate the benefits of the device.

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References


Copyright © 2004 by the American Association for the Study of Liver Diseases. Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/hep.20496

Conflict of interest: Nothing to report
Preamble

The recommendations in this article provide a data-supported approach. They are based on the following: (1) a formal review and analysis of recently published world literature on the topic (as listed in MEDLINE); (2) the American College of Physicians’ A Manual for Assessing Health Practices and Designing Practice Guidelines; (3) policy guidelines, including the American Association for the Study of Liver Diseases’ Policy Statement on Development and Use of Practice Guidelines and the American Gastroenterological Association’s Policy Statement on the Use of Medical Practice Guidelines; and (4) the authors’ years of experience in the care of patients with portal hypertension and use of transjugular intrahepatic portosystemic shunt in the management of these disorders. These recommendations are fully endorsed by the American Association for the Study of Liver Diseases and the Society for Interventional Radiology.

Intended for use by physicians, these recommendations suggest preferred approaches to the diagnostic, therapeutic, and preventative aspects of care. They are intended to be flexible, in contrast to standards of care, which are inflexible policies designed to be followed in every case. Specific recommendations are based on relevant published information. In an attempt to characterize the quality of evidence supporting recommendations, the Practice Guidelines Committee of the American Association for the Study of Liver Diseases requires a grade to be assigned and reported with each recommendation (Table 1).

Introduction

Transjugular intrahepatic portosystemic shunt (TIPS) has been in use for more than 20 years to treat the complications of portal hypertension, and TIPS have been created in thousands of patients with liver disease worldwide. Despite the extensive use of TIPS to treat the complications of portal hypertension, there initially was a lack of consensus regarding which patients should receive TIPS instead of other forms of therapy. A 1995 conference sponsored by the National Institutes of Health concluded that TIPS was effective in the acute control and prevention of recurrent bleeding from varices, but it was unclear when TIPS should be used instead of medical and surgical therapy for these complications of portal hypertension. In addition, the efficacy of TIPS to control refractory ascites or treat Budd-Chiari syndrome was unclear but promising. Since then, more than 1,000 patients have been enrolled in multiple controlled trials comparing TIPS with endoscopic and pharmacological therapy in the prevention of rebleeding from varices and with large-volume paracentesis in the treatment of refractory ascites associated with cirrhosis. Furthermore, approximately 1,000 papers have been published on TIPS in the English literature alone. This body of work allows for more definitive recommendations about in whom and when to use TIPS in the treatment of the complications of portal hypertension.

The guidelines are divided into two large categories. The first category is a review of the technical aspects of the procedure, its complications, and the data on which patients are most at risk for an adverse outcome following TIPS. The second category is a review of the indications for TIPS. The use of TIPS for primary prevention of variceal bleeding and the control of acute bleeding are discussed first. Next, the two indications for TIPS that have been subjected to controlled trials (prevention of recurrent bleeding from varices and refractory ascites) are discussed, and guidelines are developed. Lastly, all of the other indications for TIPS that have been described in the literature but have not been subjected to controlled trials are discussed, and guidelines are created.
To prepare these guidelines, a MEDLINE search was performed on papers published between 1966 and 2004. Nine hundred eight papers were found under the subject heading “transjugular intrahepatic portosystemic shunt.” Controlled trials and large series were sought. Recently published papers were also used as a source of references missed by the MEDLINE search, as were the personal files of the two authors.

The Procedure

A TIPS is created by an interventional radiologist or, in Europe, by a specially trained physician. The technique is reviewed in several publications and will not be discussed here. The procedure may be performed under conscious sedation (most common) or general anesthesia. If the procedure is going to be prolonged or the patient is hemodynamically unstable, then general anesthesia is preferred because it allows for careful monitoring by the anesthesiologist. The success rate with TIPS for the decompression of the portal vein is high—more than 90% of cases in most series. The Society of Interventional Radiology developed guidelines for creation of a TIPS in 2001, and the consensus was that a technically successful outcome (including both creation of the shunt and a decrease in portal pressure to $\leq 10$ mm Hg) should be achieved in 95% of patients, and clinical success (resolution of the complication of portal hypertension) should be achieved in 90% of cases. Failure to achieve this threshold should lead to a review of departmental policy and procedures.

Early mortality following TIPS placement was originally reported to be quite high as a result of poor patient selection, but subsequent analysis demonstrated that preprocedure clinical features (such as high model for end-stage liver disease [MELD] or APACHE II scores, high total bilirubin levels, emergent versus elective setting, or presence of pneumonia; see Mortality) accounted for this high death rate. In most situations, death is due to progressive liver disease, perhaps as a result of portal diversion, and is not due to complications of the procedure itself, such as intraperitoneal bleeding (see Mortality). In a retrospective series of 1,750 patients, the incidence of fatal complications (intra-abdominal hemorrhage, laceration of the hepatic artery or portal vein, and right heart failure) was 1.7% (range, 0.6%-4.3%). Interestingly, the risk of fatal complications was 3% in institutions that had performed fewer than 150 TIPS total compared with 1.4% in those that had performed a greater number. These data suggest that there is a learning curve associated with the safe creation of a TIPS. Major procedural complications are expected in no more than 3% of cases; if rates exceed these levels, internal quality assessment should be considered. Authors of manuscripts on TIPS have been asked by the Society of Interventional Radiology to report the approximate number of TIPS performed in their centers before instituting the reported study to obtain a better understanding of the amount of training required to perform TIPS with an acceptable morbidity and mortality, and it is hoped these data are forthcoming.

The purpose of a TIPS is to decompress the portal venous system and therefore prevent rebleeding from varices or stop or reduce the formation of ascites. Regarding varices, it is well established that if the hepatic venous pressure gradient (HVPG) can be reduced to less than 12 mm Hg, the risk of bleeding will fall significantly. More recent data suggest that achieving a HVPG of less than 12 mm Hg may not be required to prevent rebleeding. In one series, the risk of rebleeding following TIPS revision was 18%, 7%, and 1% in patients whose HVPG had been reduced by 0%, 25% to 50%, and more than 50%, respectively. In a second series, a 50% reduction in the initial HVPG was associated with a rebleeding rate at 1 year of 11%, whereas patients with a lesser reduction had a 31% probability of rebleeding during the first year. In the latter study, the only absolute value for prevention of rebleeding was an HVPG of less than 12 mm Hg, but at the cost of an increased incidence of encephalopathy. Although the gold standard for prevention of rebleeding remains an HVPG of less than 12 mm Hg, further studies are needed to determine if lesser reductions have acceptable efficacy with a lower incidence of encephalopathy.

The optimal HVPG that needs to be obtained for the control of refractory ascites associated with cirrhosis is even less clear. In one series, the degree of portal decompression did not correlate with successful treatment of refractory ascites associated with cirrhosis, and the authors suggested that a HVPG of less than 8 mm Hg should be the hemodynamic goal. The selection of a value of 8 mm Hg is based on limited data, and because the development of ascites associated with cirrhosis reflects changes in both hepatic and renal function, it may be difficult to establish an absolute value of decompression that needs to be achieved in most patients with re-

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**Table 1. Quality of Evidence on Which a Recommendation is Based**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Randomized controlled trials</td>
</tr>
<tr>
<td>II-1</td>
<td>Controlled trials without randomization</td>
</tr>
<tr>
<td>II-2</td>
<td>Cohort or case-control analytical studies</td>
</tr>
<tr>
<td>II-3</td>
<td>Multiple time series, dramatic uncontrolled experiments</td>
</tr>
<tr>
<td>III</td>
<td>Opinions of respected authorities, descriptive epidemiology</td>
</tr>
</tbody>
</table>

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...
fractory ascites. In patients with significant pre-existing encephalopathy in whom a TIPS may still be necessary for ascites control, a higher gradient may be appropriate (to limit worsening encephalopathy); this affords the opportunity to further enlarge the TIPS at a later date if diuresis is inadequate and encephalopathy is satisfactorily controlled. Further study in this area is warranted.

Finally, in the authors’ experience the effective gradient needed to prevent rebleeding from gastric varices may be lower than 12 mm Hg and, even with apparent decompression embolization of the gastric varices, may be required to minimize the risk of early rebleeding. Also, rebleeding from gastric varices may occur with small increases in portal pressure, suggesting that surveillance of this group of patients following TIPS is of particular importance.21

Further complicating the issue is the problem of how the pressures are obtained. The classic way is to measure the free and wedged hepatic vein pressure and then subtract the two values yielding the HVPG.23 The use of the free hepatic vein or inferior vena cava pressure is necessary to correct for the intra-abdominal pressure and allows for measurement of the true pressure gradient across the liver. However, most radiologists use the right atrial pressure as the reference point because the hepatic vein is now part of the shunt; thus a free hepatic vein pressure cannot be obtained after shunt creation, because the diverted portal flow artifactually raises the pressure within the outflow hepatic vein that drains the TIPS. The right atrium is of course in the chest, and the basal pressure in the chest is lower than the intra-abdominal pressure; therefore, the true HVPG is not measured using this reference point. In addition, once the TIPS has been created, the right atrial pressure tends to rise, thus complicating the measurement. One solution to this problem is to use the inferior vena cava pressure as the reference value, but this has not been adopted by the interventional radiological community. No standardization of where in the inferior vena cava the pressure should be obtained has limited this approach, and currently the right atrial pressure is used by most interventional radiologists despite the above limitations. Some of these uncertainties could be resolved with standardization of how the HVPG is measured during creation of a TIPS so that the measurements are uniform and can be used to judge hemodynamic success more accurately.

Pre-TIPS Evaluation and Contraindications

Most patients who are referred for a TIPS should be under the care of a gastroenterologist or hepatologist, who in consultation with an interventional radiologist must reach the decision that TIPS is the appropriate form of treatment for a complication of portal hypertension. As discussed in the following section, it is clear that there are predictors of a poor outcome following TIPS. However, the risk of the procedure must always be balanced with the severity of the complication from which the patient is suffering and the likelihood of the patient surviving long enough to receive a liver transplant following creation of a TIPS. Thus, the decision to perform or not perform TIPS in a high-risk patient should be reached by the gastroenterologist/hepatologist and the interventional radiologist together. Ideally, in a high-risk patient, a transplant center should also be consulted preceding the final decision. In the emergent setting of acute, uncontrolled variceal hemorrhage, contacts with transplantation centers may be secondary to the need for shunt creation.

Table 2 lists contraindications to the creation of a TIPS. These include both absolute contraindications to any form of portosystemic diversion, be it surgical or percutaneous. Absolute contraindications include congestive heart failure, severe tricuspid regurgitation, and severe pulmonary hypertension (mean pulmonary pressures > 45 mm Hg, as these patients are not candidates for a liver transplant).24 Whether patients with more mild pulmonary hypertension can receive a TIPS safely is unclear. Relative contraindications include anatomical ones that can complicate the creation of the shunt and reduce the technical success, including portal venous obstruction, large hepatic tumors, extensive polycystic liver disease, and hepatic vein obstruction. It is well established that shunts can be created in all of these cases with the right experience and under appropriate clinical circumstances, but the difficulty of creating the TIPS needs to be balanced with the need of the patient. Situations in which these relative contraindications might be outweighed by clinical necessity include palliative TIPS in patients with hepatoma and refractory variceal bleeding, recanalization of occluded portal veins in patients with recurrent variceal bleeding or refractory ascites, and a patient with Budd-Chiari syndrome and progressive liver failure in whom there are no patent hepatic veins.

<table>
<thead>
<tr>
<th>Table 2. Contraindications to Placement of a TIPS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absolute</strong></td>
</tr>
<tr>
<td>Primary prevention of variceal bleeding</td>
</tr>
<tr>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>Multiple hepatic cysts</td>
</tr>
<tr>
<td>Uncontrolled systemic infection or sepsis</td>
</tr>
<tr>
<td>Unrelieved biliary obstruction</td>
</tr>
<tr>
<td>Severe pulmonary hypertension</td>
</tr>
</tbody>
</table>

Abbreviation: INR, international normalized ratio.
Preprocedure laboratory studies include serum electrolytes, blood count, coagulation parameters, and tests of liver and kidney function. Cross-sectional liver imaging via Duplex ultrasound, computed tomography, or magnetic resonance imaging is appropriate in all but the most life-threatening situations to assess portal vein patency or the presence of liver masses. When a history of congestive heart failure, tricuspid regurgitation, cardiomyopathy, or pulmonary hypertension is present, cardiac evaluation is appropriate before a TIPS procedure. This evaluation may include an echocardiogram, cardiology consultation, and, possibly, atrial fluid challenge. In the absence of a cardiac history, the routine performance of an echocardiogram preceding a TIPS is unnecessary in the opinion of the authors; however, others feel that because up to 16% of patients referred for liver transplantation may have pulmonary hypertension, an echocardiogram should be performed on all patients before a TIPS is created. Elevated right atrial pressures (typically measured at the start of the TIPS procedure) may warrant abandonment or delay of the procedure pending diuresis or further medical evaluation. Lastly, patients with a significant coagulopathy may be able to undergo a TIPS following the use of clotting factors or platelets. The finding of a small liver during the evaluation is not a contraindication to creation of a TIPS, but it does indicate that the procedure may be difficult and prolonged.

