ORIGINAL ARTICLE

Barrier Function, Epidermal Differentiation, and Human *β*-Defensin 2 Expression in Tinea Corporis

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Tinea corporis is a superficial mycotic infection resulting in substantial epidermal changes. We determined skin barrier function, epidermal differentiation, and human-*β*-defensin 2 (hBD-2) protein expression in 10 patients with tinea corporis caused by *Trichophyton rubrum* (*T. rubrum*). We found disturbed skin barrier function as shown by a significant increase in transepidermal water loss (TEWL) and specific ultrastructural changes including disturbed formation of extracellular lipid bilayers, lamellar body extrusion, and deposit of clotted material at the stratum granulosum/stratum corneum interface. Epidermal proliferation in tinea increased several fold and accordingly, proliferation and inflammation-associated keratins K6, K16, and K17 were expressed. Expression of basal keratins K5 and K14 increased, whereas differentiation-associated K10 was reduced. Reduction of the cornified envelope proteins involucrin, loricrin, and the S100 protein filaggrin was also seen. Reduced filaggrin expression correlated with reduced skin hydration; protein breakdown products of filaggrin have been shown to be important for water binding. Surprisingly, we found pronounced epidermal protein expression of hBD-2, which may be related to disturbed epidermal differentiation and inflammation. hBD-2 showed a weak, although significant, antifungal activity against *T. rubrum* in the turbidimetric assay and the immunohistological staining was somewhat less pronounced in areas directly underneath fungal hyphae in the stratum corneum. Together, we describe profound changes in skin barrier structure and function, epidermal proliferation, and differentiation including pronounced protein expression of hBD-2 in tinea corporis.

INTRODUCTION

Superficial fungal skin infections cause a wide spectrum of diseases in humans. Dermatophytes are highly specialized pathogenic fungi, which primarily affect the stratum corneum. Clinically superficial dermatophytosis causes erythema and scaling of the skin. Histology and periodic acid-Schiff (PAS) staining, in particular, reveal that hyphae in superficial dermatophytosis are present in the stratum corneum but do not reach the living epidermis. Nevertheless, skin reactions to dermatophytes involve the entire epidermis, histologically seen as thickened epidermis with hyper-/parakeratosis and acanthosis. In addition, an inflammatory infiltrate, primarily T cells, and sometimes neutrophils, is visible in the upper dermis (Brasch, 1990; Brasch and Sterry, 1992; Brasch *et al.*, 1993).

Erythema and scaling are also signs of eczema and psoriasis. In not yet fully developed lesions, it is often clinically difficult to distinguish between tinea, eczema, or even psoriasis. Diagnosis of tinea may be difficult even with routine histology using hematoxylin-and eosin-stained slides because the epidermal architecture is sometimes similar to eczema or psoriasis, in particular if neutrophils are present. PAS staining can be necessary to detect hyphae in the stratum corneum.

The stratum corneum, the main permeability barrier, is formed from extracellular lipids and corneocytes during epidermal differentiation of the skin (Elias, 1983). The main extracellular stratum corneum lipids are cholesterol, free fatty acids, and ceramides (Lampe *et al.*, 1983; Elias and Menon, 1991). Specific ceramides are covalently bound to cornified envelope proteins, particularly to involucrin, forming the backbone for the subsequent attachment of free ceramides, fatty acids, and cholesterol (Fuchs and Green, 1980; Cline and Rice, 1983; Watt, 1983; Wertz *et al.*, 1989; Mehrel *et al.*, 1990; Hohl, 1993; Marekov and Steinert, 1998). The cornified envelope proteins are formed during epidermal differentiation. Specific basal (K5 and K14) and suprabasal (K1 and K10) keratins are also synthesized during the differentiation process (Moll *et al.*, 1982; Smack *et al.*, 1994), demonstrating that epidermal differentiation is crucially involved in skin barrier function (Ekanayake-Mudiyan-

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selage *et al.*, 1998). The antimicrobial barrier that contains antimicrobial peptides is the second line of defense beyond the permeability barrier (Zasloff, 1987; Harder *et al.*, 1997; Liu *et al.*, 2002; Braff *et al.*, 2005).

RESULTS

Fungal spectrum of superficial mycoses
Cultured fungi from superficial dermatomycoses on glabrous skin (excluding feet) were recorded in our hospital over a period of 3 years (2001–2003). Dermatophytes were identified in approximately 65% of 232 positive cultures, mostly *T. rubrum* (48%), followed by *T. mentagrophytes* in merely 6%. *Microsporum canis* (2%) was found only in samples from young patients; other dermatophytes were only rarely seen. *Candida* species were identified in more than one-quarter of the samples (29%); additional yeasts and molds were also identified (15%).

Changes in transepidermal water loss (TEWL) and stratum corneum hydration
As *T. rubrum* is by far the most common cause of superficial dermatophytosis in adults in Germany, only patients with tinea corporis and positive cultures for *T. rubrum* were chosen for further study. In lesional *T. rubrum*-infected skin, we found a highly significant increase in TEWL compared with intraindividual healthy control skin from the same region ($+243.1\%$, $P<0.0001$, $n=25$; Figure 1a). Hydration was significantly reduced in lesional epidermis compared to control (-24.3%, $P<0.0001$, $n=22$; Figure 1b). These results show that fungal infection significantly influences TEWL and stratum corneum hydration.

