The Relationships of Cathelicidin, hBD-1, hBD-2 and hBD-3 in Patients With
P. versicolor, T. inguinalis and T. pedis Infections

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Abstract: The human β-defensins and cathelicidins are peptides with a strong antimicrobial activity. Epidermal antimicrobial substances are known to play an important role in protecting the skin against dermatophytic invasion. This study aimed to investigate the expression of human β-defensins and cathelicidin in skins of patients with dermatophytes and Pityriasis versicolor. Expressions of human cathelicidin and human β-defensins-1,2,3 were assessed by immunohistochemistry for dermatophytes of patients with Tinea inguinalis, Tinea pedis and Pityriasis versicolor and a healthy control (respectively n=8,15,15,9) group. When the normal and infected tissues were compared according to their staining intensity, human β-defensin-1 and -2 expressions were found stronger in control epithelium than that in Tinea pedis and Pityriasis versicolor (p<0.05) The expression of cathelicidin was stronger in Tinea inguinalis (p<0.05). Human β-defensin-3 expression was higher in control epithelium than that in Tinea pedis (p<0.05). It can be concluded that human β-defensins and cathelicidin appears to play a role in susceptibility of patients with Pityriasis versicolor and some dermatophytes infections.

Key words: Cathelicidin • Human β-Defensins-1, -2, -3 • Pityriasis versicolor • Tinea inguinalis • Tinea pedis

INTRODUCTION

Superficial fungal skin infections are known to cause a wide spectrum of diseases in humans. Dermatophytes are highly specialized pathogenic fungi that primarily affect the stratum corneum [1-4]. The dermatophytes, also known as tinea infections, are a group of closely related fungi that have the capacity to invade keratinized tissue (skin, hair and nails) of humans and other animals to produce dermatophytosis infection, commonly referred to as ringworm. Infection is generally cutaneous and restricted to the nonliving cornified layers as the fungi are unable to penetrate the deeper tissues or organs of immunocompetent hosts [5-10]. Antimicrobial peptides (AMPs), having low risk of resistance emergence, are proposed as new antimicrobial agents. One of their potential applications is the prevention and treatment of bacterial and fungal skin infections caused, for example, by Staphylococcal species and dermatophyte fungi [11-13]. AMPs have broad-spectrum activity against bacteria, yeast, fungi and virus and they play important roles in the defense system of many organisms from insects to humans [14]. AMPs, synthesized in the skin at sites of potential microbial entry, provide a soluble barrier that acts as an impediment to infection. In the case of infection or injury, AMP expression in the skin is upregulated due to increased synthesis by keratinocytes and deposition from degranulation of recruited neutrophils. Constitutive and inducible expression of human cathelicidin (hCAP18/LL-37), as well as human β-defensins (hBDs) 1, 2 and 3 (hBD-1, hBD-2, hBD-3), have been observed in epidermal keratinocytes [15,16]. In order to demonstrate the hypothesis that cathelicidin (LL-37) and hBD-1, hBD-2
and hBD-3 expressions were increased in the skin lesion of patients with Pityriasis versicolor, Tinea inguinalis and Tinea pedis infections compared to normal skin, using immunohistochemical staining.

**MATERIALS AND METHODS**

**Patients:** In this study, 23 patients (11 women and 12 men; median age 33.82±11.28 years; range 17-57 years) with *T. inguinalis* and *T. pedis* and 15 patients (4 women and 11 men; median age 33.2±13.35 years; range 18-58 years) with *P. versicolor* infections were included. Scales were collected by scraping the affected area of the patients. Hyphae were detected on direct microscopic examination of the squamous in the scraping of the patients’ lesions with 10% potassium hydroxide and histopathological examination.

Three millimetre punch biopsies were excised from skin lesions. In addition, these included controls as follows: 9 healthy subjects (2 men and 7 women; median age 39.22±15.13 years; range: 20-68 years). A 3-mm punch biopsy was taken from normal body skin.

The study was approved by the local ethics committee. Each patient gave his or her written informed consent. The study was conducted according to the rules of good clinical practice.