Mortality

The 1-year mortality rates for TIPS are dependent somewhat on the indication for the procedure. When a TIPS has been placed for bleeding varices, 1-year survival varies from 48% to 90%. Survival rates are similar when the indication is ascites (48%-76%). In one series but not another, survival rates were significantly worse when the indication was refractory ascites compared with variceal bleeding. These differences likely reflect variations in the severity of liver disease between the different studies.

As the use of TIPS has increased, there has been interest in models that predict outcome. MELD and a number of other models have been developed to predict survival following TIPS. The modified MELD model utilizes serum bilirubin level, international normalized ratio for prothrombin time, and serum creatinine level (cause of cirrhosis was also used previously but has since been abandoned). These three variables are used to create the following equation: \[3.8 \log_e (\text{bilirubin} [\text{g/dL}]) + 11.2 \log_e (\text{international normalized ratio}) + 9.6 \log_e (\text{creatinine} [\text{mg/dL}]) \]. A second model used a bilirubin level of greater than 3.0 mg/dL (1 point), an alanine aminotransferase level of greater than 100 IU/L (1 point), pre-TIPS encephalopathy (1 point), and urgency of TIPS (2 points) and divided patients into three groups (low risk, 0 points; medium risk, 1-3 points; high risk, 4-5 points). These two models and Child-Turcotte-Pugh scores were used prospectively in a subsequent study to predict survival. All three accurately predicted 3-month survival to a similar degree, whereas 1-year survival was predicted best by the MELD model. Short-term mortality has also been predicted by using bilirubin alone or a combination of serum bilirubin, APACHE-II score, and TIPS urgency. Irrespective of which model is chosen, the short-term and 1-year survival can be predicted with some accuracy. These survival estimates can be used to advise patients about expected outcomes and can also be used to decide which patients will require referral to a liver transplant center.

Recommendations

1. TIPS should only be performed by experienced interventional radiologists (or specially trained physicians). Success and complication rates should be monitored; if they fail to meet expected rates, review of the program should be considered (evidence: grade III).

2. The decision to perform a TIPS, especially in a high-risk patient, should be reached by a team consisting of a gastroenterologist/hepatologist, interventional radiologist, and, where appropriate, a transplant physician (evidence: grade III).

3. Preceding creation of a TIPS, tests of liver and kidney function should be performed in addition to cross-sectional imaging of the liver to assess portal system patency and exclude liver masses (evidence: grade III).

4. Reduction in HVPG to less than 12 mm Hg should be achieved when the indication is bleeding esophageal varices. Embolization of gastric varices may be required despite adequate decompression of the portal venous system (evidence: grade II-2).

5. The degree of reduction in HVPG to control ascites is unclear, but at present a gradient of 8 mm Hg or less has been suggested to be a reasonable goal (evidence: grade II-2).

6. Patients with high predicted 30-day mortalities should be informed of their prognosis, and TIPS should be performed only in the absence of other options (evidence: grade II-2).

7. In high-risk patients, the need for liver transplantation should be discussed before the performance of an elective TIPS (evidence: grade III).
to 15%. The cause of the thrombosis may be leakage of the intima. Thrombosis of the TIPS usually occurs early and can happen within 24 hours of TIPS creation. Thrombosis of the TIPS can either be due to thrombosis or hyperplasia within the parenchymal tract or within the outflow hepatic vein. The occluded stents are coated by a collag- enous matrix that is covered by endothelial cells. Thrombosis of the TIPS is identified using Doppler ultrasound, and patency is re-established through repeat catheterization. In one controlled trial, use of the anticoagulant phenprocoumon was associated with a lower rate of complete occlusion within the first 3 months following TIPS placement. However, in the absence of more studies, the routine use of anticoagulation is not recommended.

The major difficulty with TIPS is the unpredictable patency of the shunts as a result of pseudointimal hyperplasia within the parenchymal tract or within the outflow hepatic vein. The occluded stents are coated by a collag- enous matrix that is covered by endothelial cells. The incidence of stenosis varies from 18% to 78% depending upon the surveillance techniques used, frequency of assessment, and definitions of failure, (e.g., elevated portasystemic gradient, ultrasound velocity criteria, or percent diameter stenosis). Most physicians rely on Doppler ultrasound to identify TIPS stenosis. Unfortunately, the earlier studies claiming greater than 90% accuracy for sonographic prediction of shunt dysfunction have failed to stand under the light of larger prospective or retrospective studies. In one series, several ultrasonographic features were used to identify TIPS stenosis, including flow reversal, jet lesion, and decreased flow in the TIPS or portal vein. The sensitivity of each of these tests varied from 10% to 26% with a specificity of 88% to 100%. Thus the negative predictive value was poor and the positive predictive value was acceptable. In a second series of 31 occluded or stenotic stents, ultrasound predicted shunt malfunction in only 11 and incorrectly predicted patency in 20; thus the sensitivity was only 35%. Many of the sonographic studies are methodologically flawed, because sonographic criteria of shunt dysfunction were used to trigger TIPS venography; however, when sonography suggested no shunt dysfunction, proof of shunt patency via venography was not performed. Part of the difficulty of using sonography is that it is an imaging technique that measures velocity, from which diameter within a conduit can be estimated. However, with TIPS it is portal decompression—not percent shunt stenosis—that is important in assessing TIPS function. One prospective study compared 151 Doppler sonograms with TIPS venograms and assessment of portal pressure. Using a success or failure definition of a portosystemic gradient of less than 15 mm Hg or 15 mm Hg or more, respectively, sonography provided a sensitivity and specificity of only 86% and 48%, respectively. Thus an abnormal Doppler ultrasound is predictive of occlusion or stenosis, whereas a normal ultrasound does not exclude TIPS dysfunction. The best indicator of TIPS dysfunction is a recurrence of the problem for which the TIPS was originally inserted: either variceal bleeding, hepatic hydrothorax, or ascites. If recurrent varices are identified by upper endoscopy, then the TIPS most likely is insufficient. Documentation of patency can only be achieved with certainty through recatheterization of the shunt.

The development of covered stents should reduce the frequency of TIPS dysfunction. Two large series have recently been published that have examined the use of polytetrafluoroethylene (PTFE)-covered stent grafts for TIPS. One of the reports is of a series of 71 patients, all of whom received the covered stents, whereas the second report is a randomized controlled trial comparing the covered stents with the standard bare stents. In the non-randomized series, a total of 8 shunt revisions were performed for an incidence of 11.3%, and primary pa-

### Table 3. Complications of TIPS

<table>
<thead>
<tr>
<th>Complication</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombosis</td>
<td>10–15</td>
</tr>
<tr>
<td>Occlusion/stenosis</td>
<td>18–78</td>
</tr>
<tr>
<td>Transcapsular puncture</td>
<td>33</td>
</tr>
<tr>
<td>Intraperitoneal bleed</td>
<td>1–2</td>
</tr>
<tr>
<td>Hepatic infarction</td>
<td>~1</td>
</tr>
<tr>
<td>Fistulae</td>
<td>Rare</td>
</tr>
<tr>
<td>Hemobilia</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Sepsis</td>
<td>2–10</td>
</tr>
<tr>
<td>Infection of TIPS</td>
<td>Rare</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>10–15</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>10–44</td>
</tr>
<tr>
<td>New/worse</td>
<td>5–20</td>
</tr>
<tr>
<td>Chronic</td>
<td></td>
</tr>
<tr>
<td>Stent migration or placement into inferior vena cava or too far into portal vein</td>
<td>10–20</td>
</tr>
</tbody>
</table>

NOTE. Data are from Boyer and Vargas and Roессle et al.
tency rates at 6 and 12 months were calculated to be 87% and 81%, respectively.\(^{51}\) Although these results are better than what would be expected with bare stents, all patients did not undergo venography and therefore the true incidence of shunt stenosis is unknown. In the randomized study, 80 patients with cirrhosis and either uncontrolled or recurrent bleeding from varices or refractory ascites were enrolled in the study. Patients were followed with Doppler ultrason, and venography was performed at 6, 12, and 24 months post-TIPS. Five (13%) of the 39 patients receiving the PTFE-covered stent grafts had shunt dysfunction, whereas 18 (44%) of those receiving the bare stent had shunt dysfunction \((P < .001)\). In addition, early thrombosis of the TIPS was observed in three patients who received the bare stents. The actuarial rates of primary patency in the covered and bare stent groups were 86% and 47%, respectively, at year 1 and 80% and 19%, respectively, at year 2. Recurrence of the complication of portal hypertension for which the TIPS was placed was also significantly more common in the bare stent group compared with the PTFE-covered stent group. The incidence of hepatic encephalopathy was less in the PTFE-covered stent group \(\text{difference not significant}\), and survival was the same.\(^{55}\) The PTFE-covered stents have recently been approved for use in the United States by the Food and Drug Administration.\(^{52}\) The use of the PTFE-coated stent grafts should decrease significantly the incidence of shunt dysfunction and recurrence of the complications of portal hypertension. It is unclear, however, whether this development will improve the cost-effectiveness of TIPS compared with other forms of therapy.

Puncture of the liver capsule is common, but serious intraperitoneal bleeding is infrequent \((1\% \text{ to } 2\% \text{ of cases})\). Similarly, creation of a biliary venous or hepatic artery–portal vein fistula is rare. The development of jaundice or sepsis following TIPS suggests the former, whereas pulsatile flow in the portal vein suggests the latter.\(^{53,54}\) Hemolysis may occur following TIPS placement and appears to be due to damage to the red cells by the stent.\(^{55-57}\) Recognition that the rise in bilirubin levels is due to hemolysis is an important diagnosis, because an alternative diagnosis is liver failure following TIPS, which carries a poor prognosis.\(^{58}\) Hepatic infarction is a rare complication of TIPS and is generally related to injury and/or thrombosis of the hepatic artery that supplies the affected segment.\(^{59}\)

Hepatic encephalopathy and TIPS dysfunction are the two complications that have limited the effectiveness of TIPS most significantly. The incidence of new or worsening encephalopathy following TIPS is 20% to 31%.\(^{25,60,61}\) In controlled trials comparing TIPS with alternative forms of therapy, the incidence of encephalopathy is always greater in those who received a TIPS \(\text{see sections Esophageal Variceal Bleeding and Ascites Associated With Cirrhosis})\). Pre-TIPS factors associated with an increased risk of post-TIPS encephalopathy in one study included etiology of liver disease other than alcohol, female sex, and hypoalbuminemia.\(^{61}\) In a second series, increasing age, past history of encephalopathy, and evidence of encephalopathy at the time of TIPS were predictive of post-TIPS encephalopathy.\(^{60}\) It is important to note that if encephalopathy is precipitated by variceal bleeding, prevention of rebleeding should make it less likely that the patient will have recurrent encephalopathy. Only if the hepatic encephalopathy is uncontrollable is a TIPS contraindicated.\(^{15}\) In most patients, the encephalopathy responds to standard therapy, and only rarely \((\sim 5\%\) ) must the TIPS be occluded to control the encephalopathy.\(^{52,63}\) A TIPS also can be reduced in caliber should excessive encephalopathy prove difficult to control and yet allow for continued portal decompression.\(^{64}\) There is no data supporting the use of lactulose in all patients following a TIPS to reduce the incidence of encephalopathy.

