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Expression of alpha-defensin-1 in chronic hyperplastic candidosis

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BACKGROUND: Chronic hyperplastic candidosis (CHC) represents a chronic opportunistic candida infection. We clarified the presence, localization and participation of α-defensin-1 in host response against chronic candidal stimulus.

METHODS: Immunohistochemically stained CHC biopsies ($n = 10$) were compared to candida negative idiopathic leukoplakia ($n = 10$).

RESULTS: In CHC α-defensin-1 was detected in neutrophils intravascularly, in lamina propria and in the epithelium, in part in intraepithelial microabscesses. Staining intensity of individual neutrophils varied and was associated with peri- and extracellular staining, in particular in the superficial epithelial cell layers. In controls only very few homogeneously staining neutrophils were detected intravascularly without any extracellular α-defensin-1 deposition.

CONCLUSIONS: Neutrophils form microabscesses and respond to *Candida* by activation and release of α-defensin-1 to peri- and extracellular matrix. This together with the epithelial cell migration from the basal layer to epithelial surface leads to α-defensin-1 rich protective shield in the most superficial epithelial cell layers.

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Introduction

Leukoplakia (LP) is defined as a white patch or plaque that cannot be rubbed off or characterized clinically or pathologically as any other disease (1). Chronic hyperplastic candidosis (CHC) is defined as oral LP related to chronic candidal infection and in which appropriate antifungal therapy leads to resolution of the condition. The prevalence of all oral LP has been reported to range from 0.2 up to 11%. In approximately 10% of these cases the tissues harbor candidal hyphae and meet the other pathological criteria of CHC (2). In addition to the fungal virulence factors many local and systemic host factors allow the conversion of this harmless commensal to a pathogenic organism.

Host cell derived antibiotic peptides termed defensins form an important antifungal factor of the innate immunity (3, 4). In humans, four of the α-defensins (HNP1-4) are expressed by neutrophils as a means of non-oxidative antimicrobial killing, which probably supplements the short-term and short-range action of oxygen radicals (5). All α-defensins are synthesized and stored intracellularly in primary or azurophil granules and released upon activation (5, 6). α-Defensins seem to be active against a broad range of microorganisms. However, an opportunistic hyperplastic candidosis forms a particularly long-lasting stimulus, due to its relatively low pathogenicity and non-destructive nature. Some other defensins including β-defensin have been discovered in skin epithelium, some mucous membranes, e.g. oral and nasolacrimal (7). This study focused on α-defensin-1.

Materials and methods

Samples

Twenty biopsy samples of oral mucosal lesions were obtained from patients with CHC or LP ($n = 10$ for each) undergoing examination of mucosal lesions. Patient samples meeting pathological criteria of CHC were taken as the subjects to be tested and samples from patients with LP of idiopathic origin were used as candida negative controls. Periodic Acid Schiff (PAS)

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Table 1 Clinical and demographic data of the patients with chronic hyperplastic candidiasis (CHC) and leukoplakia (LP)

Number	*Gender*	*Age (years)*	*Location of the lesion*	*Clinical presentation*	*Additional information*
1 CHC	M	59	Tongue	Diffuse keratinization	Heavy smoker
2 CHC	F	45	Tongue	Red/white lesion	Smarting pain
3 CHC	F	59	Tongue	Homogenous	
4 CHC	F	67	Palate	Verrucous	Prosthesis
5 CHC	F	55	Cheek/commissure	Hyperplastic	Heavy smoker
6 CHC	F	53	Tongue	Nodular, indurated	Sharp tooth edges, heavy smoker
7 CHC	M	44	Tongue	Ulcerative	
8 CHC	M	53	Palate	Verrucous	
9 CHC	F	81	Cheek	Papular leukoplakia	Carcinoma of tongue, operated
10 CHC	F	85	Tongue	Hyperplastic, verrucous	
1 LP	F	51	Alveolar ridge	Leukoplakia	
2 LP	F	55	Tongue	Leukoplakia	
3 LP	F	52	Cheek	Striated leukoplakia	
4 LP	F	73	Floor of the mouth	Exophytic	Prosthesis, heavy smoker
5 LP	F	66	Palate	Striated	
6 LP	F	64	Floor of the mouth	Homogenous, smooth	
7 LP	F	50	Alveolar ridge	Exophytic	
8 LP	F	48	Cheek	Striated	
9 LP	F	84	Alveolar ridge	Exophytic, pigmented	Prosthesis
10 LP	M	49	Alveolar ridge	Leukoplakia	

