MYCOLOGY

In-vitro proteinase production by oral *Candida albicans* isolates from individuals with and without HIV infection and its attenuation by antimycotic agents

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In-vitro proteinase production by oral *Candida albicans* isolates from patients with and without HIV infection (18 isolates from each group) was assessed by image analysis of a plate assay, with bovine serum albumin (BSA) as a substrate. The effect of sub-minimal inhibitory concentrations (sub-MICs) of nystatin, amphotericin B, clotrimazole and miconazole on in-vitro proteinase production by these yeast isolates was also investigated. Proteinase production by *C. albicans* isolates from patients with HIV infection was significantly greater than production by those from individuals without infection. All 18 isolates from HIV-infected individuals produced proteinase, in comparison to 56% of isolates from uninfected individuals. Pre-exposure of *C. albicans* isolates (seven proteinase producers from each group) to 1/4 and 1/16 MICs of nystatin, amphotericin B, clotrimazole and miconazole resulted in decreased proteinase production in all isolates tested. However, after exposure to the four antimycotic agents, proteinase production was decreased to a significantly greater extent in isolates from uninfected individuals than in those with HIV disease. Furthermore, when the relative concentration effect of antimycotic agents on proteinase production was compared, *C. albicans* isolates from the HIV-free group demonstrated a salient dose-response relationship compared with the HIV-infected group. These results indicate that *C. albicans* from patients with HIV infection are significantly more proteolytic than those from individuals without the infection, and that polyenes and imidazoles curtail the proteolytic activity of all *C. albicans* isolates, albeit to a lesser extent in those from HIV-infected patients. It appears that HIV disease favours oral colonisation by more proteolytic *C. albicans* isolates, with resilient proteolytic activity.

Introduction

Extracellular proteinase is considered to be a virulence factor of *C. albicans* [1–3], the most common agent of human candidosis. For example, immunoglobulin A, one of the main mucosal defence barriers, can be rapidly and thoroughly cleaved by proteinase [4]. It has also been reported recently that complement C₃ and some specific endogenous proteinase inhibitors involved in regulating inflammation can also be degraded by *C. albicans* proteinase [5]. Proteinase of *C. albicans* can digest, *in vitro*, several physiologically important substances including albumin, keratin and skin proteins [3, 6, 7]. The in-vivo secretion of proteinase has been demonstrated in infected tissues by histochemical

methods [8, 9]; moreover, secretion of proteinase correlates strongly with other virulence traits of *C. albicans*, such as adherence to epithelia [10]. The foregoing suggest the importance of *Candida* proteinase in the pathogenesis of candidosis.

The extracellular proteinase of *C. albicans*, first described by Staib [11], is not a single entity but is known to consist of three distinct enzymes [4], a complete proteinase and two that are partially proteolytic. Although the latter enzymes differ from the complete proteinase in substrate specificity and other properties, they cross-react immunologically with each other. These enzymes are classified as aspartate proteinases (EC 3.4.23.6) and are related to pepsin, renin and cathepsin D. Their mol. wt is *c.* 45 kDa, with optimal activity in the pH range 3.5–4.0 [12].

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It is recognised that oral candidosis caused by

C. albicans is one of the most common infections associated with human immunodeficiency virus (HIV) infection [13, 14]. Nevertheless, little is known of the production profile of the extracellular proteinase of *C. albicans* from HIV-infected patients in comparison with those from disease-free individuals. Very recently it has been observed that some isolates of *C. albicans* from HIV-infected patients are more proteolytic than those from HIV-uninfected individuals [15, 16].

Furthermore, the effect of major antimycotic agents – polyenes and imidazoles – on in-vitro proteinase production by *C. albicans* has not been studied systematically. Angiolella *et al.* [17] investigated this phenomenon and noted that methyl partricin, fenticonazole and 5-fluorocytosine suppressed proteinase production by *C. albicans*, but no other reports of this phenomenon are documented.

Accordingly, two related investigations were performed in the current study, with two groups of 18 oral isolates of *C. albicans*, from patients with and without HIV infection. Firstly, in-vitro proteinase production by oral *C. albicans* isolates from these two groups was compared, and secondly, the effect of antimycotic agents on proteinase secretion of a randomly selected group of 14 isolates was assayed by exposing them to 1/4 and 1/16 sub-MICs of two polyenes (nystatin and amphotericin B) and two imidazoles (clotrimazole and miconazole).

Materials and methods

Candida isolates and growth conditions

Two groups comprising 18 oral *C. albicans* isolates each were used in the study. The isolates in the first group were from patients with HIV infection whose CD4 cell counts were < 400 cells/mm³, attending the HIV Research Clinic of the Faculty of Dentistry, University of Hong Kong, Hong Kong. The second group of 18 isolates was from HIV-free patients with angular cheilitis, obtained by the Microbiology Research Services of the same institution. These isolates were identified by sugar assimilation and fermentation tests with the API 20C system (API products, bioMérieux, Lyon, France) and the 'germ tube' test. Stock cultures were maintained at 4°C on Sabouraud dextrose agar (SDA). All isolates were first screened for proteinase production by the bovine serum albumin (BSA) assay; proteinase-positive isolates (seven from each group) were subsequently selected at random for tests with antimycotic agents.