**TIPS in the Transplant Candidate**

Patients awaiting liver transplantation frequently bleed from varices or have refractory ascites associated with cirrhosis and therefore are candidates for a TIPS. Because these patients will subsequently undergo a hepatectomy, there are complications involved with TIPS that are unique to this population. A TIPS is created within the substance of the liver, and most interventional radiologists attempt to place the stent as close as possible to the hepatic vein/inferior vena cava ostium to reduce the risk of developing stenosis within the hepatic vein. With the exception of cases of benign or malignant portal vein thrombosis, the stent should extend the shortest possible distance into the main portal vein, both to allow creation of a durable shunt and yet not complicate the portal-to-portal vein anastomosis performed during transplantation. When the stent extends into the inferior vena cava (or atrium) or deep into the main portal vein, transplantation difficulties can arise. In one series of 12 patients who had a TIPS preceding liver transplantation, 4 patients had portal vein stents near the coronary vein or extending into the superior mesenteric vein, and venous reconstruction was required in 1 patient.\(^{65}\) In a second series of 24 patients who had a TIPS created before transplantation, 8 patients had more complicated surgeries that were attributable to the presence of a TIPS. Four of the stents were in the inferior vena cava, one was in the superior mesenteric vein, and in three the portal vein was thrombosed. Despite being able to complete the trans-
plant in all 8 patients, patient and graft survival were somewhat worse in those with complications related to the presence of the TIPS. However, in other series, despite the technical issues that arose during the transplant because of the presence of the shunt, operative time and patient and graft survival were the same in patients who were transplanted in the presence and absence of a TIPS. All patients who have a TIPS created should be considered possible liver transplant candidates; thus care should be taken to not extend the stents beyond the minimum necessary portions of the portal and hepatic vein/inferior vena cava junction required to insure a functioning shunt. If the patient is being considered for living related transplantation, then lining the entire hepatic vein to the inferior vena cava may complicate transplantation, because a cuff of hepatic vein is required to complete the transplant in these patients.

**Recommendations**

8. Physicians who perform TIPS need to be aware of both the procedural complications and the complications due to portal diversion and must be experienced in their management (evidence: grade II-3).

9. Each center performing TIPS should have an established program of TIPS surveillance, and although there are no established guidelines, Doppler ultrasound should be performed before the patient is discharged from the hospital and at specified intervals following the procedure and the yearly anniversary of the TIPS thereafter (evidence: grade II-1).

10. Ultrasonographic findings suggesting TIPS dysfunction or recurrence of the complication of portal hypertension that lead to the initial TIPS should lead to repeat shunt venography and intervention, as indicated. The recurrence of symptoms in the face of a “normal” ultrasound does not eliminate the need for TIPS venography (evidence: grade II-2).

11. TIPS stenosis is common, especially in the first year, and Doppler ultrasound lacks the sensitivity and specificity needed to identify many of these patients. Therefore, repeat catheterization of the TIPS or upper endoscopy should be performed at the 1-year anniversary of placement, especially in those patients who bled from varices (evidence: grade II-3).

**Indications**

Table 4 lists the variety of conditions for which TIPS has been used. It is recognized that a number of listed indications, such as hepatorenal syndrome or Budd-Chiari syndrome, may never be assessed in larger prospective randomized controlled trials because of their low incidence. Accordingly, for these conditions recommendations will be based on review of uncontrolled series and expert opinion.

**Primary Prevention of Variceal Bleeding**

The development of varices is a common sequela of portal hypertension. The frequency of esophageal varices varies from 30% to 70% in patients with cirrhosis, and 9% to 36% will have so-called “high-risk” varices. Esophageal varices will develop in patients with cirrhosis at a yearly rate of 5% to 8%, but in only 1% to 2% will the varices be large enough to pose a risk of bleeding. In patients with small varices, approximately 4% to 30% of the patients will develop large varices each year and will therefore be at risk of bleeding. Use of treatments to prevent bleeding from these varices that have never bled is termed “primary prophylaxis,” and beta-blockers are currently considered the best approach to prevent bleeding in this group of patients. Previously, when surgical shunts were used as primary prophylaxis bleeding from varices was prevented, but this occurred at the unacceptable cost of increased mortality in the shunted patients compared with the control patients. No trials comparing TIPS with other forms of therapy in the prevention of the first bleed from varices have been performed. Because TIPS, like a surgical shunt, brings with it the risks of hepatic encephalopathy, liver failure, and procedural complications, it cannot be recommended for primary prophylaxis, and its use should be limited to unique situations.

**Acutely Bleeding Esophageal Varices Refractory to Medical Treatment**

Most patients who present with actively bleeding varices can be controlled with pharmacological and endoscopic therapy. However, an occasional patient will rebleed or continue to bleed despite aggressive management, and these patients become candidates for portal decompression. Previous experience with surgical shunts
was poor because of the high mortality (31%-77%) associated with urgent or emergent shunting.\textsuperscript{69,70} Although TIPS has now been used in this situation successfully, it is important to note that its urgency is an independent predictor of early mortality.\textsuperscript{26,28} One report analyzed 15 studies in which TIPS was used to control bleeding in patients who had failed medical therapy. TIPS controlled bleeding in 93.6% ± 6.7% of patients, and early rebleeding was seen in only 12.4% ± 6.1% of the patients; however, hospital mortality at 6 weeks was high (35.8% ± 16%).\textsuperscript{74} It is clear that the preprocedural condition of the patients (MELD score, APACHE II score, urgent indication) predict the 30-day survival after TIPS in this group of patients. Although TIPS has not been compared with alternative treatments in the acutely bleeding patient, nonselective portacaval shunts have been compared with endoscopic therapy. Shunts were more effective than endoscopic therapy in the control of bleeding, but mortality rates of 31% to 77% were observed.\textsuperscript{70} Similar results would be expected if TIPS were compared with endoscopic therapy in the acute control of bleeding, but these studies are unlikely to occur given the desperate state of many of these patients. Pending the development of alternative therapies, TIPS will remain the only alternative to control acute variceal bleeding that is refractory to medical therapy.

**Esophageal Variceal Rebleeding**

Once varices have bled, the risk of rebleeding is at least 50% and many of these patients will die.\textsuperscript{75,76} Hence, a number of therapies have been used to prevent rebleeding in these patients, most of which have been subjected to controlled trials.\textsuperscript{70} When surgical shunts were compared with endoscopic therapy, rebleeding rates were reduced, whereas the incidence of hepatic encephalopathy was increased in the surgical groups and mortality was unaffected (Table 5).\textsuperscript{69,70} When TIPS was first developed, it was hoped that the effect on rebleeding would mirror that of surgical shunts but with lower rates of encephalopathy because of the ability to tailor shunt size to the minimum necessary diameter required to decompress the portal system. This has not proven to be the case for a variety of reasons, including the unpredictable patencies of uncovered stents and the lack of controlled trials using stents of different diameters to prevent rebleeding. In 1999, a meta-analysis of the 11 published controlled trials comparing TIPS with endoscopic therapy was reported.\textsuperscript{77} The results with TIPS mirror the results with surgical shunts—that is, there is less rebleeding compared with endoscopic therapy, but at the price of more encephalopathy without an improvement in survival (Table 5). As has been seen in the trials comparing surgical shunts with endoscopic therapy, the rate of crossover between treatment groups was greater for endoscopic therapy (17%) than with TIPS (2%). The cost of treating the patients with TIPS was greater than the cost of endoscopic therapy because of the need for frequent reintervention to maintain TIPS patency.\textsuperscript{78} TIPS has also been compared with pharmacological therapy in a small number of patients. In one series of approximately 90 patients, the risk of rebleeding during 2 years of follow-up was 39% in those who received pharmacological therapy and 13% in those receiving TIPS. The frequency of encephalopathy was approximately twice in the patients treated with TIPS. Child-Turcotte-Pugh class improved in 72% of the drug group but in only 45% of the TIPS group. The 2-year probability of survival was the same in both groups (72%). Endoscopic reintervention was required in 12 of the drug-treated patients, and in 5 patients portal decompression, either via TIPS or surgery, was required for variceal rebleeding. The cost of therapy for patients receiving TIPS was twice that of the pharmacological group, in part because 70% of the TIPS patients required reintervention.\textsuperscript{79} It is important to note the variation in the cohorts among the different trials, because in some studies patients were medical failures with several bleeds, whereas in others they had a single bleed before being randomized.

It is clear from the above studies that both TIPS and surgical shunts are the most effective method for the prevention of rebleeding. There has been one published trial in which TIPS was compared with a surgically placed

<p>| Table 5. Surgical Shunts and TIPS vs Endoscopic Therapy in the Prevention of Rebleeding |</p>
<table>
<thead>
<tr>
<th>Number of Patients</th>
<th>Rebleeding Rate</th>
<th>Encephalopathy</th>
<th>Mortality</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Endo</td>
<td>PCS</td>
<td>TIPS</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>376</td>
<td>49.8%</td>
<td>12.4%*</td>
<td>8.6%</td>
</tr>
<tr>
<td>811</td>
<td>46.6%</td>
<td>18.9%*</td>
<td>18.7%</td>
</tr>
</tbody>
</table>

NOTE. Data are from D’Amico et al.\textsuperscript{70} and Papatheodoridis et al.\textsuperscript{77}

Abbreviations: Endo, endoscopic therapy; PCS, portacaval shunt.

*Meta-analysis revealed that rebleeding was significantly less with PCS or TIPS compared with endoscopic therapy.

†Meta-analysis revealed that incidence of encephalopathy was greater with PCS or TIPS compared with endoscopic therapy.
H-graft shunt. The patients were not randomized but were done as pairs (i.e., one receiving a surgical shunt and the second receiving a TIPS). A total of 132 patients were in the study. The frequency of rebleeding was 16% in the TIPS group and 3% in the surgical group. The patients undergoing TIPS required frequent interventions to maintain TIPS patency. Thirty-day and total mortality were 15% versus 20% and 43% versus 30% in the TIPS and surgery groups, respectively. Another randomized controlled trial comparing TIPS with distal splenorenal shunt has been completed. Rebleeding was seen in 5.5% of the distal splenorenal shunt patients and 9% of the TIPS patients (difference not significant). However, only 11% of the distal splenorenal shunt patients required reintervention to maintain patency, whereas 82% of the TIPS patients required reintervention. Survival was the same in both groups (J. M. Henderson, personal communication). Thus, both TIPS and distal splenorenal shunt are effective in preventing rebleeding in patients who have failed pharmacological or endoscopic therapy, but TIPS patients require more frequent reintervention to prevent rebleeding.

Bleeding From Gastric Varices

The efficacy of TIPS in the control of rebleeding from gastric varices has been reported in a number of small series. In most of the series, the outcome of patients with bleeding gastric varices was compared with those who had bled from esophageal varices. In none of the trials were the patients randomized to alternative therapies, and in most the TIPS was performed because of refractory bleeding. In some series, the initial HVPG in patients with gastric varices was lower than that of patients with esophageal varices, whereas in other series no differences were observed. In these small series, TIPS was equally effective at controlling bleeding from gastric as well as esophageal varices. Controlled trials comparing surgical shunts or glue in the treatment of these patients would help to better define the role of TIPS in the management of patients with bleeding from gastric varices. In the authors’ opinion, TIPS is an important tool in the control of gastric variceal bleeding, though the final portal systemic gradient required to achieve variceal decompression may be lower than what is required for esophageal variceal bleeding, and embolization of the varices also may be required.

Prevention of Bleeding From Portal Hypertensive Gastropathy and Gastric Antral Vascular Ectasia

The diagnosis of portal hypertensive gastropathy (PHG) and gastric antral vascular ectasia (GAVE) are made endoscopically. The mucosa in PHG may show a mosaic-like pattern (“snake skin”), or, in more severe cases, cherry red and black-brown spots. The changes are usually seen in the fundus or body of the stomach. GAVE is localized to the antrum and is characterized by red patches or spots that may be diffuse or linear in appearance. PHG is limited to patients with portal hypertension, whereas GAVE can be seen in a variety of different disorders, including cirrhosis. The effect of TIPS on PHG and GAVE has been examined in several small series. In one report, 75% of patients with severe PHG showed both endoscopic improvement and a decrease in the need for transfusions. In another series, 9 of 10 patients showed endoscopic improvement in PHG following TIPS. In contrast, bleeding from GAVE in patients with cirrhosis was unaffected by TIPS.

Recommendations

12. The use of TIPS to prevent bleeding from varices that have never bled is contraindicated because of the risk of increasing morbidity and mortality (evidence: grade III).

13. TIPS is effective in controlling acute bleeding from varices that is refractory to medical therapy and is preferred to surgery in this situation (evidence: grade II-3).

14. TIPS should not be used for the prevention of rebleeding in patients who have bled only once from esophageal varices, and its use should be limited to those who fail pharmacological and endoscopic therapy (evidence: grade I).

15. TIPS is effective in the prevention of rebleeding from gastric and ectopic varices (including intestinal, stomal, and anorectal varices) and is the preferred approach for the prevention of rebleeding in this group of patients (evidence: grade II-3).

