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ARTICLE *in* FEMS MICROBIOLOGY LETTERS · SEPTEMBER 1997

Impact Factor: 2.12 · DOI: 10.1111/j.1574-6968.1997.tb12595.x · Source: PubMed

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FEMS MICROBIOLOGY LETTERS

Degradation of human subendothelial extracellular matrix by proteinase-secreting *Candida albicans*

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Received 9 May 1997; revised 9 June 1997; accepted 9 June 1997

Abstract

Candida albicans infections in severely immunocompromized patients are not confined to mucosal surfaces; instead the fungus can invade through epithelial and endothelial layers into the bloodstream and spread to other organs, causing disseminated infections with often fatal outcome. We investigated whether secretion of the *C. albicans* acid proteinase facilitates invasion into deeper tissues by degrading the subendothelial basement membrane. After cultivation under conditions that induce the secretion of the acid proteinase, *C. albicans* degraded radioactively metabolically labeled extracellular matrix proteins from a human endothelial cell line. The degradation was inhibited in the presence of pepstatin A, an inhibitor of acid proteinases. Pepstatin A-sensitive degradation of the soluble and immobilized extracellular matrix proteins fibronectin and laminin by proteinase-producing *C. albicans* was also detected, whereas no degradation was observed when the expression of the acid proteinase was repressed. Our results demonstrate that the *C. albicans* acid proteinase degrades human subendothelial extracellular matrix; this may be of importance in the penetration of *C. albicans* into circulation and deep organs.

Keywords: *Candida albicans*; Secreted acid proteinase; Extracellular matrix; Tissue invasion

1. Introduction

The opportunistic fungus *Candida albicans* is a major cause of infections in immunocompromized patients. Infections by *C. albicans* range from superficial infections of the oral and vaginal mucosa to life-threatening disseminated disease. Various characteristics of the fungus have been implicated to contribute to its virulence, for example the ability to grow both as a yeast as well as in a hyphal form within the host, the phenotypic switching between different colony morphologies, and the adherence to a variety of host surfaces [1]. Another suggested virulence property is the secretion of acid proteinases as this ability correlates with the relative virulence of different *Candida* species or *C. albicans* isolates in animal models [2,3]. In addition, proteinase-deficient *C. albicans* mutants are less virulent [4]. Several roles of the acid proteinase during colonization and infection have been proposed, e.g. digestion of host proteins for nutrient supply [5], evasion of host defenses by degrading immunoglobulines and complement proteins [6,7], adherence [8,9] and invasion [10,11]. The proteolytic degradation of keratin and collagen

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PII S0378-1097(97)00273-5

may facilitate the destruction of tissue barriers in cutaneous infection [12,13]. To establish systemic infection, *C. albicans* must penetrate the mucosal barrier, gain access to the bloodstream and evade again for dissemination into internal organs. Basement membranes underlying the host epithelium and endothelium form a tight network of extracellular matrix (ECM) proteins and a tissue barrier that the invading microorganisms must degrade to penetrate into circulation and secondary infection foci. Therefore, we assessed the potential of the *C. albicans* secreted acid proteinase to degrade human subendothelial ECM as well as individual ECM proteins.

2. Materials and methods

2.1. C. albicans culture

C. albicans strain ATCC38696 was used throughout this study. The strain was routinely propagated on YPD-agar plates (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose, 1.5% (w/v) agar) at 30°C. For induction of acid proteinase secretion the strain was grown in YCB-BSA (2.34% (w/v) yeast carbon base, 0.2% (w/v) yeast extract, 0.4% (w/v) BSA, pH 5.0) at 37°C; growth in YPD-liquid medium was used to inhibit proteinase expression. Under these conditions the strain grew exclusively in the yeast form.