**Immunohistochemical Staining:** Punch biopsies were fixed in 10% buffered formalin and embedded in paraffin blocks. Sections that were 4µm thick were cut and one section was stained with haemotoxylin - eosin to observe the tissue morphology. For immunohistochemistry, endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide (v/v) in methanol for 10 minutes at room temperature (RT). The sections were subsequently washed in distilled water for 5 minutes and antigen retrieval was performed for 3 minutes using 0.01M citrate buffer (pH 6.0) in a domestic pressure cooker. The sections were transferred in 0.05M Tris-HCl (pH 7.6) containing 0.15M sodium chloride (TBS). After washing in water, the sections were incubated at RT for 30 minutes with normal swine serum (for anti-hBD-1, 2, 3 and LL-37) (1:20) diluted in TBS to block nonspecific binding. The sections were then covered with the primary antibodies diluted 1:500 for anti-hBD-1, 2, 3, LL-37 in TBS at 4°C overnight (anti-hBD-1, 2, 3 and LL-37 were from Phoenix, USA). After washing in TBS for 15 minutes, the sections were incubated at RT for one hour with secondary antibody (swine-anti-rabbit Ig-biotinylated) at a dilution of 1:100. Then, treatment was followed with avidin-biotin peroxidase complex (Dakopatts, Denmark). Diaminobenzidine was used to visualise peroxidase activity in the tissues. Nuclei were lightly counterstained with haemotoxyline and then the sections were dehydrated and mounted. Both positive and negative controls were included in each run. Positive controls consisted of sections of tonsillitis tissues for hBD-1, 2, 3 and LL-37. TBS was used in place of the primary antibody for negative controls.

Light microscopy of immunohistochemically stained sections was performed by a pathologist and a biologist who were blinded to the clinical information of the patients. Distribution, localization and characteristics of immunostaining were recorded. Brown colour in cytoplasm and/or nucleus of epithelial cells of the basal layer of the epidermis was evaluated as positive staining. Scoring was also performed by observers blinded to patient data. Scoring differences between observers were resolved by consensus. For each antibody, the intensity of the reaction [negative (-), weak (1+), moderate (2+), or strong (3+)] was determined in order to describe the immunoreaction.

**Statistical Analysis:** For each peptide, staining scores in control and infected epithelium were compared statistically. Statistical analyses were performed with SPSS software (Statistical Package for the Social Sciences, version 15.0, SSPS Inc, Chicago, III, USA). The differences between the expressions of the hBD-1, 2, 3 and LL-37 in normal and *T. inguinalis*, *T. pedis* and *P. versicolor* tissues were analyzed by independent-t test, Tukey and Tamhane T2 tests. A p value of less than 0.05 was considered as statistically significant.

**RESULTS**

The 47 examples of *P. versicolor*, *T. inguinalis*, *T. pedis* and control from 47 patients were examined (Table 1).

The stronger LL-37 was observed in infected epithelium than control epithelium in *T. inguinalis* (Table 1, p=0.006<0.05). The 22% of the strong LL-37 expression was control epithelium and 87.5% of *T. inguinalis* epithelium was considered to have LL-37 expression (Table 1). Fig. 1 shows strong LL-37 protein expression in skin from patients with *Tinea inguinalis* infection cells.

The hBD-1, hBD-2 and hBD-3 expressions were higher in control epithelium than those in infected epithelium in *T. pedis*. There were statistically significant differences in hBD-1, hBD-2 and hBD-3 expressions between control and epithelium in *T. pedis* (p<0.05).
Table 1: Immunohistochemical staining and statistical results of hBD-1, hBD-2, hBD-3 and LL-37 in *T. inguinalis* and *T. pedis* infections and control group.

<table>
<thead>
<tr>
<th></th>
<th><em>T. inguinalis</em> (n=8)</th>
<th><em>T. pedis</em> (n=15)</th>
<th>control (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-) n</td>
<td>%n</td>
<td>(+1)n</td>
</tr>
<tr>
<td>hBD-1</td>
<td>2</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>hBD-2</td>
<td>2</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td>hBD-3</td>
<td>2</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>LL-37</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*p value for comparison with control group.

Table 2: Immunohistochemical staining and statistical results of hBD-1, hBD-2, hBD-3 and LL-37 in *P. versicolor* infections and control group.

<table>
<thead>
<tr>
<th></th>
<th><em>P. versicolor</em> (n=15)</th>
<th>Control (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-) n</td>
<td>%n</td>
</tr>
<tr>
<td>hBD-1</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>hBD-2</td>
<td>9</td>
<td>60</td>
</tr>
<tr>
<td>hBD-3</td>
<td>4</td>
<td>26,6</td>
</tr>
<tr>
<td>LL-37</td>
<td>1</td>
<td>6,6</td>
</tr>
</tbody>
</table>

*p value for comparison with control group.