16. Pending further studies, in patients with good liver function, either a TIPS or a surgical shunt are appropriate choices for the prevention of rebleeding in patients who have failed medical therapy (evidence: grade II-2).

17. In patients with poor liver function, TIPS is preferred to surgical therapy in the prevention of rebleeding in patients who have failed medical therapy (evidence: grade III).

18. The use of TIPS in the management of PHG should be limited to those who have recurrent bleeding despite the use of beta-blockers (evidence: grade II-3).

19. TIPS is ineffective in controlling bleeding from GAVE in patients with cirrhosis and should not be used in this situation (evidence: grade II-3).
Ascites Associated With Cirrhosis

Ascites develops in patients with cirrhosis because of the development of portal hypertension in concert with splanchnic vasodilation, renal sodium retention, and active renal vasoconstriction. As the liver disease progresses, the ascites becomes more resistant to diuretic therapy, and refractory ascites develops. Ascites is said to be refractory to medical treatment when it is unresponsive to sodium restriction and the use of high doses of diuretics (400 mg/d spironolactone and 160 mg/d furosemide) or when the patient is intolerant of diuretic therapy. Once refractory ascites develops, prognosis is poor; approximately 50% of patients die within 12 months. A number of approaches have been taken in the management of patients with refractory ascites, including peritoneo-venous shunts, repeated large volume paracentesis (LVP), and TIPS. Peritoneo-venous shunts have been abandoned because of a lack of efficacy and high rate of complication except in unusual circumstances. TIPS has been compared with LVP in the treatment of patients with refractory ascites associated with cirrhosis. The data from five published controlled trials are shown in Table 6. There were a total of 330 patients enrolled in these five trials. In the TIPS groups, the percentage (mean ± SD) of patients who showed improvement in their ascites (lack of need for paracentesis) was 62.0% ± 19.2%, while in the LVP groups improvement was seen in 23.6% ± 18.5% of patients. The transplant-free 2-year survival in three of the studies was similar (37% ± 17.7% for the TIPS patients and 40.1% ± 16.8% for the LVP patients), and in the fourth study survival was also similar in the two groups. Only in the most recently published report was survival significantly better in the TIPS group. Encephalopathy occurred somewhat more frequently in the TIPS groups compared with the LVP groups (39.4% ± 20.9% and 22.6% ± 13.9%, respectively). Somewhat surprisingly, there was no difference in the quality of life between the two groups in one of the studies. Cost-effectiveness was not examined in any of the studies.

Refractory Hepatic Hydrothorax

Hepatic hydrothorax develops in patients who have ascites associated with cirrhosis when there is direct communication between the abdominal and thoracic cavities. It may develop in patients with or without clinically apparent ascites. In most patients, the defect is in the diaphragm that overlies the dome of the liver. Once refractory ascites develops, prognosis is poor; approximately 50% of patients die within 12 months. A number of approaches have been taken in the management of patients with refractory ascites, including peritoneo-venous shunts, repeated large volume paracentesis (LVP), and TIPS. Peritoneo-venous shunts have been abandoned because of a lack of efficacy and high rate of complication except in unusual circumstances. TIPS has been compared with LVP in the treatment of patients with refractory ascites associated with cirrhosis. The data from five published controlled trials are shown in Table 6. There were a total of 330 patients enrolled in these five trials. In the TIPS groups, the percentage (mean ± SD) of patients who showed improvement in their ascites (lack of need for paracentesis) was 62.0% ± 19.2%, while in the LVP groups improvement was seen in 23.6% ± 18.5% of patients. The transplant-free 2-year survival in three of the studies was similar (37% ± 17.7% for the TIPS patients and 40.1% ± 16.8% for the LVP patients), and in the fourth study survival was also similar in the two groups. Only in the most recently published report was survival significantly better in the TIPS group. Encephalopathy occurred somewhat more frequently in the TIPS groups compared with the LVP groups (39.4% ± 20.9% and 22.6% ± 13.9%, respectively). Somewhat surprisingly, there was no difference in the quality of life between the two groups in one of the studies. Cost-effectiveness was not examined in any of the studies.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of Patients</th>
<th>Ascites Improved</th>
<th>Survival*</th>
<th>New or Severe Encephalopathy</th>
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<tbody>
<tr>
<td></td>
<td>TIPS</td>
<td>LVP</td>
<td>TIPS</td>
<td>LVP</td>
</tr>
<tr>
<td>Lebrec et al.90</td>
<td>13</td>
<td>12</td>
<td>38%</td>
<td>0%</td>
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<tr>
<td>Rossle et al.91</td>
<td>29</td>
<td>31</td>
<td>84%†</td>
<td>43%</td>
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<tr>
<td>Gines et al.92</td>
<td>35</td>
<td>35</td>
<td>51%†</td>
<td>17%</td>
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<tr>
<td>Sanyal et al.22</td>
<td>52</td>
<td>57</td>
<td>58%†</td>
<td>16%</td>
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<tr>
<td>Salerno et al.93</td>
<td>33</td>
<td>33</td>
<td>79%‡</td>
<td>42%</td>
</tr>
</tbody>
</table>

*Transplant-free survival after 2 years for first three studies.
†Significant difference between two groups.
‡End point was failure, which was defined as the need for at least four LVPS for recurrent ascites.

Hepatorenal Syndrome

Hepatorenal syndrome (HRS) is a dreaded complication of cirrhosis, because its development is associated with a poor prognosis. HRS exists in two forms. Type 1 HRS is defined as the rapid (over a 2-week period) development of renal failure, whereas in type 2 HRS the renal failure develops more slowly. The prognosis for patients with type 1 HRS is significantly worse than for those with type 2 HRS. TIPS has been used in a number of patients with HRS. In these small series, the use of TIPS has been associated with improvements in glomerular filtration rates and renal plasma flow, as well as falls in serum creatinine and plasma aldosterone levels. However, because none of the trials was controlled, no comparative survival benefit has been shown. In one series, only 20% of the patients with type 1 HRS were alive 1 year after TIPS creation, whereas with type 2 HRS approximately 45% were alive after 1 year. These results are somewhat better than expected based on the experience of others; however, care must be exercised in comparing uncontrolled studies, because severity of disease may not be the same across studies. In one of the controlled trials in which
TIPS was compared with LVP in the control of refractory ascites associated with cirrhosis as discussed above, a reduced incidence of HRS in those receiving a TIPS was observed.22 Similar to the findings when TIPS has been used for other complications of portal hypertension, pre-TIPS bilirubin levels were predictive of survival in these patients as well.98 Finally, creation of a TIPS in HRS patients can be difficult because of concerns about fluid overload and the need to limit the volume of contrast used. TIPS needs to be compared with other therapies such as terlipressin and other vasactive compounds before its role in the treatment of HRS is determined, and currently its use should be considered investigatory.88,101

Recommendations

20. Although TIPS will decrease the need for repeated large-volume paracentesis in patients with refractory ascites associated with cirrhosis, it should be used only in those patients who are intolerant of repeated large-volume paracentesis (evidence: grade I).

21. TIPS is effective in the control of hepatic hydrothorax, but it should be used only in patients whose effusion cannot be controlled by diuretics and sodium restriction (evidence: grade II-3).

22. TIPS is not recommended for the treatment of HRS, especially type 1 HRS, pending the publication of controlled trials (evidence: grade II-3).

Budd-Chiari Syndrome

Budd-Chiari syndrome (BCS) results from blockage of exit of the blood from the liver as a result of hepatic vein thrombosis or obstruction of the inferior vena cava.102,103 Liver injury results from hepatic congestion, and side-to-side portacaval shunts were used previously for the management of this disorder. More recently, the prognosis for these patients has been examined based on a number of variables, and although it is clear some of the patients require no intervention, for others the only solution appears to be a liver transplant. A model has been created using the following equation that allows for the prediction of survival of patients with BCS: 1.27 × encephalopathy + 1.04 × ascites + 0.72 × prothrombin time + 0.004 × bilirubin.104 Based on this model, patients can be separated into three groups with good, intermediate, and poor 5-year survivals. Only in patients with an intermediate prognosis was a side-to-side portacaval shunt shown to have a positive impact on survival.104 Although side-to-side portacaval shunts have been used effectively in this group of patients, operations within the portal space are to be avoided, if possible, because many of these patients may eventually require a liver transplant. There have been a number of case reports and two small series on the outcome of patients with BCS who have received a TIPS.105,106 In one series, patients with good prognostic indices were treated symptomatically and with anticoagulation and did well.105 In both series it was the patients with progressive disease who underwent a TIPS. Patients with acute hepatic failure due to BCS did poorly; half of the patients died in the immediate postprocedure period. Patients with more chronic disease did much better and had relief of symptoms, improvement in liver function, and a good intermediate (mean follow-up: 2-4 years) survival. Most of the patients had an underlying prothrombotic disorder and required long-term anticoagulation.106 The frequency of TIPS insufficiency and thrombosis in the BCS patients did not differ from the frequency of these events in patients with cirrhosis. Despite these results, it remains unclear whether or not TIPS improves survival; but if TIPS is going to have an impact, it most likely will be in the patients with an intermediate prognosis.102 Performing a TIPS in a patient with BCS can be difficult if the hepatic vein is completely occluded. In this situation, a transmesenteric TIPS may be performed, but this approach is limited to a few centers with extensive experience in creating a TIPS.107,108

Veno-occlusive Disease or Sinusoidal Obstruction Syndrome

Sinusoidal obstruction syndrome is seen most commonly following hematopoietic stem cell transplantation, but it can also occur following exposure to toxins in plants such as bush tea.109 Symptoms vary from mild sodium retention to progressive liver failure leading to death. In patients with the severe form of the disease, ascites is common as a result of the development of portal hypertension. TIPS has been used in a small number of these patients.109-113 In these series, TIPS improved ascites and lowered levels of aspartate aminotransferase and alanine aminotransferase but did not affect serum bilirubin levels. Most of the patients died despite the creation of the TIPS.

Hepatopulmonary Syndrome

Hepatopulmonary syndrome is a complication of cirrhosis in which shunts develop in the lung, leading to the development of hypoxia.114 Six patients have been reported who had hepatopulmonary syndrome and received a TIPS; 5 of the 6 showed improvement in oxygenation, and some but not all showed a decrease in intrapulmonary shunts.115 The mechanism through which TIPS may improve intrapulmonary shunting in patients with portal hypertension is unclear.
Recommendations

23. The decision to create a TIPS in a patient with Budd-Chiari syndrome should be based on the severity of disease, and only patients with moderate disease appear to be reasonable candidates for a TIPS (evidence: grade II-3).

24. Patients with BCS and mild disease can be managed medically, whereas those with more severe disease or acute hepatic failure are best managed by liver transplantation. (evidence: grade II-3).

25. The use of TIPS to treat sinusoidal obstruction syndrome cannot be recommended (evidence: grade II-3).

26. The use of TIPS to treat hepatopulmonary syndrome cannot be recommended (evidence: grade II-3).

Conclusions

TIPS is an important part of the current armamentarium used to treat the complications of portal hypertension. Most fellowship-trained interventional radiologists are capable of creating a TIPS in a patient with patent hepatic and portal veins. Creation of a TIPS ranks among the more complex procedures performed by interventional radiologists, and it is important that each physician monitor their success and complication rates. As with any complex intervention, the decision to create a TIPS should be reached by a gastroenterologist or hepatologist who is experienced in the management of these patients in concert with an interventional radiologist. Pre-TIPS evaluation includes routine tests of liver and kidney function as well as Doppler ultrasound, contrast-enhanced abdominal computed tomography, or magnetic resonance imaging of the liver. Once a TIPS is created, it cannot be forgotten—the patient requires frequent monitoring by Doppler ultrasound and clinic visits to look for the development of TIPS dysfunction. The use of PTFE-covered stents may reduce the risk of TIPS dysfunction, but this will not eliminate the need for continued surveillance.

TIPS will effectively prevent rebleeding from varices and decrease the need for repeat thoracentesis in patients with hepatic hydrothorax or for large-volume paracentesis in patients with refractory ascites. However, TIPS will increase the incidence of hepatic encephalopathy and will not improve survival in any of these patients. Hence, TIPS should not be considered as primary therapy for any complication of portal hypertension with the exception of bleeding gastric or ectopic varices. In all other situations, TIPS should only be created when the patient has failed other forms of medical therapy (i.e., pharmacological or endoscopic therapy, diuretics, or repeated large-volume paracentesis or thoracentesis). In patients with good liver function and recurrent bleeding from varices despite medical treatment, it is unclear whether a surgical shunt or TIPS is the better form of therapy pending the publication of additional controlled trials. Which patients with BCS are best managed by TIPS remains undefined, although creation of a TIPS in select patients may be of benefit. Creation of a TIPS for the treatment of HRS or hepatopulmonary syndrome is of unproven benefit and should be considered investigatory.