2.2. Preparation of radioactively labeled subendothelial extracellular matrix (ECM)

Radiolabeled subendothelial ECM for degradation studies was prepared with the continuous human endothelial cell line EA.hy926 [14]. EA.hy926 cells were cultivated to subconfluence in 24-well tissue culture plates (Nunc) in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (Gibco), 2 mM L-glutamine (Nordcell), 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The cells were washed with phosphate buffered saline (PBS), and then glucose-free RPMI medium with 2 mM L-glutamine and 1% (v/v) FCS was added. After incubation for 1 h at 37°C with 5% CO_2, the medium was replaced with a new one containing 2.5 μCi ml^{-1} of D-[2-^{3}H]-mannose (Amersham, Buckinghamshire, UK) and incubation was continued for 24 h. ECM was prepared by detergent treatment of the cells as described previously [15]. After the procedure, the absence of endothelial cells on the bottom of the wells was confirmed by microscopy. Incorporation of ^{3}H-mannose into the subendothelial ECM (1×10^6 cpm per well) was determined by subtracting the combined radioactivity in the supernatant after the labeling period (5.8×10^6 cpm per well) and in the removed cells (0.2×10^6 cpm per well) from the total radioactivity added (7×10^6 cpm per well).

2.3. Radioactive labeling of fibronectin and laminin

Fibronectin and laminin (Collaborative Research, Bedford, MA) were labeled with ^{125}I (Amersham) by the iodogen method [16]. The activity obtained was 4×10^6 cpm μg^{-1} of protein.

2.4. Degradation assays

C. albicans cells were grown for 20 h at 37°C in YCB-BSA medium to induce acid proteinase secretion [17] and diluted 1:50 in YCB medium without BSA. To one half of this suspension (4×10^6 cells ml^{-1}) 5 μg ml^{-1} pepstatin A was added from a stock solution of 1.25 mg ml^{-1} pepstatin A in methanol. An equivalent amount of methanol without pepstatin A was added to the other half of the yeast suspension.

For the human subendothelial ECM degradation assay, 600 μl of the diluted yeast suspensions with or without pepstatin A were added to the microtiter wells with the ^{3}H-labeled ECM preparation (see above) and incubated at 37°C. As a negative control, 600 μl of YCB medium without yeasts were incubated with the ECM proteins. 50 μl samples were taken at different time points and the degradation of the proteins was determined by measuring the radioactivity released into the supernatant using an LKB 1215 Rackbeta liquid scintillation counter (Wallac). The experiment was performed in quadruplicate to ensure reproducibility.

To investigate the degradation of immobilized fibronectin and laminin, Lab-Tek Chamber slides (Nunc, Roskilde, Denmark) were incubated over-

night at room temperature with 1×10^6 cpm of protein in 250 μl PBS per well. After removing the supernatant the wells were washed three times with 500 μl PBS and incubated with 400 μl of the yeast suspension with or without pepstatin A, or YCB medium without cells. 20 μl samples were taken at different time points and the degradation of the subendothelial ECM was determined by measuring the radioactivity released into the supernatant in an LKB 1272 Clinigamma counter (Wallac, Turku, Finland). The assay was performed in triplicate (samples with yeast cells) or duplicate (medium control) to ensure reproducibility.

Degradation of soluble fibronectin and laminin was also analyzed by SDS-PAGE. For these assays, 250 μl of the yeast suspensions or YCB medium without cells were mixed with 1.5×10^6 cpm of radioactively labeled protein and incubated at 37°C. In a control experiment yeast cells grown in YPD medium to repress proteinase expression were incubated with the radioactive proteins. 30 μl samples were taken at various times, mixed with an equal amount of Laemmli buffer [18] and stored at −20°C until further use. Twenty μl of the samples were loaded onto 5–15% gradient gels and, after electrophoresis, visualized by autoradiography.

3. Results

3.1. Secretion of the acid proteinase enables C. albicans to degrade extracellular matrix produced by human endothelial cells

We first analyzed the degradation by proteinase-producing *C. albicans* cells of metabolically labeled ECM proteins from the human endothelial cell line EA.hy296 (see Section 2). This method measures proteolysis as the release of radioactivity from ^{3}H-mannose-labeled ECM of cultured endothelial cells. As can be seen in Fig. 1, *C. albicans* cells induced for proteinase production degraded the subendothelial ECM. This degradation was due to the secretion of the acid proteinase as the addition of pepstatin A, an inhibitor of acid proteases, repressed the release of radioactivity from the ECM preparation close to the level seen in the control assay without yeast cells.