Table 3: Statistical analysis of hBD-1, hBD-2, hBD-3 and LL-37 in *T. inguinalis*, *T. pedis* and *P. versicolor* infections.

<table>
<thead>
<tr>
<th></th>
<th><em>T. pedis</em> (n=15)</th>
<th><em>T. inguinalis</em> (n=8)</th>
<th><em>P. versicolor</em> (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(I) factor</td>
<td>(J) factor</td>
<td>Sig.</td>
</tr>
<tr>
<td>hBD-1</td>
<td>hBD-1</td>
<td>hBD-2</td>
<td>NS</td>
</tr>
<tr>
<td>hBD-2</td>
<td>hBD-3</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LL-37</td>
<td>0,003</td>
<td>LL-37</td>
<td>0,000</td>
</tr>
<tr>
<td>T. inguinalis (n=8)</td>
<td>hBD-1</td>
<td>hBD-2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>hBD-3</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>LL-37</td>
<td>0,001</td>
<td>LL-37</td>
</tr>
<tr>
<td>T. versicolor (n=15)</td>
<td>hBD-1</td>
<td>hBD-2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>hBD-3</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>LL-37</td>
<td>0,000</td>
<td>LL-37</td>
</tr>
</tbody>
</table>

Fig. 1: Immunohistochemical analysis of strong LL-37 protein expression in skin from patients with *Tinea inguinalis* infection.

Fig. 2: Immunohistochemical analysis of negative HBD-3 protein expression in human skin infected with *Tinea pedis*.

(Table 1). The 73.3% of the negative hBD-2 expression was infected epithelium, but 66.6% of control was considered to have weak hBD-2 expression (Fig. 2). Similarly, 53.3% of the negative hBD-3 expression was epithelium in *T. pedis*, but 55.5% of control epithelium was considered to have moderate hBD-3 expression (Table 1).

The 46.6% and 40% of the weak HBD-1 and hBD-2 expressions were infected epithelium (Fig. 3) and 44.4% and 66.6% of the control were considered to have weak hBD-1 and hBD-2 expressions (Table 2). Thus, stronger hBD-1 and hBD-2 were observed in control epithelium than infected epithelium in *P. versicolor* (Table 2, p<0.05).
Fig. 3: Brown staining shows moderate HBD-1 protein expression in Pityriasis versicolor (haematoxylin counterstain).

When *T. inguinalis, T. pedis* and *P. versicolor* infections with the levels of hBD-1, hBD-2, hBD-3 and LL-37 expressions were correlated separately, LL-37 expression was higher than hBD-1, hBD-2, hBD-3 peptides in all infected tissues types (Table 3, p<0.05).

**DISCUSSION**

The innate immune defense function of skin is greatly enhanced by a soluble AMP barrier that is activated when physical barriers fail to prevent microbial entry. Even under resting conditions, low levels of AMPs are synthesized at the sites of potential microbial entry into the skin and provide a further impediment to infection [17]. After injury, AMPs levels in the skin rise rapidly due to increased synthesis by keratinocytes and deposition from degranulation of recruited neutrophils. The chemoattractant properties of cathelicidins and defensins may further amplify this process through their functional interactions with leukocyte surface receptors. The growing number of multifunctional peptides suggests that the host AMPs defense system acts both directly and indirectly to prevent cutaneous infection [18-20].

The expression was studied by the immunohistochemistry of different forms of AMP in dermatophytes and *P. versicolor*. Using immunohistochemistry, the specific cell types containing the different AMP peptides can be identified and, in particular, those cells that express different AMP peptides can be specifically recognized. There have been several biochemical studies of AMP in various fungal infections [21-25]. However, biochemical studies of dermatophytes have used tissue homogenates that contain a mixture of cell types including lesional and non-lesional epithelium and stromal cells and all these cell types may express AMP.

In this study, the skins of patients with *P. versicolor* and *T. pedis* showed significantly lower hBD-1 and hBD-2 expressions and significantly higher LL-37 expression in *T. inguinalis* as compared to healthy controls. In this conditions the infected skin is highly susceptible to bacterial infection. *T. inguinalis* keratinocytes expressed LL-37 as a protective response against bacterial superinfection.