Acknowledgment: The authors thank Mr. L.T. Tucker for his skilled secretarial assistance and the Practice Guidelines Committee of the AASLD for all of their help in the preparation of this manuscript. The members of the Practice Guidelines Committee include K. Rajender Reddy, M.D., Chair, Robert L. Carithers Jr., M.D., Stanley M. Cohen, M.D., Thomas W. Faust, M.D., Steven L. Flamm, M.D., Gregory J. Gores, M.D., Elizabeth Hespenheide, R.N., B.S.N., Michael R. Lucey, M.D., David R. Nelson, M.D., F. Fred Poodrad, M.D., Margaret C. Shuhart, M.D., M.S., Brent A. Tetri, M.D., Zobair M. Younossi, M.D., M.P.H., and Nizar N. Zein, M.D.

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Reduced Plasma Adiponectin in NASH: Central Obesity as an Underestimated Causative Risk Factor

To the Editor:

We read with interest the recent article on adiponectin in nonalcoholic steatohepatitis (NASH) by Hui et al. The authors reported that plasma adiponectin levels (1) are reduced in patients with NASH and (2) are inversely correlated with the histologically assessed degree of necroinflammation. These observations are highly relevant and bear therapeutic potential for the treatment of NASH; however, we feel that a potentially important point regarding the underlying basis of reduced adiponectin in NASH is underestimated in this report. In a number of studies, plasma adiponectin is negatively associated with body fat mass. Although body mass index and waist/hip ratio were given, Hui and colleagues did not provide a direct assessment of body fat mass.

We measured plasma adiponectin levels using enzyme-linked immunosorbent assay (B-Bridge International, San Jose, CA) in 34 patients with a histological diagnosis of NASH and in 23 controls (all values are given as the mean ± SD) matched for age (46.9 ± 12.2 vs. 46.0 ± 13.5 years), sex (female/male: 23:11 vs. 15:8), body mass index (29.8 ± 5.2 vs. 28.1 ± 3.6 kg/m²), and body fat mass (37.9% ± 10.0% vs. 37.4% ± 8.7%) as determined via bioelectrical impedance analysis. Although body fat mass was identical, the waist-to-hip ratio as measure of central obesity was significantly higher in the subjects with NASH compared with controls (0.95 ± 0.09 vs. 0.88 ± 0.13, respectively; P < .05). In the study by Hui et al., the waist-to-hip ratio was also significantly higher in the patients presenting with NASH compared with the control group, despite having a similar body mass index. In our study population, plasma adiponectin was significantly decreased in patients with NASH compared with controls (6.0 ± 2.7 vs. 10.7 ± 5.1 µg/mL; P < .001), consistent with the report by Hui et al. Taken together, although the control group had exactly the same degree of obesity, the patients with NASH had a significantly altered body fat distribution toward central obesity and significantly lower plasma adiponectin levels.

Data in the literature indicate that different fat stores might have different metabolic and inflammatory activity and that central obesity, as indicated by a high waist-to-hip ratio, is associated with unfavorable factors. Obesity is an established risk factor for the development of hepatic steatosis. Based on our data and the literature, we propose that the transition toward a hepatic inflammatory response and the development of NASH within a fatty liver are dependent on a shift in body fat distribution. Increasing visceral obesity results in (1) increased production of proinflammatory cytokines and adipokines such as leptin, tumor necrosis factor α, and interleukin 6 and (2) decreased production of protective adipokines such as adiponectin. This abnormal balance might ultimately lead to the clinical and histopathological occurrence of NASH.

In conclusion, visceral obesity might be an important causative risk factor for NASH. Prospective multicenter studies with long-term follow-ups are necessary to further investigate the role of visceral obesity in the pathogenesis of NASH to identify patients at risk and thus provide early treatment for them.

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of visceral obesity compared to control subjects matched by percent body fat. Similar results have been published previously in a study which used single slice computed tomography (CT) to directly measure visceral obesity. However, the comparison between subjects with NASH and matched controls does not justify the proposition by Tietge et al. that "transition towards a hepatic inflammatory response and the development of NASH within a fatty liver are dependent on a shift in body fat distribution." A more appropriate study to assess the role of central obesity in the development of the necroinflammatory response is to compare patients with NASH to those with simple steatosis who are matched by percent body fat. We measured body composition using dual energy x-ray absorptiometry in 37 of our 109 subjects (28 with NASH and 9 with simple steatosis). The subjects with NASH were of similar age (47.2 ± 13.0 vs. 44.9 ± 14.6 years), gender proportion (male/female: 20/8 vs. 7/1), body mass index (31.1 ± 4.8 vs. 30.9 ± 4.0 kg/m²), and percent body fat (35 ± 8 vs. 35 ± 9 %) compared with those with simple steatosis (results expressed as mean ± SD). Despite the similar degree of overall obesity, there was a trend toward increased central obesity in subjects with NASH compared to those with simple steatosis as indicated by the waist-to-hip ratio (WHR) (Fig. 1, P = .1, Mann-Whitney U test). These data support the hypothesis that visceral obesity may underlie the metabolic alterations which precipitate a necroinflammatory response in the fatty liver. Our findings need to be validated in a larger cohort. Direct measurement of visceral mass by computed tomography or magnetic resonance imaging will provide a more accurate assessment of the relationship between the fat compartments, the biochemical milieu and the histological features of NASH.

Preprocedure Coagulation Tests Are Unnecessary Before Abdominal Paracentesis in Emergency Departments

To the Editor:

We read with great interest the article of Grabau et al. regarding performance of therapeutic abdominal paracentesis, because the authors highlight no significant procedure-related complications even in patients with marked thrombocytopenia or prolongation in the prothrombin time (PT) in an outpatient setting. We have launched a comparable study in an emergency setting and would like to share our results.

For a 1-year period starting in August 2003 in an emergency department of a tertiary center, a total of 186 abdominal paracenteses were carried out in 60 patients. The number of procedures carried out in a single patient ranged between 1 and 17. All patients underwent complete blood cell counts, biochemistries, and PT before the procedure. In the absence of a cutoff for coagulation parameters that would restrict paracentesis, all patients were eligible. The emergency physicians performed the procedures using ultrasonography to define the puncture site in the outer-left lower abdomen and an 18-gauge aspirating catheter (Surflo, Terumo Corporation, Tokyo, Japan) using sterile technique. Their age (mean ± SD) was 59.0 ± 14.0 years. The underlying diseases were hepatocellular carcinoma in 46, viral-related liver cirrhosis in 107, alcoholic cirrhosis in 7, and other malignancies in 9. The preprocedure mean international normalized ratio (INR) for PT was 1.6 ± 0.5 (range, 0.9-4.7), and the mean platelet count was 124 ± 103 × 10⁹/µL (range, 6-641 × 10⁹/µL). Details of the data are given in Table 1.

References


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Conflict of interest: Nothing to report.
Table 1. Preprocedure Prothrombin Times and Platelet Counts, and Complications

<table>
<thead>
<tr>
<th>PT (INR)</th>
<th>Diagnostic Paracentesis†, n</th>
<th>Therapeutic Paracentesis‡, n</th>
<th>Complications, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤1.4</td>
<td>27</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>1.5–1.9</td>
<td>1</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>2.0–2.4</td>
<td>0</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>2.5–2.9</td>
<td>0</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>≥3.0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Platelet count (×10^5/μL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥100</td>
<td>28</td>
<td>69</td>
<td>0</td>
</tr>
<tr>
<td>50–99</td>
<td>0</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>40–49</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>30–39</td>
<td>0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>20–29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>≤19</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

*The volume (mean ± SD) of ascites removed for diagnostic paracentesis (n = 28) was 157 ± 105 mL.
†The volume (mean ± SD) of ascites removed for therapeutic paracentesis (n = 158) was 3.020 ± 1.570 mL.

There were no procedure-related complications that required hospitalization, transfusions, or plasma volume expansion. Only two of 186 procedures (incidence, 1.1%; 95% CI, 0.3%-3.8%) were associated with minor complications in the same patient (incidence, 1.7%; 95% CI, 0.3%-8.9%) at different visits. One minor complication with removal of 1,200 mL of ascitic fluid for this 45-year-old male, a patient with hepatitis B virus-related cirrhosis, was local ecchymosis at the puncture site, with a platelet count of 81 × 10^5/μL and an INR of 2.6. The diameter of ecchymosis was 3.5 cm. The other episode of cutaneous bleeding (estimated 10 mL) occurred with removal of 4,000 mL of ascitic fluid when he had a platelet count of 51 × 10^5/μL and an INR of 2.9. It was promptly controlled within 10 minutes with local compression.

From our data, it appears that bleeding complications of abdominopelvic paracentesis in an emergency department are rare, and even if present, appear to be very mild, regardless of preprocedure INR or platelet count. Considering the results of Grabau et al. in an outpatient setting, we propose these tests are unnecessary before abdominal paracentesis in an emergency setting. A limitation to the procedure is clinically evident fibrinolysis or disseminated intravascular coagulation.3 Otherwise, our data should translate into the avoidance of unnecessary transfusion and related complications, cost savings, and shortening of the length of stay for patients in emergency departments.

References


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Conflict of interest: Nothing to report.

A Challenge on the Use of the Words Embryonic and Perinatal in the Context of Biliary Atresia

To the Editor:

Zhang et al.1 report an elegant study looking at the expression of a large number of genes and gene products in infants with biliary atresia, the main aim being to demonstrate gene or gene expression differences between two distinct clinical phenotypes. However, both the underlying general assumption and the specific nature of the patients in this study need to be challenged before one can accept the results as conclusive.

Use of the terms embryonic and perinatal in association with biliary atresia is widespread in the literature. Both terms imply an explicit assumption regarding the timing of the etiological cause; however, for a number of reasons this approach may be somewhat simplistic. The “perinatal” form of biliary atresia implies that there is destruction of an already fully formed biliary tree by a virus (presumably) at or around the time of birth. The two elements of this assumption are controversial. First, many viral studies in humans are not referenced in the article that are entirely negative but still perfectly valid.2,3 Second, the assumption that the timing of an etiological insult is perinatal has little actual evidence to support it. Antenataly detected biliary atresia, although it represents a small proportion of most series (≈5%) and has an unusual biliary appearance (cystic), has implications on the timing of biliary atresia occurrence. In our recently reported series of 9 infants with biliary atresia, all occurrences were detected between 18 and 20 weeks’ gestation, with 8 of 9 being nonsyndromic.4 Furthermore, the key studies of Francoise Muller, who measured various gastrointestinal enzymes (specifically γ-glutamyltranspeptidase) in serial samples of amniotic fluid, have also shown that in those cases of nonsyndromic biliary atresia detected “incidentally,” there was definite evidence of bile obstruction early in the second trimester.5-7

In the current study, the authors have chosen very unusual examples of biliary atresia and classified them as “embryonic.” Infants with the embryonic form of biliary atresia typically have a constellation of extrahepatic anomalies characterized by splenic anomalies (100%), situs inversus (50%), preduodenal portal vein (60%), absence of the inferior vena cava (40%), and cardiac anomalies (50%) (all percentages are based on the King’s College series, currently n = 50). We have used the term biliary atresia “spenic malformation syndrome,”8 and others, polysplenia9 or polysplenia syndrome when describing such infants.

So why were the infants in Zhang’s study exceptional? Of the 5 “embryonic” infants, only one was a typical example, with polysplenia and a preduodenal portal vein (infant 2). Infant 3 did not have a splenic anomaly but had other typical features (preduodenal portal vein, annular pancreas, and malrotation). Infant 5 had congenital cardiac abnormalities but only an interrupted inferior vena cava to suggest syndromic biliary atresia. Infant 1 was extremely abnormal and very atypical with diaphragmatic hernia, vaginovescicular fistula, cleft lip and palate, and so forth. Finally, the preterm infant (infant 4) had...
absolutely no features to suggest an embryonic cause for its biliary atresia, certainly not with a patent ductus arteriosus or hydronephrosis.