3.2. Degradation of the ECM proteins laminin and fibronectin by proteinase-secreting C. albicans

We next investigated the degradation of two ECM proteins, fibronectin and laminin, the latter of which is one of the main components of human basement

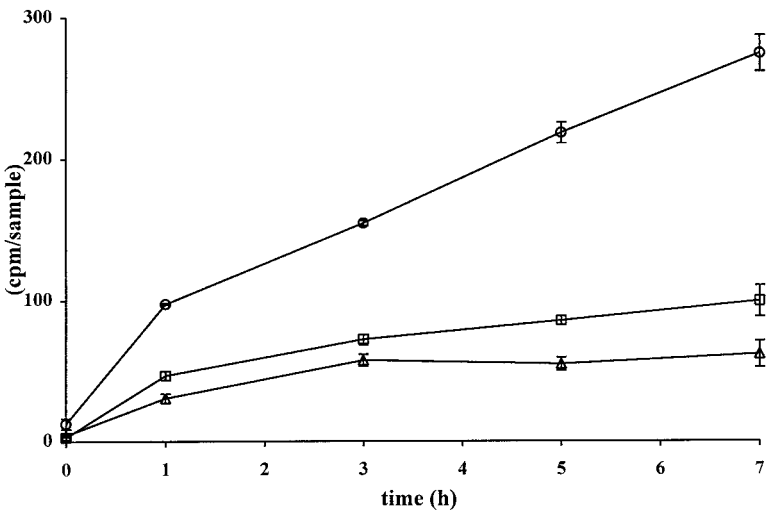

Fig. 1. Degradation of subendothelial ECM by *C. albicans* induced for proteinase secretion. Degradation of the radiolabeled ECM preparation was analyzed by taking samples at different times and measuring the radioactivity released into the supernatant. The figure shows the means and the standard deviation from one experiment performed in quadruplicate. ○, induced cells, no pepstatin present; □, induced cells, pepstatin present; △, control (medium without cells).