M, Male; F, Female.

staining of the biopsy samples and fungal culture of saliva samples on Dentocult CA® culture medium (Orion Diagnostica, Espoo, Finland) were used to detect fungal infection (Table 1). The local ethical committee approved the study protocol.

Immunohistochemistry

Paraffin embedded sections were deparaffinized, dehydrated and washed in Tris 0.02 M, NaCl 1.5 M, pH 7.5. The antigen epitopes hidden by cross-links caused by formalin fixation were disclosed by immersing the slides in Antigen Retrieval Buffer followed by 10 min heating in a microwave at 600 W. ChemMate staining robot was used for the immunostaining procedure. Slides were run through three washes in buffer containing carrier protein, detergent and preservative and contributing to blocking of non-specific tissue binding of the immunological reagents used in the protocol. Then the sections were incubated in 20 μg/ml goat anti-human α-defensin-1 IgG (Chemicon, Temecula, CA, USA) in antibody diluent for 30 min at room temperature, 2 μg/ml biotinylated rabbit anti-goat IgG for 25 min, endogenous peroxidase block solution for 8 min and finally with streptavidin conjugated highly purified horse radish peroxidase and 0.03% H_2O_2 with concentrated diamino benzidine (DAB) as chromogen (160 μl of DAB) in horse radish peroxidase substrate buffer. Sections were counter-stained for 30 s with Mayer's haematoxylin solution before mounting. For antigen absorption control polyclonal goat anti-human α-defensin-1 IgG was incubated with recombinant human α-defensin-1 peptide (GF 099 Chemicon, Temecula, CA, USA) in phosphate buffered saline containing 1% bovine serum albumin, at 1:10 on a molar basis, for 1 h at room temperature. Antigen preabsorption test confirmed specificity of the staining. All staining reagents were from Dako (Glostrup, Denmark) if not otherwise mentioned.

Results

Staining of sections from lesions of both chronic hyperplastic candidosis (CHC) and leukoplakia (LP) showed parakeratinized epithelium with clear, broad and bulbous rete ridges. In some areas the keratinized layers had eroded and the underlying epithelium had become exposed. All 10 leukoplakia samples harboring candidal hyphae showed typical histological features of chronic hyperplastic candidosis. PAS staining was used to detect the hyphae and confirm the CHC diagnosis (Fig. 1a and b).

α-Defensin-1 was mainly located in neutrophils. Intravascular α-defensin-1 containing neutrophils were more often present in the CHC than in the LP lesions (Fig. 1c). Staining of intravascular neutrophils was intense and uniform. α-Defensin-1 containing neutrophils were also found in lamina propria, in particular in CHC (Fig. 1c). However, no peri- or extracellular α-defensin-1 staining was detected in the lamina propria. There was an apparent gradient or stratification of α-defensin-1 positive neutrophils in the epithelium (Fig. 2a and b). This accumulation was particularly intense in heavily candida infected and neutrophil infiltrated samples, which also contained intraepithelial neutrophil microabscesses (Fig. 2b). In CHC epithelia, cytoplasmic staining of many individual neutrophils was relatively weak, but associated with peri- and extracellular staining. This was particularly prominent in areas of microabscesses. In addition, CHC epithelia contained an intense superficial band or rim of α-defensin-1 deposition in the uppermost