Differential expression of antimicrobial peptides appears to play a role in the susceptibility of patients with chronic inflammatory skin disorders to infectious complications. For example, LL-37 is induced in human keratinocytes during psoriasis, lupus erythematosus and contact dermatitis [15]. The hBD-2 and hBD-3 are also upregulated in keratinocytes of inflamed psoriatic lesions [26, 27]. The increased expression of antimicrobial peptides in psoriasis correlates with a low rate of secondary infection. In contrast, the expression of LL-37 and hBD-2 is not upregulated in those individuals with atopic dermatitis who are highly susceptible to bacterial and viral infections [21]. The differences in antimicrobial peptide expression between these two disorders gain immunological relevance in the view of the antimicrobial activity of LL-37 against *S. pyogenes* [16] and its synergistic activity with b-defensins against *S. aureus* [18], a leading agent of human skin infections. Gambichler *et al.* [18] showed that expression of hBD-2 and hBD-3 was significantly reduced in dermatophyte as compared to inflammatory conditions such as psoriasis. On the other hand, Kawai *et al.* [23] demonstrated that the stratum corneum infected by dermatophytes contained a larger amount of hBD-2 than that of normal individuals.

Jensen and coworkers [22] found a significantly impaired permeability barrier in tinea corporis. A disturbance in barrier function is accompanied or caused by changes in epidermal proliferation and differentiation. Increased epidermal proliferation is often linked to disturbed differentiation, as there may not be sufficient time for proper differentiation during exaggerated cell renewal. Epidermal differentiation is of crucial importance for the integrity of the permeability barrier [28]. Jensen *et al.* [22] showed strong staining intensity of the AMP hBD-2 in the spinous and granular layers of tinea corporis. Similarly, the expression of hBD-2 in candidal leukoplakia [29-31] and in Malassezia furfur infection has been described [27]. However, they showed that expression appeared to be most pronounced in areas with only scattered fungal elements. The hBD-2 expression was somewhat less pronounced in areas beneath a high fungal hyphae load, meaning that fungal hyphae most
probably do not directly cause increased hBD-2 protein expression, but instead suppress defensin expression. It has recently been found that hyphae invasion of *Candida albicans* inhibits the expression of human β-defensins in experimental oral candidiasis [28]. Jensen and coworkers [22] showed that natural and recombinant hBD-2 have a weak antifungal activity against *T. rubrum*, compared with fluconazole. Therefore, it is most likely that hBD-2 expression is a secondary event in fungal infection and may be related to disturbed differentiation and induction of cytokines. Keratinocytes may express hBD-2 as a protective response against bacterial superinfection, because tinea can be colonized by bacteria [29, 30]. The hBD-2’s antibacterial activity is already well known [32]. Thus, increased hBD-2 expression may be an attempt to strengthen antibacterial activity and substitute for the impaired physical barrier in tinea. In conclusion, they propose that superficial dermatophytosis attributable to infection with *T. Rubrum* results in disturbed skin barrier function, reduced stratum corneum hydration, enhanced proliferation and changes in epidermal differentiation including induction of hBD-2 expression.

Belen and coworkers showed that there was an increase in LL-37 protein expression in epidermis of skin from patients with *Tinea corporis* or *P. versicolor* compared with healthy skin [20]. While LL-37 are typically expressed at low levels in normal keratinocytes, they are induced after injury or bacterial infection and increase rapidly as a consequence of direct synthesis by the epidermal keratinocytes and deposition from recruited granulocytes [19]. Similarly, in the present study, the immunohistochemical experiment suggests that lesional skin of *T. inguinalis* patient’s exhibit significantly higher LL-37 expression than healthy skin, predominantly indicating an increased antimicrobial activity due to disturbance of skin barrier function. Moreover, Ong and coworkers [21] showed that LL-37 increased in the superficial epidermis of all patients with psoriasis, while it decreased in atopic dermatitis patients.

Differential expression of AMPs appears to play a role in the susceptibility of patients with chronic inflammatory skin disorders to infectious complications. These studies indicate potential roles for AMPs in host immune defense against skin infection.

In summary, the current observations support the hypothesis that cutaneous fungal infections by a wide variety of organisms have relationship with the expression of AMPs. This hypothesis suggests new avenues for therapy and new approaches to understanding individual susceptibility to these unique dermatologic diseases.

**REFERENCES**