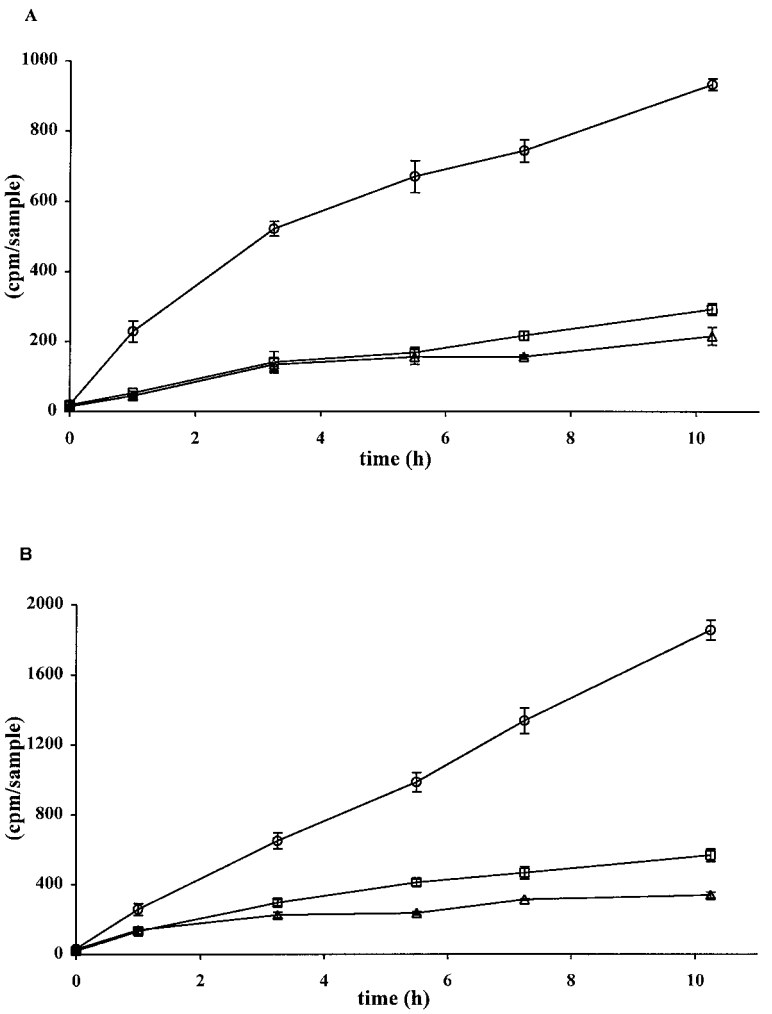

Fig. 2. Degradation of immobilized fibronectin (A) and laminin (B) by *C. albicans* induced for proteinase secretion. Degradation of the radiolabeled proteins was analyzed with samples collected at different times of incubation and measuring the radioactivity released into the supernatant. The figure shows the means and the standard deviation from one experiment performed in triplicate (samples with cells) or duplicate (control samples without cells). ○, induced cells, no pepstatin present; □, induced cells, pepstatin present; △, control (medium without cells).

membranes where it forms a tight three-dimensional network together with other ECM proteins [19]. ^{125}I-labeled fibronectin or laminin was immobilized in the wells of Lab-Tek Chamber slides and incubated with *C. albicans* cells in the presence or absence of pepstatin A. Fig. 2 shows that both ECM proteins were degraded by *C. albicans* due to the action of its secreted acid proteinase.

To confirm that the release of radioactivity indeed was due to proteolysis we analyzed the degradation of soluble fibronectin and laminin by *C. albicans* by SDS-PAGE and autoradiography of the radiolabeled proteins after incubation with yeasts grown under proteinase-inducing (YCB-BSA medium) or -repressing (YPD medium) conditions. Samples were taken at various time intervals and the proteins and degradation products, after separation by SDS-PAGE, visualized by autoradiography. The degradation of the ECM proteins by proteinase-producing *C. albicans* can be seen in Fig. 3A and B. The 250 kDa