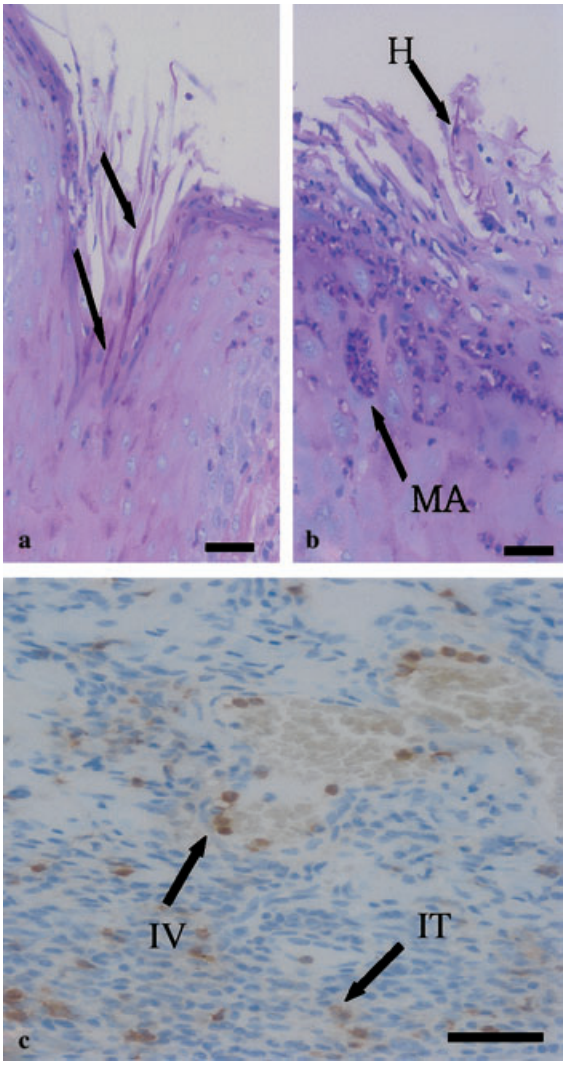

Figure 1 Periodic Acid Schiff (PAS) staining of chronic hyperplastic candidosis shows candidal hyphae in a crypt-like area, where hyphae (arrows) can be seen with variable depths of penetration into the epithelium (a). PAS staining of chronic hyperplastic candidosis (CHC) shows candidal hyphae (H) and many microabscesses (MA) in the epithelium (b). α-Defensin-1 immunoreactive neutrophils in interstitial tissues (IT) and intravascularly (IV) in lamina propria of CHC. Neutrophils stain relatively strongly and have not released their α-defensin-1 to peri- or extracellualr matrix (c).

epithelial cell layers (Fig. 2c). However, in CHC the number of candidal hyphae did not correlate with the number of α-defensin-1 positive neutrophils in connective tissue ($P = 0.58$) or in the epithelium ($P = 0.35$) (Table 2).

In contrast, the candida-negative control LPs contained few neutrophils and very little immunoreactive α-defensin-1, mostly in intravascular neutrophils. Occasionally such cells were also found in extracellular matrix of lamina propria. LP epithelium was not infiltrated by neutrophils and no epithelial microabscesses or extracellular α-defensin-1 staining was observed. Occasional individual α-defensin-1 immunoreactive neutrophils in blood vessels and lamina propria confirmed the success of the staining protocol also in LP slides (Fig. 2a). All such neutrophils in LP samples were intensely stained with no variation in the apparent staining intensity and no peri- or extracellular α-defensin-1 staining in association to such cells.

Discussion

Protection against *Candida albicans* has been considered to be mediated predominantly, besides epithelial covering and epithelial cell-derived β-defensin, by neutrophils and macrophages (8). From our present results a step-by-step process can be envisioned. Whenever the oral mucosa is invaded by *Candida albicans*, the host innate immunity is alarmed and a local inflammatory reaction is triggered. It starts probably with rolling, adhesion and transmigration of neutrophils which are recruited from intravascular compartment. Once they get outside the blood vessel, they set off their journey through the lamina propria toward the epithelium where the offending agent and probably some chemokines are located. Cells keep on migrating till they reach the epithelium where they accumulate. As they get inside the epithelium they also start to organize themselves into microabscesses. As a sign of local activation they degranulate their contents into their extracellular surrounding where α-defensin-1 can exert its effect on candidal pseudohyphae, which as very long and extended branching structures several folds exceed the diameter of individual neutrophils. However, α-defensin-1 does not only stay where it is released but instead continues towards the epithelial surface probably as a result of normal epithelial growth, migration and shedding cycle (epithelial flow), leading to heavy accumulation of neutrophil-derived α-defensin-1 in the most superficial layer of the epithelium. This forms an antimicrobial α-defensin-1 rich frontier shield against candidal ingrowth. We speculate that this process is responsible for the appearance of the strong α-defensin-1 positive rim as shown in Fig. 2d.

