MYCOLOGY

Different isoforms of secreted aspartyl proteinases (Sap) are expressed by *Candida albicans* during oral and cutaneous candidosis *in vivo*

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Distinct isoforms of secreted aspartyl proteinases (Sap) of *Candida albicans* are important virulence factors for different types of candidosis. Predominant expression of Sap1–3 has been shown to be crucial for superficial infections in experimental mucosal and cutaneous candidosis, whereas Sap4–6 might be important for systemic disease. This in-vivo study investigated Sap expression in two samples from patients with oral candidosis and from cutaneous infection. Two different polyclonal antibodies directed against Sap1–3 and Sap4–6 were used for ultrastructural characterisation of protein localisation and expression. Post-embedding immuno-electron microscopy revealed Sap1–3 and Sap4–6 immunoreactivity in all samples. All *C. albicans* cells expressed predominantly the proteinases Sap1–3 which were evenly distributed within the cell wall and cytoplasmic membrane. In contrast, Sap4–6 labelling was only evident in a few fungal cells. In particular it was localised at the tips of hyphal cells during invasion. These data suggest a different pathogenetic role for Sap1–3 and Sap4–6 during host–fungal interaction.

Introduction

Superficial infections by *Candida albicans* are common diseases in immunocompromised patients. Among the putative virulence factors of *C. albicans*, secretion of hydrolytic enzymes such as aspartyl proteinases seems to be an important determinant for pathogenicity [1, 2]. Ten members of a gene family encoding different secreted aspartyl proteinases (Sap1–10) have been isolated to date [3–5]. In-vitro and in-vivo studies have demonstrated that these genes are differentially regulated (A. Felk, W. Schäfer, B. Hube, unpublished data) [3, 5, 6–13]. Present evidence suggests that the success of the fungal pathogen in different types of infections depends partly on the expression of distinct virulence factors such as members of the *SAP* gene family [12–22]. Whereas *SAP4–6* has been shown to facilitate systemic infections, expression of *SAP1–3* appears critical for the virulence of *C. albicans* in superficial candidosis. These results were obtained by studying the virulence phenotype of *C. albicans* mutants defective in distinct *SAP* genes in comparison

to the parental strain during infection. Models for testing were mainly experimental animal infections [12, 14–19] or in-vitro test systems representing specific types of candida infections or distinct stages of the infection process [20–22]. Previous investigations used models of oral and cutaneous candidosis based on reconstituted human epithelium and epidermis (RHE) for virulence studies [9–11]. At the ultrastructural level, different patterns of Sap localisation have been demonstrated in these models by immuno-electron microscopy with polyclonal antibodies directed against Sap1–3 and Sap4–6 [23]. To date, the ultrastructural localisation of Sap immunoreactivity *in vivo* has been investigated only in experimental rat vaginitis [24] and oral candidosis in HIV-infected patients [25]. The antibodies used in both studies recognised only the gene products of *SAP1–3* as they were generated by immunisation with Sap2 [24] or with Sap1–3 [25].

In the present investigation, two different polyclonal antibodies specifically directed against Sap1–3 and Sap4–6 were used. Immuno-electron microscopy was used to examine different ultrastructural expression patterns for Sap1–3 and Sap4–6 during both oral and cutaneous candidosis *in vivo*, possibly reflecting distinct roles of these proteinases during the invasion process.

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Materials and methods

Clinical parameters of patients

Samples of pseudomembrane were removed by scraping from the buccal oral mucosa (male, female), from the left groin (male) and from the fold under the left breast (female) of four volunteer HIV-negative patients. The patients were 44, 58, 64 and 76 years old, untreated and all had been suffering from pseudomembranous candidosis for at least 2 weeks. Parts of the clinical material from all four patients were used for microbiological culture, biochemical characterisation and immuno-electron microscopy.

Mycological examination

The specimens were inoculated on Kimmig's Agar (Merck, Darmstadt, Germany) and incubated for 72 h at 37°C. Biochemical identification of *C. albicans* was based on the ATB 32 C system (API System, bioMérieux, La Balme-les-Grottes, Montalieu Vercieu, France).

Immuno-electron microscopy

Post-embedding immunogold labelling was done as described previously [10]. After fixation, specimens were embedded in LR-White. Sections, 80–100 nm thick, were mounted on nickel grids. Grids were then incubated with anti-Sap polyclonal rabbit antibodies directed against Sap1–3 (α -Sap2) or against Sap4–6 (α -Sap6F) [22]. After washing overnight with PBS, grids were incubated with 10 nm gold-conjugated goat anti-rabbit IgG (Auroprobe EM Immunogold reagents, Amersham, UK). Grids were then fixed with glutaraldehyde 2% and stained with uranyl acetate 0.5% for 10 min and lead citrate 2.7% for 5 min (Ultrastainer, LKB, Sweden) at 20°C. A Zeiss EM 902 transmission electron microscope (Zeiss, Oberkochen, Germany) operating at 80 kV was used for examination, at magnifications between 3000 $\times$ and 85 000 $\times$.