Quantification of proteinase production by the BSA assay

Extracellular proteinase production by *C. albicans* was induced with BSA according to the technique described

by Staib [11]. Briefly, an 18-h yeast suspension of 1×10^6 cells/ml was prepared, and 5 µl of this yeast suspension were spot inoculated on to a BSA 1% agar plate. The plate was incubated aerobically in a humidified container for 5 days at 37°C, flooded with naphthalene black 1.25% solution in methanol/water 90% v/v for 15 min and decolourised for a further 36 h with several changes of the latter solution. The diameter of the zone proteolysis and the colony were examined and quantified by Quantimet 500+ Image Analysis System (Quantimet Image System, Leica Instruments, Hong Kong). Proteinase production (Pr_d value) was calculated as follows:

$$Pr_d \text{ value} = D_z/D_c$$

(where D_z was the diameter of the zone of proteolysis and the colony, D_c was the diameter of the colony).

All the experiments were repeated on three separate occasions with duplicate determinations on each occasion.

Preparation of antimycotic agents

Antimycotic agents used were as follows: nystatin, amphotericin B, clotrimazole and miconazole (Sigma). Nystatin and amphotericin B were dissolved in a mixture of dimethyl sulphoxide and ethanol (3:2); clotrimazole and miconazole were dissolved in N-dimethylformamide. MICs of antimycotic agents used were (µg/ml): nystatin 2.0, amphotericin B 0.4, clotrimazole 10.0, miconazole 4.0 [18]. Dilutions of 1/4 and 1/16 of the above concentrations were used in the experiments.

Evaluating the effect of antimycotic agents on proteinase production by *C. albicans*

To test the effect of antimycotic agents on proteinase production by *C. albicans*, a loopful of 18-h yeast growth was transferred to 10 ml of yeast nitrogen base medium supplemented with 50 mM glucose with or without subinhibitory concentrations of 1/4 and 1/16 MIC dilutions of antimycotics, and grown aerobically at 37°C in a shaking water bath for 18 h. The cells were harvested by centrifuging at 1500 g for 10 min, washed twice in 10 ml of sterile phosphate-buffered saline (0.1 M, pH 7.2) and resuspended in the same buffer to a final concentration of 1.0×10^6 yeasts/ml as determined by microscopic counting in a haemocytometer counting chamber. The subsequent procedure for inducing proteinase production on BSA was as described above.

Statistical analysis

All the numerical data obtained were analysed by the Wilcoxon matched-pairs signed-ranks test.

Results

Assessment of proteinase activity by the image analysis system

In pilot studies both visual and computerised image analysis measurement were used to record the diameter of the zone of proteolysis and the fungal colony. As highly concordant and accurate results were obtained once the image analysis system was standardised, the latter was used subsequently throughout the study. The replicate Pr_d data for each isolate were highly reproducible, as shown by the very low standard deviation (range 0.0–8.4%); the highest coefficient of variation was <9%.

In-vitro proteinase production by *C. albicans* isolates from patients with and without HIV infection

C. albicans isolates from HIV-infected patients were significantly more proteolytic than those from HIV-uninfected individuals ($p = 0.002$, Fig. 1). The mean Pr_d value for the HIV group (1.73) was 25% higher than that for the HIV-free group (1.29). It was also noticeable that all 18 isolates in the former group were proteinase positive, in contrast to 56% isolates in the latter group.

Fig. 1. Proteinase production by *C. albicans* isolated from (A) HIV-infected (mean 1.73) and (B) -uninfected (mean 1.29) individuals ($p = 0.002$). Proteinase production (Pr_d) was expressed as a function of diameter of the zone of proteolysis and the colony (see text).

Effect of antimycotic agents on in-vitro proteinase production

The effect of four different antimycotic agents on proteinase production of *C. albicans* from individuals with or without HIV infection is shown in Fig. 2. Compared with the control groups (no antimycotic treatment) exposure to sub MICs of nystatin, amphotericin B, clotrimazole and miconazole significantly decreased proteinase production by all 14 *C. albicans* isolates tested ($p = 0.008$; Fig. 2, right-hand column). Of the four antimycotic agents tested, exposure to sub-MICs of miconazole resulted in the greatest reduction in proteinase production (22.35%), followed by nystatin (21.18%), amphotericin B (16.47%) and clotrimazole (15.88%).

When the effect of antimycotic agents on isolates from the HIV-infected and non-infected groups was compared, it was clear that proteinase production was reduced to a significantly greater extent in the isolates from the latter group (except exposure to 1/16 MIC of nystatin) (Table 1).

Generally, there was a good dose-response relationship between enzyme production and exposure to all four antimycotic agents in isolates from the disease-free group, i.e., exposure to 1/4 MIC suppressed proteinase production to a greater extent than exposure to 1/16 MIC ($p < 0.01$, Fig. 3). However, this phenomenon was less apparent in the isolates from the HIV group exposed to nystatin and clotrimazole. There was no significant difference in proteinase production between 1/4 and 1/16 MICs exposure of isolates to the two drugs ($p > 0.05$, Fig. 3).