Neutrophils are capable of killing and digesting smaller invading microorganisms such as individual, planctonized yeast cells in intracellular phagolysosomes. When a neutrophil faces a candidal pseudohyphen, it degranulates letting its granular content with α-defensins come in contact with the candidal yeast membrane and this can result in inhibition of the growth or even killing of the microorganism (9). The second and somewhat unexpected conclusion is that the effects of epithelial β-defensins are apparently in CHC also in the epithelium *per se* supplemented with neutrophil-derived, imported α-defensin-1. We conducted our study with the first class or α-defensin-1 but believe that its behavior indicates also that of the two other neutrophil granule α-defensin subclasses.

Neutrophils can produce oxygen radicals via activation of the cell surface NADPH oxidase. The final product, hydroxyl radical, is a very short-lived intermediate, which is able to diffuse only twice its own molecular diameter before reactive decomposition to

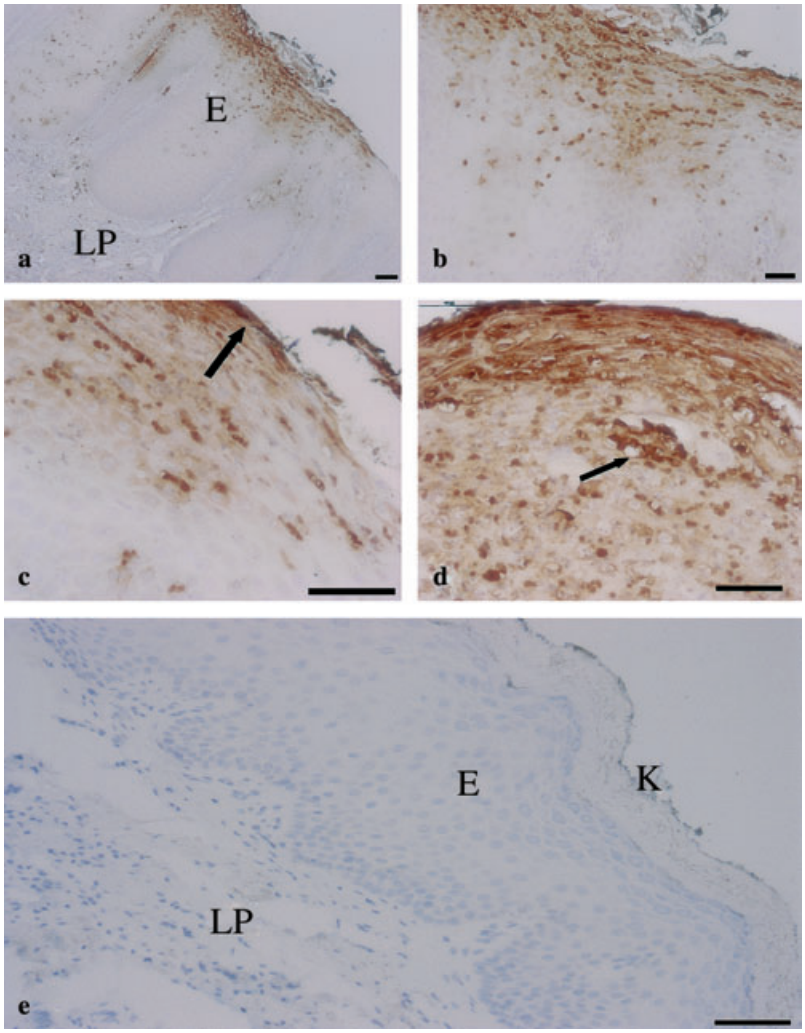

Figure 2 Strong α-defensin-1 staining of neutrophils in the epithelium (E) (a). A higher magnification of chronic hyperplastic candidosis (CHC) section shows some cytoplasmic staining of α-defensin-1 in neutrophils in the epithelium, but also staining in the associated pericellular and extracellular matrix (b). α-Defensin staining of chronic hyperplastic candidosis showing immunoreactivity of neutrophils in the epithelium infected with *Candida*. Note also staining in the associated peri- and extracellular matrix and some concentration of extracellular α-defensin-1 towards the keratinized surface layer of the epithelium (arrow) (c). A higher magnification of CHC section shows an epithelial microabscess (arrow) containing and surrounded by many neutrophils which show intensive staining reaction to α-defensin-1. α-defensin deposits are also seen in the superficial epithelial cell layers (d). In leukoplakia neither neutrophils nor α-defensin-1 staining can be seen. The epithelium is intact without clefts or microabscesses (K, keratin layer; E, epithelium; LP, lamina propria) (e). (Scale bar for all figures = 50 μm).