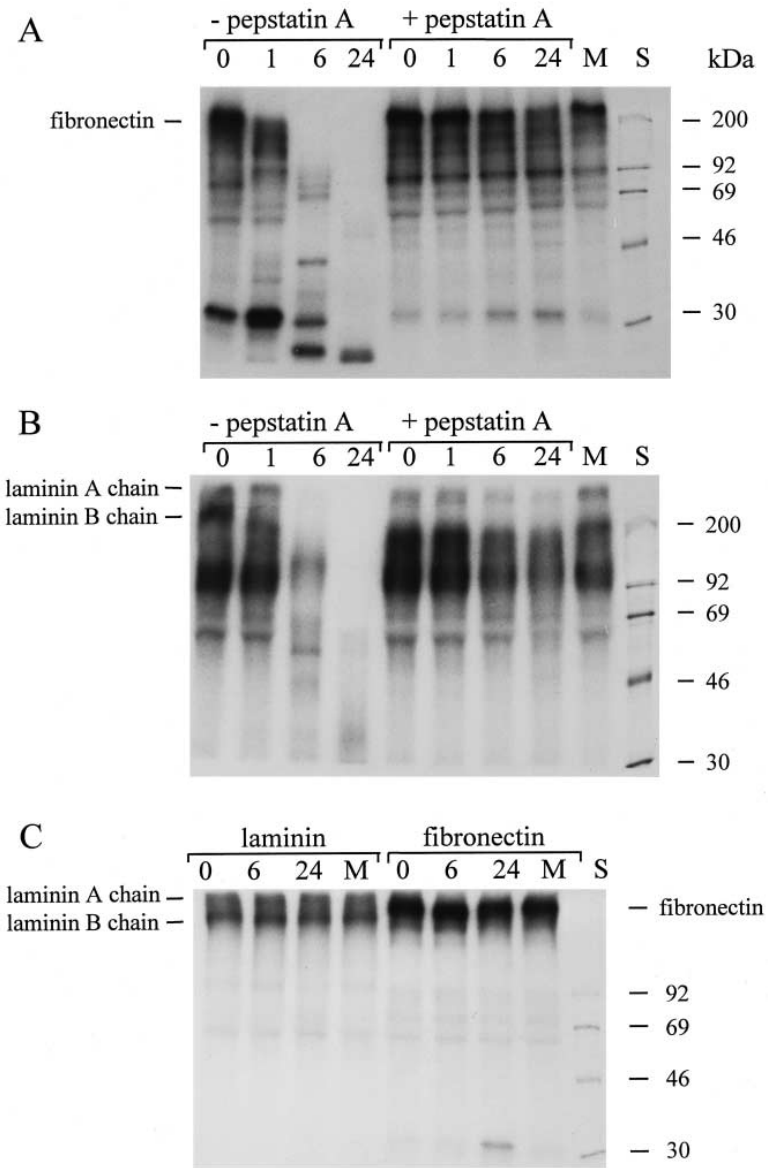

Fig. 3. Degradation of ^{125}I-labeled soluble fibronectin (A and C) and laminin (B and C) by *C. albicans* induced (A and B) or repressed (C) for proteinase secretion in the presence or absence of the inhibitor pepstatin A. Samples were taken at different times of incubation (given in hours above the lanes) and analyzed by SDS-PAGE and subsequent autoradiography. In the medium control samples (M), the proteins were incubated for 24 h with the respective growth medium YCB-BSA (A and B) or YPD (C) without yeasts. The positions of the 250 kDa fibronectin peptide and the 400 kDa and 200 kDa laminin A and B chains are indicated. Size markers (S) are shown on the right.

fibronectin and both laminin A and B chains (400 kDa and 200 kDa, respectively) were degraded following incubation with the yeasts induced for proteinase production. In contrast, these peptides remained intact when the activity of the acid proteinase was blocked by the addition of pepstatin A to the incubation mixture. Even after 24 h of incubation, there was only little difference to the con-

trol sample without yeasts. When *C. albicans* was grown under proteinase-repressing conditions, no degradation of fibronectin and laminin could be observed, in agreement with this activity being dependent on the secreted acid proteinase (Fig. 3C).

4. Discussion

The transition from commensal colonization of the mucosal surface by *C. albicans* to disseminated disease involves invasion of the epithelium and systemic spread through the bloodstream. To establish a metastatic site of infection, *C. albicans* must adhere to the endothelial cell surface and evade from the bloodstream into the internal organs [20]. Following adherence, *C. albicans* may destroy the underlying cell [21] or, after being phagocytosed, cross the endothelium by transcytosis [22]. The fungus may also directly come into contact with the extracellular matrix underlying the endothelial cells when the endothelium is damaged, e.g. in risk patients who receive cytotoxic drugs [20]. In any case, the subendothelial basement membrane which is made up of a tight interconnected protein network represents a barrier to further spread. Invasive pathogenic bacteria have adopted strategies to destroy this barrier, for example by activating host tissue proteinases like plasmin, either directly or by capturing host plasminogen activators on their surface [23,24]. Many microorganisms also produce their own proteinases to circumvent host defense mechanisms [25]. Secretion of the acid proteinase by *C. albicans* has been demonstrated to occur in vivo [8] and may therefore be a mechanism to facilitate the spread of the fungus by degrading the host ECM. In this study, we have demonstrated for the first time that *C. albicans* cells induced for proteinase secretion were able to degrade human subendothelial ECM. In separate experiments, fibronectin and laminin, the latter being a main structural component of human basement membranes, were also shown to be degraded by proteolytic *C. albicans*. *C. albicans* adheres efficiently to these ECM proteins [20], suggesting that in vivo the proteolytic activity can be directed by the adherent yeasts at the ECM to promote subsequent invasion.

It has been shown that *C. albicans* secretes a variety of isoenzymes of the acid proteinase which differ in their in vitro induction conditions [26,27]. The conditions used in our assays favor the expression of the SAP2 isoenzyme but not that of any of the others. It will therefore be interesting to define the in vivo conditions that favor the induction of the proteinase isoenzymes as well as their role in tissue penetration.

Acknowledgments

This study was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF Grant O1 K1 8906-0).

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