Results

C. albicans was identified in all four patients. There were no obvious differences apparent in the localisation and distribution of Sap1–3 and Sap4–6 immunoreactivities between samples from oral and cutaneous candidosis. To avoid repetition, the different stages of interaction between *C. albicans* and host cells (without contact, adhesion, invasion, within keratinocytes following invasion) are not demonstrated in detail for both mucosal and cutaneous candidosis. Labelling with the Sap1–3 antibody showed a regularly distributed intensive immunoreactivity in all *C. albicans* cells of the mucosal (Fig. 1A–C) and the cutaneous samples (Fig. 1D). Sap1–3 immunoreactivity was mainly localised adjacent to the cell wall and the cytoplasmic membrane of all *C. albicans* cells (Fig. 1A–D). No

significant difference in the density and distribution of Sap1–3 expression was observed between yeast (Fig. 1A, B, D) or hyphal cells (Fig. 1C). The pattern of enzyme labelling of *C. albicans* not in direct contact with the keratinocytes (Fig. 1A) was similar to that of fungal cells at the adhesion stage (Fig. 1B), invading (Fig. 1C) or within keratinocytes following cell invasion (Fig. 1D).

In contrast, labelling with the antibody directed against Sap4–6 revealed gold particles only in a few *C. albicans* cells of the mucosal (Fig. 2A, B, D) and cutaneous samples (Fig. 2C). In particular, enzyme labelling was found at the tips of those hyphal elements which penetrated host tissue (Fig. 2C). Where *C. albicans* was not in direct contact with (Fig. 2A), or adhering to host cells (Fig. 2B) or inside keratinocytes following cell invasion (Fig. 2D) there were very few or no gold particles after labelling with the Sap4–6 antibody. No specific gold labelling was seen in control experiments without the addition of the polyclonal antibodies Sap1–3 or Sap4–6.

Discussion

Immuno-electron microscopy studies with two polyclonal antibodies specifically directed against Sap1–3 and Sap4–6 demonstrated different ultrastructural patterns of enzyme expression for Saps during oral and cutaneous candidosis *in vivo*. Sap1–3 was expressed by all fungal cells and may be relevant for the infection process in general. Sap4–6 seems to have a particular function during direct interaction with the host, especially during hyphal invasion or penetration. Earlier studies used reconstituted human epithelium or epidermis for establishing in-vitro models of oral or cutaneous candidosis [9–11]. At the ultrastructural level, labelling with antibodies demonstrated a similar expression pattern for Sap1–3 and Sap4–6 during experimental oral and cutaneous infections [9–11]. The patterns of Sap secretion in the present study correspond to the results of the in-vitro models and confirm the usefulness of these infection systems for mimicking the situation *in vivo*. To date, in-vivo investigations of Sap secretion have been performed only in a rat vaginitis model and during oral candidosis of HIV-infected patients [23, 24]. Both studies clearly demonstrated protein secretion by *C. albicans*, but labelling was directed only against Sap1–3 and, therefore, failed to recognise Sap4–6 immunoreactivity. The results of the present study imply that the majority of Saps important for pathogenicity during oral or cutaneous infections with *C. albicans* are Sap1–3 and not Sap4–6. In contrast, a high production of Sap4–6 by *C. albicans* cells was detected during interaction with murine peritoneal macrophages [22]. These results imply a direct role of Sap1–3 for tissue damage during superficial infections, while Sap4–6 seems to be more

Fig. 1. Electron micrograph with post-embedding immunogold labelling against Sap1–3, with 10-nm gold particles in samples of *C. albicans* from oral and cutaneous candidosis showing regular distribution of Sap1–3 antigen within the cell wall of all *C. albicans* cells. (A) Fungal cell not in direct contact with host cells; (B) adherent *C. albicans* cell; (C) invasion of a keratinocyte by *C. albicans*; (D) *C. albicans* within a keratinocyte following cell invasion. Bars = 0.5 μ m.

important for penetration into deeper tissues and interactions with the cellular defence.

The relevance of *SAP1–3* for mucosal and cutaneous

candidosis has also been illustrated by gene expression studies [6, 9–11] and infection experiments with *SAP* null mutants [10, 11, 14, 15, 21]. The importance of *SAP1–3* for infection *in vivo* is supported by analysis of

Fig. 2. Electron micrograph with post-embedding immunogold labelling against Sap4–6, with 10-nm gold particles in samples of *C. albicans* from oral and cutaneous candidosis. The great majority of *C. albicans* cells remain unlabelled. Very few if any gold particles are observed (A) in fungal cells not in contact with host cells, (B) at the adhesion stage and (C) in *C. albicans* within the keratinocytes following cell invasion. (D) Significant labelling can be only detected at the tips of a few invading hyphal cells. Bars = 0.5 μ m.

SAP expression in human oral candidosis. Transcripts for *SAP1* and *SAP3* were found only in patients with clinical manifest infections but not in asymptomatic carriers [9, 25]. At the protein level expression of Sap of *C. albicans* was recently demonstrated in human saliva [26].

In summary, these results suggest differing roles for Sap1–3 and Sap4–6 in pathogenicity during oral and cutaneous candidosis *in vivo*.

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