Nevertheless, using these infants, the authors then extrapolate their molecular and genetic findings and conclusions based on the more usual syndromic variant described above. For example, they searched for and found abnormal expression of laterality genes (i.e., Sprouty-4 like, Zinc family member-3-heterotaxy-1), when in fact not one of the "embryonic" infants had any clinical evidence of axial determination defects.

Searching for the roots of biliary atresia lies in unraveling the key molecular differences between the syndromic and nonsyndromic forms. The groups to be discriminated, however, need far better definition and a much higher degree of within-group homogeneity before we can speculate on any difference in their genes.

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DOI 10.1002/hep.20549
Conflict of interest: Nothing to report.

Reply:

We welcome Dr. Davenport’s interest in our article reporting hepatic transcriptional signatures that differentiate two clinical phenotypes in infants with biliary atresia. His comments underscore two important issues that need to be carefully considered in any patient-based studies addressing the pathogenesis of biliary atresia: etiology and time of onset of disease. Both issues are interrelated and, when used in conjunction with the presence or absence of nonhepatic malformations, have been used in the literature to identify clinical forms. In regards to etiology, there are sufficient data to suggest that viral insults are a plausible etiology despite the variable identification of specific viruses in published studies from different populations of patients with biliary atresia. This is further supported by an experimental mouse model of rotavirus-induced biliary atresia, in which the virus is efficiently cleared from the liver after obstruction of the extrahepatic bile duct.

The inability to detect viral elements in these mice even when sensitive techniques are used (such as PCR) underscores the concept that a negative result does not rule out a previous infection by a virus, which may have triggered an inflammatory and obstructive injury to the bile ducts. Therefore, patient- and animal-based studies support the existence of a “perinatal” form of acquisition in a group of infants with biliary atresia, perhaps due to an infectious insult. The exact timing of the proposed viral insult is currently unknown.

Dr. Davenport’s comments about the time of onset of disease and the use of the term “embryonic” to describe a group of patients included in our study highlight an important gap in our understanding of pathogenic mechanisms of disease and the need to develop a uniform system to classify clinical subtypes of biliary atresia. In regards to the time of onset of disease, although experimentally the administration of rotavirus to pregnant mice at term results in biliary obstruction in their offspring, an association between a viral insult to the developing human fetus and the postnatal diagnosis of biliary atresia has not been fully established. However, the early onset of jaundice and the coexistence of congenital nonhepatic malformations in a subset of infants with biliary atresia imply, at least in part, a perinatal onset of disease. As described in the literature cited by Dr. Davenport, the high prevalence of unique malformations in these infants, especially splenic malformations and laterality defects, allows for the grouping of these patients into the biliary atresia–splenic malformation or polysplenia syndrome. However, the nomenclature to describe infants with biliary atresia presenting with other types of nonhepatic malformations is far from clear or uniformly accepted. For example, two additional phenotypic groups have been proposed based on the presence of associated anomalies that do not follow any recognizable syndromic pattern/sequence or the presence of intestinal malrotation and atresia; notably some of the abnormalities included minor cardiovascular and urogenital malformations. These differences notwithstanding, common to all patients with nonhepatic malformations is the coexistence of one or more congenital malformations. In this context, we applied the term “embryonic” to all five infants with biliary atresia who also had nonhepatic malformations. Despite the phenotypic heterogeneity among these infants, they shared unique transcriptional profiles, as demonstrated by the coordinated expression of regulatory genes and the overexpression of imprinted genes when compared to infants with biliary atresia without our phenotypic abnormalities (termed “perinatal”).

Our experimental design and the limited number of subjects were not adequate to analyze the hepatic transcriptome in search of molecular signatures that are unique to subtypes of infants with biliary atresia and nonhepatic malformations, such as those with the biliary atresia–splenic malformation syndrome. These studies will need a population size that allows for much greater discriminatory (statistical) power of the gene expression profiling, and enable the testing of hypotheses relating to the expression of laterality genes in the subgroup of infants with laterality defects. We agree with Dr. Davenport that critical to these analyses is a rigorous phenotypic definition of clinical groups and a much higher degree of within-group homogeneity. When such a population is assembled, it will be equally important to use mathematical models to explore the existence of novel subtypes based on molecular signatures, to determine how they correlate with clinical phenotypes, and to explore their impact on long-term outcome.

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References

To the Editor:

Morishita and colleagues\(^1\) reported an interesting study investigating the expression of the cell cycle inhibitor p18(INK4c). The authors demonstrated that loss of p18 expression occurred especially in lower-differentiated hepatocellular carcinomas (HCCs) and correlated with an unfavorable prognosis. A decreased expression in advanced tumor stages and a very similar prognostic role has been demonstrated in previous studies for p27(Kip1), but not for p21(CIP1/WAF1).\(^2,3\) The association of a lack in p18 and p27 expression in human liver tumors reflects data gained in basic animal studies. In contrast to p21\(^{−/−}\) mice, p18\(^{−/−}\) and p27\(^{−/−}\) mice display increased body size and develop the same tumors at a higher age.\(^4\) The induction patterns of p18 and p27 messenger RNA in mouse embryonic development are strikingly similar.\(^5\) We recently performed a study using knockout mice for p18, p21, p27, or p18/p21 and p18/p27 in combination.\(^6\) In partial heptectomy experiments, we demonstrated that the effect of a p18 knock-out on hepatocyte cell cycle progression is similar to a p27 knock-out, whereas it differs from a p21 knock-out. The present study by Morishita and colleagues therefore highlights the relevance of observations made in the partial heptectomy model for liver carcinogenesis in humans.

However, the authors suggest in their article that the loss of p18 in the development of HCC is mediated through an upregulation of cyclin-dependent kinase 4 (CDK4) activity. In our study, we showed that loss of p18 expression alone did not influence CDK4 activation after partial heptectomy, whereas lack of p21 lead to an earlier activation of CDK4. This phenotype was enhanced in p18/p21 double knockout animals. Moreover, combined p18/p27 knockout mice displayed increased amounts of hepatocytes entering S phase after partial heptectomy compared with the respective single knockouts.\(^6\) Because p21 and p27 expression are frequently downregulated in HCC,\(^7\) it remains unclear if the effect on CDK4 activity observed by Morishita and colleagues might be caused by a simultaneous loss of p18 and p21 or p27 expression. Although p18 single mutant mice develop liver tumors in a model of chemical carcinogenesis,\(^8\) these tumors were not HCCs but hemangiosarcomas from the hepatic sinusoidal endothelial cells, suggesting that a single lack of p18 might not be sufficient for increased carcinogenesis in hepatocytes. Additionally, it is unclear why the authors observed changes in CDK4 but not CDK6 activity depending on the p18 status, whereas it was shown that p18 in vivo preferentially associates with CDK6.\(^9\) Therefore, further studies should be conducted evaluating the expression status of p18, p21, and p27 in parallel to examine their functional collaboration in HCC development. Because mutations or increased promoter methylation appear not to be involved in the downregulation of p18 expression in HCC, an additional analysis of p21 and p27 expression might even reveal a functional dependency between these proteins with regard to their expression during liver carcinogenesis, as suggested previously for p16(INK4a) and p27.\(^10\) A thorough understanding of the role of CDK inhibitors in hepatic cell cycle regulation may ultimately provide new insights into molecular hepatocarcinogenesis and may uncover new targets for therapeutic approaches.


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Reply:

We appreciate the comments of Luedde et al. We read the interesting article reported by Luedde et al. In the partial hepatectomy (PH) experiments using knockout mice for various cyclin-dependent kinase (Cdk) inhibitors, the authors have shown that a loss of p18\(^{\text{INK4C}}\) alone did not influence Cdk4 activity, but the lack of p21\(^{\text{CIPI/WAF1}}\) led to Cdk4 activation. On the other hand, we have reported that the kinase activity of Cdk4 was higher in p18\(^{\text{INK4C}}\)-negative hepatocellular carcinomas (HCCs) than in p18\(^{\text{INK4C}}\)-positive ones. Based on the results, we concluded that p18\(^{\text{INK4C}}\) expression might play an important role in the development of HCC through the up-regulation of Cdk4 activity. These two reports appear to be contradictory. However, our experiments are different from the study of Luedde and colleagues. The reasons are as follows. Liver regeneration after PH reflects the proliferation of normal hepatocytes, and the process is completed after one week. Thus, changes induced by liver regeneration after PH are quite short, and are reversible. On the other hand, proliferation of HCC is a chronic and continuous process in transformed cells (cancer cells), and it is irreversible. Therefore, although the kinetic changes of cell cycle-related molecules in liver regeneration after PH provide important hints for the mechanism of hepatocarcinogenesis, they may not be directly applied to the proliferation of cancer cells. To solve this problem, it may be necessary to study Cdk4 activity in carcinogen-induced HCC of p18\(^{\text{INK4C}}\)-knockout animals.

It has been shown that the expression of p21\(^{\text{CIPI/WAF1}}, \text{p27 KIP1}\) and p57\(^{\text{KIP2}}\) were frequently downregulated in HCC. Therefore, we agree with the suggestion that the effect on Cdk4 activity in HCC may be caused by a simultaneous loss of p18\(^{\text{INK4C}}, \text{p21 CIPI/WAF1}\) and/or p27\(^{\text{KIP2}}\). In the future, it will be needed to determine what Cdk inhibitors influence Cdk4 activation in HCC. In addition, we would like to address the following point. Luedde et al. have stated that a loss of p18\(^{\text{INK4C}}\) expression alone did not influence Cdk4 activation after PH. However, a clear band corresponding to the retinoblastoma protein phosphorylated at Ser-780 was visible 36 and 48 hours after PH in p18\(^{\text{INK4C}}\)-knockout animals (Fig. 5C in the text of Luedde et al.). This result suggests that a loss of p18\(^{\text{INK4C}}\) alone may also influence Cdk4 activation in liver regeneration after PH. We previously reported that the overexpression of Cdk4 was detected in HCC of Long Evans cinnamon rats and human. In addition, Pascale et al. have also demonstrated that the overexpression of cyclin D1/Cdk4 complex occurred in chemically induced HCC of Fischer 344 rats. Conversely, it has been shown that Cdk6 protein was not increased in HCC. Therefore, we assume that the amount of Cdk4 (cyclin D/Cdk4) may be higher than that of Cdk6 (cyclin D1/Cdk6), and p18\(^{\text{INK4C}}\) may dominantly interact with Cdk4 rather than with Cdk6 in p18\(^{\text{INK4C}}\)-positive HCCs, suggesting that p18\(^{\text{INK4C}}\) in HCC may contribute only to the up-regulation of Cdk4. Finally, expression levels of p18\(^{\text{INK4C}}, \text{p21 CIPI/WAF1}, \text{p27 KIP1}, \text{p57 KIP2}\) and other Cdk inhibitors should be evaluated to examine their functional collaboration in the development of HCC.

To the Editor:

I read with interest the article on measurement of hepatic venous pressure gradient in patients with active variceal bleeding. I would like to mention one therapeutic aspect in this article that I suggest is not appropriate. The authors randomized the patients with cirrhosis and active bleeding into two arms—injection sclerotherapy and band ligation—and measured the hepatic venous pressure gradient before and after the procedure until the 5th day of admission. They concluded that injection sclerotherapy had caused a sustained increase in hepatic venous pressure gradient, which is followed by a higher rebleeding rate. The authors did not administer any pharmacological treatment to the patients who exhibited signs and symptoms of acute variceal bleeding within 12 hours of admission.

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Measurement of Hepatic Venous Pressure Gradient in Patients With Active Variceal Bleeding

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I believe it is inappropriate to withhold vasoactive drugs from patients who experience active variceal bleeding. The authors claim that at the time they designed the study, endoscopic treatment was the treatment of choice for acute variceal bleeding. However, in an American Association for the Study of Liver Diseases single-topic symposium in 1998, pharmacological treatment was established as an effective treatment for variceal bleeding. Furthermore, D’Amico et al. published an elegant meta-analysis in 1999 in which they concluded that pharmacological treatment should be started immediately even if variceal bleeding is suspected before endoscopic confirmation.