water. Although myeloperoxidase catalyzed hypochlorite HClO compounds are more long-lived, all reactive oxygen species are characterized by short-term and close-range effects. This poses a problem, when the offenders are long-lived and almost macroscopic organisms, candidal pseudohyphae. Therefore, β-defensins produced *in situ* by resident epithelial cells and α-defensins released by recruited immigrant cells play an important role in anti-candidal defense (6, 10). Formation of a neutrophil-derived, epithelially deposited α-defensin-1 barrier to the superficial mucosal epithelium at the interface with well oxidized atmosphere may lead to an effective anti-candidal shield against new ingrowth. As might go against logical reasoning, we did not find a significant correlation between prevalence of candidal hyphae and number of α-defensin-1 expressing neutrophils (Table 2). A possible reason for this correlation discrepancy is that β-defensins may be playing a significant role in host defense against candida and that α- and β-defensins are acting together. In addition, it is not known if the lesions improve or become worse, and this could make a difference in the cytokine release to attract neutrophils, and in the expression of β-defensins by the epithelial cells.

This may imply that the host responses of the patients did not match the candidal challenge and that the host defense is for some systemic or local reason, compromised. In CHC this inability to combat candidal insults at the required eradication level could explain the tendency of the lesion to persist for a long time and to develop to CHC.

Table 2 α-Defensin-1 in chronic hyperplastic candidosis (CHC) and leukoplakia (LP)

Sample	*Hyphae*	*Microabscesses*	*Neutrophil infiltrates*	*Dysplasia*	*α-Defensin-1*
1 CHC	+ + +	−	+ + +	Dysplasia	E +, CT ±
2 CHC	+	+	+	No dysplasia	E−, CT ±
3 CHC	+ + +	+	+	No dysplasia	E +, CT ±
4 CHC	+	+	+ + +	No dysplasia	E +, CT ±
5 CHC	+	+	+	No dysplasia	E +, CT ±
6 CHC	+ + +	+	+	No dysplasia	E +, CT−
7 CHC	+	+	+ + +	Dysplasia	E +, CT−
8 CHC	+	+	+	No dysplasia	E +, CT−
9 CHC	+ + +	+	+	No dysplasia	E +, CT−
10 CHC	+ + +	+ + +	+ + +	No dysplasia	E +, CT−
1 LP	−	−	−	Dysplasia levis	−
2 LP	−	−	−	No dysplasia	−
3 LP	−	−	−	No dysplasia	−
4 LP	−	−	−	No dysplasia	−
5 LP	−	−	−	No dysplasia	−
6 LP	−	−	−	No dysplasia	−
7 LP	−	−	−	Dysplasia levis	−
8 LP	−	−	−	No dysplasia	−
9 LP	−	−	−	No dysplasia	−
10 LP	−	−	±	No dysplasia	−

(−) Negative, (±) only occasional, (+) some, (+ +) moderate numbers of and (+ + +) high numbers of hyphae, microabscesses, neutrophil infiltrates and α-defensin-1 positive cells.
E, epithelium; CT, connective tissue.

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