Discussion

Candida infections are found in at least 80% of patients with acquired immunodeficiency syndrome (AIDS) and in about one third of those with HIV infection [13, 14, 19]. Oral candidosis is one of the most common fungal infections, appearing mainly in the oral cavity as erythematous or pseudomembranous lesions [13, 14]. *C. albicans* is undoubtedly the most common causative agent of this disease; its extracellular acid proteinase is a recognised virulence attribute, which facilitates tissue penetration, while proteinase-deficient strains are less invasive [3]. Whereas some have cast doubt on the relevance of candida proteinases in oral candidosis [20], others have demonstrated convincingly that conditions may prevail intra-orally that facilitate its activity [21, 22]. Indeed, the acidic proteinase of *C. albicans*, which requires low pH for optimal activity, is active in acidic salivas that persist in certain physiological and pathological states (e.g., xerostomia, Sjögrens syndrome) [23]. Interestingly, xerostomia and accompanying changes in salivary composition and lower pH

Fig. 2. Percentage reduction in proteinase production by *C. albicans* isolates from HIV-infected (■) and -uninfected (▨) individuals, and the total study population (□) after exposure to four different antimycotic agents.

Table 1. Differences in proteinase production between *C. albicans* isolates from HIV-infected and non-infected individuals, after exposure to sub-MICs of four antimycotic agents

Patient status	Mean Pr _d * by <i>C. albicans</i> strains exposed to							
	Nystatin		Amphotericin B		Clotrimazole		Miconazole	
	1/4 [†]	1/16	1/4	1/16	1/4	1/16	1/4	1/16
HIV ⁺	1.42	1.46	1.47	1.68	1.58	1.62	1.40	1.56
HIV ⁻	1.16	1.29	1.13	1.36	1.14	1.36	1.05	1.25
p value	<0.05	NS	<0.05	<0.01	<0.05	<0.05	<0.05	<0.01

NS, no significant difference.

*Seven isolates, each tested in duplicate on three occasions.

[†]Sub-MIC concentration.

values have been recorded recently in HIV-infected patients [24–27].

Several methods, including BSA plate assay, spectrophotometry, SDS-PAGE and ELISA, can be used to evaluate *C. albicans* extracellular proteinase activity. Although proteinase activity assay on BSA plates has, up to now, been considered as a semi-quantitative method with visual estimation of the area of proteolysis [28], this technique has been complemented with an image analysis system to generate accurate and reproducible data, as shown in this investigation, where the highest coefficient of variation in replicate experiments was <9%.

The present results indicate that *C. albicans* isolates from HIV-infected individuals are more proteolytic than those from uninfected individuals. There are two recent reports on this phenomenon. Bernardis *et al.* [15], studied *C. albicans* isolates from 155 HIV-infected subjects by scoring the proteinase activity on BSA agar and by monitoring the secreted proteinase antigen by an immunoenzymatic assay. They found that *C. albicans* isolates from patients with advanced HIV infection were more proteolytic than those from either earlier stages of HIV infection or HIV-uninfected subjects [15]. Others have found that genetically atypical *C. albicans* strains existed in HIV-infected individuals that could produce greater

Fig. 3. Effect of pre-exposure to sub-MICs of four different antimycotic agents on proteinase production by *C. albicans* from (a) HIV-infected and (b) -uninfected individuals. Significant differences in proteinase production between isolates exposed to 1/4 (■) and 1/16 (▨) MICs is denoted by asterisks, * $p < 0.05$; ** $p < 0.01$; NS, not significant; □, controls without the antimycotic agent.

amounts of extracellular proteinase than typical *C. albicans* strains [16].

The results of the effect of polyenes (nystatin and amphotericin B) and imidazoles (clotrimazole and miconazole) on extracellular proteinase of *C. albicans* from individuals with and without HIV infection indicate that sub-MICs of these antifungal agents may modulate the proteolytic activity of *C. albicans*. This conclusion differs from that of the report by Angiolella *et al.* [17]. They found that only the polyene derivative methyl partricin suppressed proteinase production, but not the azoles miconazole, fluconazole and ketoconazole. This disparity is likely to be due to the fact that only a single isolate was used by the latter workers; 14 isolates were used in the current study.

The present results indicate that exposure to polyenes and imidazoles tends to have less inhibitory effect on proteinase production by *C. albicans* isolates from patients with HIV infection, when compared with isolates from patients without HIV infection. This suggests that proteinase production in *C. albicans* isolates in HIV disease is not only stronger but also more recalcitrant than in isolates from uninfected groups. Indeed, genetically atypical *C. albicans* isolates less susceptible to antimycotic agents have been described in HIV-infected patients [17], indicating that response to antifungal agents may be modulated in association with HIV disease. In clinical terms, this sequence of events might then be one reason for the well known treatment failure of oral and systemic antimycotic therapy of oral candidosis in HIV-infected and AIDS patients [14, 29].

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