The present study was performed between 1998 and 2001, the period in which the pharmacological treatment consensus had already been accepted. I believe that researchers should either stop or modify a study when a consensus on treatment modalities is altered completely during the study period.
Reply:

We are grateful to Dr. Ozdogan for his interest in our article aimed to investigate the possible influence of emergency endoscopic treatment on HVPG in patients with cirrhosis and with bleeding esophageal varices (BEV). We completely agree with him that the investigators should stop a trial if during the study period a consensus has been reached regarding the treatment modalities under evaluation. However, this does not appear to be the issue in our study. The author refers to a single topic symposium on portal hypertension and variceal bleeding reported in HEPATOLOGY in 1998. At that period there was consensus suggesting only that endoscopic measures are the first choice of treatment for the control of BEV. It is true that the metaanalyses of D’Amico et al. (1999) and de Franchis and Primignani (1999) have been seriously criticized. Hence it is obvious than even in 2003 more trials were required in order to determine further potential advantages of combined therapy in the management of these patients. Therefore, our study which was conducted (1998-2001) under the guidelines of good clinical practice is absolutely documented according to the recommendations for the management of patients with BEV. As we have shown, in BEV endoscopic therapy increases HVPG which is sustained after sclerotherapy, but not band ligation, and this resulted in a higher rebleeding rate. Taking into consideration (1) the above results; (2) the available data suggesting that vasoactive drugs may reduce HVPG; and (3) the safety, efficacy and easy of administration of these drugs compared to endotherapy, we consider that the early administration of vasoactive drugs is mandatory in all patients with cirrhosis and with BEV. Therefore, in the forthcoming era of future trials, we believe that withholding vasoactive drugs in patients with BEV is not justified.

References

LANDMARKS IN HEPATOLOGY

The Liver has a Body—A Cook’s Tour

Just as there are fashions, fads and fancies in Art, Music, Literature and Dress, so there are in the more academic pursuits of Science, Economics, Engineering and Architecture. Even Medicine is vulnerable to the vagaries of the vogue. “Fashion” sneered Ambrose Bierce cynically, “is a despot whom the wise ridicule and obey.” in Physic, a prime practice that has swung like a pendulum in and out of fashion is the performance of a physical examination as part of the diagnostic algorithm. Now, perhaps more than ever, with the availability of elaborate laboratory tools to analyze bodily fluids, the perception of high resolution imaging with sonic and electromagnetic vibrations, and the intimate glimpses afforded by microscopic, ultramicroscopic and molecular dissection of the tissues of the body, there seems to be little room for the tradition of inspection, percussion, auscultation and palpation. Auscultation of the heart has yielded to echocardiography; percussion of the chest to computer-assisted tomography; careful evaluation of the neural network with flashlight, pin, cotton wool, tuning fork and tendon hammer to magnetic resonance imaging of the brain and spinal cord; and inspection and palpation of the abdomen to endoscopic invasion of the gastrointestinal tract by flexible light-emitting tubes that propel charged-coupled devices, and with wireless capsules that relay their reconnaissance by radio transmission to the examiner. Whereas no one regrets the passing of gustation of sweat and urine in the diagnosis of jaundice and diabetes, respectively, is it any wonder that residents and fellows show little fascination or even interest, let alone respect, for the physical examination as part of the diagnostic algorithm. Now, physicians of the Ancient World, in the East and the West, did not have the virtually unlimited access that we have today to the patient’s body, except perhaps at the time of the “ultimate physical examination.” Nonetheless, by carefully scrutinizing the accessible, i.e., the face, hands, body posture and movement, the rhythm and noise of breathing, behavior, demeanor and mood, together with some limited intrusive approaches, like feeling the pulse, inspecting the tongue, hearing the rumbles of the abdomen and even shaking the patient, they made many astute observations. Witness Hippocrates and his facies, fingers and succussion. Hippocrates recognized jaundice and hepatic coma, and he could hear the succussion splash of fluid moving in the pleural space in his patients with pleurisy, whom he jolted to make the diagnosis. Galen, in 2nd Century CE Rome, could fill 16 volumes with his writings on observations, interpretations and prognostications of the pulse, a practice incidentally much favored in Ancient China too. Wang Shuhe, who lived during the Western Jin dynasty in the 3rd Century CE, compiled all available knowledge on pulse diagnosis in his manual on the pulse, Mai Jing. Galileo timed his pendulum from his pulse and vice-versa, while his Paduan contemporary Sanctorius Sanctorius (who had also improved on Galileo’s thermometer and dabbled in paracentesis) invented the pulsilogium, a pulse-watch dedicated to that purpose. The pulse, tongue, eyes, nails and skin were favorites of the Ayurvedic physicians too, dating from the early centuries of the current era in India, but here great emphasis was placed on the patient’s age, constitution, body proportions, and capacity for food and exertion, which were evaluated chiefly to allow them to estimate life expectancy. Yet though the practice of medicine in most cultures was vaunted as relying on the five senses, physical examination literally remained at a superficial level, by and large, until well into the late 18th Century. As Richard Gordon has sardonically remarked, the 17th Century physician was useless but decorative. With his satin gilt-decorated coat, buckskin breeches, silk stockings and buckled shoes, lace ruffles and full bottomed wig, he swung a long cane with a hollow gold head filled with Marseilles vinegar that he sniffed repeatedly to ward off infection. To Richard Mead (1673-1754), physician to Queen Anne and George II and Fellow of the Royal College of Physicians, the gold-headed cane was a badge of office to be carried with pride. To caricaturists, like Thomas Rowlandson and William Hogarth, it was an icon with which to identify individuals with medical pretensions, as in Hogarth’s 1736 cartoon “The Company of
Undertakers” that ridiculed leading physicians and quacks of the day who, with expressions that range from sour to stupid, are depicted either sniffing their canes or absorbed by the contents of a urinal. Successful and sought-after clinicians of the era, like Mead and Scotsman William Cullen,11,12 were often consulted by letter to which they replied in kind with a diagnosis, a prescription and a bill for services rendered. All of this was to change following the intellectual ferment of the French Revolution in 1789, which Iain Bamforth identifies as the event that turned medicine into a public utility.13 In place of mail order consultations, and diagnoses made by inference from a detailed, almost Freudian, interrogation and a Sherlock Holmes-like scrutiny of the patient’s face, physicians in the 19th Century added a structured physical examination to the narrative to help identify disease, using all the senses. There was at large a new doctrine of organ-based disease, arising from the discoveries of pathologists, microbiologists, physiologists and pharmacologists of the age, like Giovanni Morgagni, Rudolf Virchow, Robert Koch, Louis Pasteur, Claude Bernard, William Beaumont, Oswald Schmiedeberg, Paul Ehrlich, and so many others too numerous to tally here. And there were also innovations in the clinic that enhanced patient evaluation. The prevailing no-touch approach that was a product of social propriety and traditional diagnostic reasoning, gave way to the thoroughness of a physical examination that would have appeared impudent and embarrassing to patient and doctor alike in the 18th Century. In 1808, Jean Nicholas Corvisart, Napoleon’s personal physician, popularized percussion by publishing a translation of the 95-page booklet on the technique that had been produced in Latin almost 50 years previously in 1761, by the Graz-born Austrian Joseph Leopold Auenbrugger.14 Auenbrugger, who learned the percussion technique by watching his innkeeper father knock on barrels to assess how much wine was left in them, did not invent the percussion of patients as it had already been practised to some extent by Hippocrates, Galen, and even his teachers,15,16 and veterinarians in Switzerland were already percussing the heads of cattle to diagnose cysticerci.17 Auenbrugger’s contribution, and later that of Corvisart, was that he considered percussion to be an essential component of the physical examination. The second boost to physical diagnosis was, of course, the introduction by René-Théophile-Hyacinthe Laennec of the stethoscope, which he developed from the rolled up quire of paper he had used to listen, from a respectable distance, to the heart of a buxom young woman.18 These devices for examination, together with the introduction of others such as the sphygmomanometer, the spirometer, the roentgenograph, the electrokardiogram, the ophthalmoscope and various forms of endoscopy, were seminal contributions made in the 19th Century to the practical science of clinical medicine.

In the realms of physical diagnosis, hepatology is a goldmine of lesions that typify moderate or advanced derangement of liver structure and/or function, granted, admittedly, that no physical sign is unique or pathognomonic. However, liver disease can affect each and every organ system, and since we have seen in the language of many cultures that “The body has a liver,”19 in the framework of the physical examination we can now safely say that, by its widespread effects, “The liver has a body” too. An organ system-oriented classification of the physical signs of liver disease would at first thought seem logical mechanistically, but a more practical tack would be to order the various signs as we come across them during the traditional conduct of the physical examination. For descriptive purposes, this is akin to taking a sightseeing expedition of the body, and here the analogy of another popular innovation of the 18th and 19th Centuries comes to mind, namely that of the Grand Tour. Whereas history is replete with the exploits of individual wayfarers who journeyed to distant lands to learn the terrain and the culture of the peoples there, like Benjamin of Tudela and his 12th Century itinerary from Saragossa to 300 cities in Greece, Mesopotamia, Syria, Palestine and Persia; Ibn Battutah and his 13th Century journey from Tangiers to India, China, Africa, Asia and Europe; Marco Polo and his trip from Venice to China in the 14th Century; and Cabeza de Vaca’s 16th Century foray into the interior of America, historians generally regard the so-called Grand Tour as having its beginnings in the late Renaissance and reaching its high point in the 18th Century. That prolonged sightseeing pastime abroad of the affluent continued well into the 19th Century, however, as travel became easier and more lands were colonized, and it is exemplified by the entrepreneurial activities of the 19th Century temperance campaigner Thomas Cook,20 whose name is now synonymous with the guided but cursory tour. Parenthetically, it was also implicit once that a Cook’s tour ensured home-away-from-home comfort for the well-to-do English while they were “touring” Europe, the Mediterranean, the Middle East, the Orient or the Far East, but that is hardly the case today.

In our Cook’s tour of the physical signs of the liver patient, we must first take in a colorful scenic view of the face before making our major stop at the hands. Is the patient conscious and coherent, or confused and even comatose? Are the eyes, mucus membranes and skin yellow, and if so shall we call it jaundice or icterus?21 Is there the bronzing of hemochromatosis or the hyperpigmentation of cholestasis or the whiff of fetor hepaticus22, the
glauntness of temporal wasting, and “paper money” skin
where tiny superficial vessels resemble the finely chopped
red and blue silk threads embedded in U.S. dollar bills;
and are creamy periorbital excrescences, xanthelasmas,
present?23 Is the corneal pigmentation visible that Drs.
Kayser24 and Fleischer25 espied before Wilson described
his eponymous disease,26 and do sunflower cataracts ob-
scure our gaze into the patient’s soul? Are the teeth green
from childhood cholestasis,27 or the lips, mucus mem-
branes and tongue blue from hypoxemia that could hint
at the hepatopulmonary syndrome? Is the tongue vitamin
B deficiency red in the alcoholic or decorated like the skin
elsewhere with the white lace-like pattern of lichen planus
that sometimes accompanies chronic hepatitis C,28 pri-
mary biliary cirrhosis,29 primary sclerosing cholangitis,30
and other liver disorders too?31 Are we struck by the alco-
holic’s *elephantiasis des buveurs*, i.e., the rhinophyma that
is so popular in literature and the arts32; do his cheeks
bulge from parotid enlargement33 and, of course, are spi-
der nevi present too?34 (Fig. 1A). Does a flat forehead,
widely-set eyes and a pointed chin suggest Alagille syn-
drome? There is much to delay us in the face before we
move on to the hands.

Clubbing of the fingers (Fig. 1B,C), which occurs in
liver disease and a host of other disorders, has fascinated
physicians of all persuasions ever since Hippocrates de-
scribed curvature of the nails and hot finger tips, the so-
called “Hippocratic fingers,” in his patients with
empyema more than 2000 years ago.24 When digital club-
bning, palmar erythema and spider nevi are in conjunction
(Fig. 1A-C), the presence of liver disease and especially
cirrhosis is almost assured, yet the cause of clubbing (as-
suming that we even know how to recognize it without
the use of an unguisometer35,36) remains enigmatic. As
Samuel West wrote presciently in 1897,37 “Clubbing is
one of those phenomena with which we are all so familiar
that we appear to know more about it than we really do.”
It has been hypothesized that clubbing occurs because of a
noninflammatory vasodilatory hyperemia of the finger
tips and nail beds, which results from a neurocirculatory
reflex that may originate in the cholinergic sympathetic autonomic innovation of the digits and the vagus nerve; transection of the latter does not reliably reverse clubbing but it has been known to resolve its painful bony counterpart, hypertrophic osteoarthropathy. Many cytokines and growth factors, like platelet-derived growth factor, vascular endothelial growth factor, growth hormone, and hepatocyte growth factor, have been impeached but the real culprit has yet to be caught. Some digitally-orientated philosophers have even suggested that these humors activate dormant genes that return the hand to an embryonic claw, or even restore the claws that humans have lost during evolution. Irrespective of the pathogenesis, however, when liver or lung disease is the cause, replacing the spent organ with a new one returns the digits to their pristine condition, unless rejection supervenes.

Even if the fingers are not clubbed and the hands are not disfigured with vitiligo or the rash of cutaneous porphyria, the nails may be thickened, ridged, brittle, flat or concave to indicate the presence of liver disease. Streaks of green in the nails testify to previous cholestasis, whereas if the lunulae are deep sky-blue, the suggestion is of Wilson disease, and if red, then the specter of alcoholism is raised. In cirrhosis and other conditions too, an opaque whitening that involves the whole nail usually including the lunulae, with distal red or brown discoloration, defines Terry’s nails (Fig. 1C), whereas the paired transverse white lines of Muercké that appear when there is hypoalbuminemia of any cause, must be distinguished from the transient white bands of alcohol abuse and from Mees’s white lines, those sinister marks of arsenic poisoning that transform hepatologists into criminologists. Inasmuch as impressions from onychomancy — divination from finger nails — conjure suspicions of liver disease and its cause, so much more does elicitation of the peculiar tremor known as asterixis that almost invariably indicates liver failure, even though this distinctive movement disorder occasionally stems from renal failure, respiratory failure, drug reactions and other nonhepatic causes.

That dramatic mental and neurological manifestations disturb patients with liver failure, has been known since the time of Hippocrates and Galen. Reference was made to the neuropsychiatric phenomena of what is now called hepatic encephalopathy (or portal-systemic encephalopathy) in all the more important writings on liver disease in the 18th and 19th Centuries, as appraised in detail by John Walsh more than 50 years ago, including his review of reports by such liver luminaries as Richard Bright, George Budd and Friedrich Theodor von Frerichs. Irregular jerky movements of the limbs, or jactitations, have been noted but the significance of the so-called “liver flap,” as it was colloquially called in the lingo of hepatic coma aficionados, was not appreciated until the publication in 1953 by Raymond Adams and Joseph Foley, of their landmark chronicle of the neurological disaster associated with liver disease. This publication, which was a comprehensive detailed extension of the preliminary account that they had reported a few years earlier, also contained biochemical, electrophysiological and exhaustive neuropathological data. Joe Foley, now a grand octogenarian but then a junior faculty member in neurology, performed repeated detailed neurological examinations on 60 patients who had been admitted with severe liver injury, two-thirds of whom had alcoholic cirrhosis, as he was looking for a harbinger of impending coma. More than 100 other patients were examined at least once too. Foley, who conducted these clinical studies initially at Boston City Hospital in collaboration with the renowned hepatic comatologist Charles Davidson and his colleagues, and later at the Massachusetts General Hospital, noticed that the liver flap appeared early in the course of the disease and in several cases was the first sign of looming coma. Foley considered this idiosyncratic movement disorder to be one of the most characteristic features of hepatic coma and one of the most useful in predicting disaster, since the vast majority of their deeply comatose patients died. Foley’s original description of the involuntary movement that some have hyperbolically likened to the beating of a bird’s wing, or flügelschlagen, as well as his description of hepatic coma itself, are unsurpassed. He described the appearances at irregular intervals of 1-7 seconds of rapid arrhythmic lateral deviations of the fingers, flexion-extensions at the metacarpophalangeal joints and flexion-extensions at the wrist, when the patient was asked to hold the arms and hands outstretched with the fingers spread apart. Flexion was always the most rapid of the movements that occurred in bursts every second or two, although there were also movement-free intervals. Comparable movements could be seen in the arms, legs and feet, tightly closed eyelids, corners of the retracted mouth; pursed lips and during sustained grasping by the hands; though bilateral, the movements were asymmetrical and asynchronous. The protruded tongue has picturesquely been described as showing “tromboning.” Foley also described a fine 6-9 per second tremor of the outstretched fingers that has only recently been termed “mini-asterixis,” which is thought by some to originate in the cortex, reflecting a pathologically slowed and synchronized motor cortical drive. Asterixis itself is technically-speaking not a tremor but rather a form of negative myoclonus in which there are irregular myoclonic lapses of posture caused by involuntary 50-200msec silent peri-
ods in muscles that are tonically active, as shown by electromyography.\textsuperscript{54,66} Notwithstanding, the exact mechanism remains elusive and many postulated explanations are yet to be explored.\textsuperscript{68} As for the term asterixis itself (which incidentally does not actually appear in the landmark publication\textsuperscript{64} but soon entered the neurological vernacular\textsuperscript{69}), this was Joe Foley’s invention too as he sought to substitute a universal neurologically-egalitarian nomenclature for the partisan term liver flap. He and another classics scholar, his Jesuit priest friend Father Cadigan, had repaired to a local hostelry in Boston, the Athens Olympia Café, to discuss neurological semantics. There, inspired by a splendid meal and fortified by an unspecified volume of the famous grape brandy invented just 60 years previously by the Greek silk trader Spyros Metaxas, Foley contrived the term \textit{an-iso-sterixis} (later shortened to asterixis for \textit{Hoi Polloi}) from the Greek, which means “a lack of [maintenance] of position,” which indeed it is.

Before we leave the bounding pulse and warm hands, flapping in dorsiflexion as if hesitantly bestowing a blessing, we cannot help but notice a curious flexion deformity of some of the digits, caused by shortening of the palmar fascia. Known as Dupuytren’s contracture, after the legendary but arrogant 19th Century French surgeon Baron Guillaume Dupuytren, Chief of Surgery at l’ Hôtel Dieu in Paris and personal surgeon to both Louis XVIII and Charles X, this hand deformity had already been described around 1200 CE in the Icelandic sagas\textsuperscript{70} and was well known in Scotland too since the 16th Century, as the curse of the MacCrimmons of the Western Isles, whose preeminent bagpipe players were increasingly thwarted in their performances by progressive finger contraction deformities that were a frequent inheritance amongst members of that clan.\textsuperscript{71} Felix Plater, a Swiss anatomist in Basel, published the first account in the medical literature in 1614,\textsuperscript{72} when he described the contractures of the ring and little fingers of a master mason, and though Henry Cline at St. Thomas’s Hospital in 1808, and his famous former apprentice, Sir Astley Paston Cooper, in 1818, described the malformation too,\textsuperscript{70} it was Dupuytren who earned eponymous immortality after he delivered a lecture on December 5, 1831, on the topic of permanent retractions of the digits of the hand. Dupuytren later published in English in the Lancet\textsuperscript{73} without acknowledging the work of Cline and Cooper, about which he cannot have been oblivious. Yet, for all its time-honored history, neither the cause nor the pathogenesis of Dupuytren’s disease are clear, but fibroblast proliferation, chromosomal aberrations, immunological abnormalities, growth factor activity and androgen-responsiveness of Dupuytren palmar tissue are all thought to play a role.\textsuperscript{71} Dupuytren’s contracture occurs in both genders and all ethnic groups, but far and away it is predominantly an affliction of older men of northern European ancestry and, by repute, it is another genetic gift from the Vikings.\textsuperscript{70} Although typical liver patients with Dupuytren’s contracture have alcoholic cirrhosis,\textsuperscript{74} it is a feature of the alcoholism rather than the liver disease as such, and it also complicates diabetes, seizure disorders, cigarette smoking and probably vibration-induced hand injury too, especially in genetically-prone individuals.\textsuperscript{70}

Transfer to the chest by way of the arm, shoulder and neck offers more sights that bespeak of underlying liver disease. Muscle wasting is common in the upper arms, shoulder and around the scapula, tattoos give a clue to a high-risk lifestyle, xanthomas appears at the elbow and the ear, and this is definitely spider nevus country. Careful inspection of the neck, in the right light and at the right angle, may reveal portopulmonary hypertension-induced jugular venous pressure elevation, which can be enhanced by applying gentle manual pressure to the abdomen, anywhere but over a congested tender liver, to elicit an hepatojugular reflux. On the pruritic back, “butterfly distribution” sparing from hyperpigmentation, shows the limits of scratching.\textsuperscript{75} In the chest, gynecomastia\textsuperscript{76} may be the most prominent part of the feminization syndrome (together with sparse beard and soft skin),\textsuperscript{77} often painfully exacerbated or caused by a side effect (or should that be front effect?) of spironolactone treatment,\textsuperscript{78} and occasionally it may give an inkling of an underlying fibrolamellar hepatocellular carcinoma that synthesizes aromatase.\textsuperscript{79} Auenbrugger’s percussion confirms the presence of an hepatic hydrothorax without the need to shake the patient. Combining palpation of the precordium with auscultation through Läännec’s invention, gives further evidence of the hyperdynamic circulation and/or pulmonary hypertension, both being relatively common in patients with cirrhosis. The abdomen has always been the domain of the hepatologist, and there is surely no need to wax lyrical to this readership on the virtue of bulging flanks, an everted umbilicus and shifting dullness (executed by Auenbrugger’s technique), nor to recall that even if the 3-hand trick of eliciting a “fluid wave” is possible, it is probably scarcely necessary because the presence of ascites is already obvious. And please, spare the patient the indignity of demonstrating the puddle sign. How fitting that the music of hepatology — the bruit of an hepatic tumor, the hum of Cruveilhier and Baumgarten,\textsuperscript{80} and the rare rush of a splenic arteriovenous fistula— can be heard, above the borborygmi, through Läännec’s stereophonic device. Visible dilated superficial abdominal wall veins with cephalad flow are common in patients with cirrhosis but it is a rare chance to observe caudal flow in...
these vessels, which is a sure sign of superior rather than inferior vena cava obstruction. Some say that the caput Medusa is as much a myth as the Greek legend from which its name derives. Abdominal hernias may not be all they seem\textsuperscript{81}; they may actually represent ascites rather than bowel extrusion, and in the inguinal region surgeons must beware of operating on what will turn out to be ascites or worse, a collateral vein filled sac.\textsuperscript{82}

And so to legs and feet, as 17th Century diarist Samuel Pepys might have said, for the culmination of the tour. In many ways the lower limbs are an anticlimax and simply mirror the upper ones, with their muscle wasting, sparse hair, and xanthomas on the knee. Spider nevi are never sighted here but the legs are, instead, the preferred location for edema. Dependency has its price, but perhaps therein lies the solution to the age-old riddle of how to grade and stage peripheral edema. Rather than being nonplussed by the usual arbitrary scale of “pluses”\textsuperscript{83} that some authors shun as meaningless,\textsuperscript{84} or foolishly trying to estimate the depth of the pit\textsuperscript{84} or the time that it takes to fill in\textsuperscript{83} without regard to the size or strength of the prodding fingers, why not grade the squelch of the waterlogged tissues as trace, mild, moderate or severe, and stage the encroachment of the legs by edema, plotting the extent upwards from the feet to the abdominal wall (with a correction for redistribution due to recumbency or loss of compressibility due to brawnniness). Muscle wasting occurs in the thighs, asterixis may be observed by simply letting them abduct as Foley recommended,\textsuperscript{64} and clonus may be elicited too. Petechiae may be seen when the high venous pressure in the legs conspires with thrombocytopenia, and the feet and ankles, the furthest cool reaches of the body, are also prone to the palpable purpura of cryoglobulinemia, which is related to Peyronie’s disease by some.\textsuperscript{10}

Nowadays fashion in medicine is evidence-based and a few killjoys try to convince us that, despite the many landmarks we have seen on our tour, the physical examination is no longer worthwhile in patients with liver disease.\textsuperscript{85-87} However, neither the author nor skilled examiners like Joe Foley would agree (personal communication, November 2004). Admittedly, some arcane maneuvers in liver diagnosis, like the scratch test for detecting the liver edge,\textsuperscript{88} are unreliable, and clinicians often disagree about their physical findings.\textsuperscript{89} Why some cannot even find the mid-clavicular line.\textsuperscript{90} Yet, for all that, with practised hand and trained eye, seasoned clinicians looking for the physical signs described above, such as spider nevi, facial telangiectasias, white nails, abdominal veins, liver consistency, ascites, and the size of the spleen,\textsuperscript{91} are able to diagnose advanced liver disease and decompensated cirrhosis with a fair degree of certainty.\textsuperscript{89,92,93} Far from being a fad or a fancy, it seems most likely that a carefully performed physical examination will still be the fashion in clinical hepatology for many years to come.

Acknowledgment: The author thanks Dr. Kenneth J. Bergmann (Department of Neurology, MUSC) for advice about movement disorders, and Dr. Joseph Foley for his reminiscences on the discovery of asterixis. The author also acknowledges the manuscript and literature retrieval skills of Margie Myers and Aretha Williams.

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