Increased proliferation in the epidermis
It is well known that fungal infection results in scaling of the skin. Scaling is very often the result of epidermal hyperproliferation. Therefore, we examined epidermal proliferation using the Ki-67 marker. We found that lesional skin exhibited a highly significant sevenfold increased amount of labeled cell nuclei in the basal cells and, to a lesser degree in the

lower spinous layers (involved epidermis: 16.0 ± 2.21 cells/visible field; uninvolved epidermis: 2.3 ± 0.8 cells/visible field; $+695\%$, $P<0.001$, $n=10$; Figure 2). This demonstrates a highly significant increase in proliferation in tinea corporis.
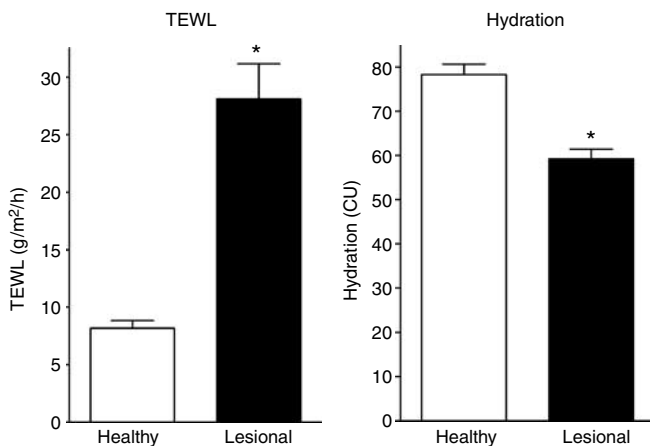

Figure 2. **Changes in epidermal proliferation rate and differentiation.** Using Ki-67 antibodies, an increase in proliferation was seen in infected epidermis ($+695\%$, $P<0.001$, $n=10$). Epidermal differentiation showed changes in involucrin, loricrin, filaggrin, anti-microbial hBD-2, and keratins. Bar = 100 µm.

Figure 1. **TEWL and hydration in healthy and lesional skin.** TEWL significantly increased in superficial dermatophytosis, indicating skin permeability barrier impairment ($+243.1\%$, $P<0.0001$, $n=25$). Stratum corneum hydration was reduced (24.3%, $P<0.0001$, $n=22$).
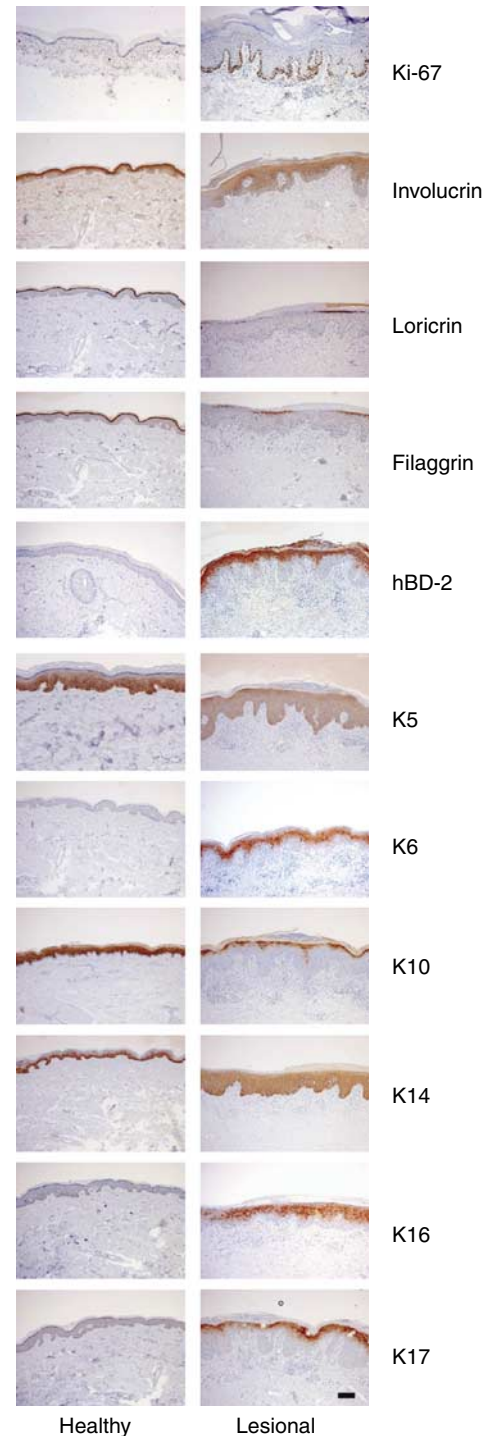
Altered distribution of involucrin, loricrin, filaggrin, β-defensin 2, and keratins in the epidermis

Changes in epidermal proliferation are usually accompanied by changes in epidermal differentiation. Therefore, we examined epidermal keratins, cornified envelope proteins, and filaggrin as markers of epidermal differentiation. In addition, human-β-defensin-2 (hBD-2) expression was examined as an important antimicrobial peptide and also as a marker of differentiation. Patterns of immunohistochemical staining were similar in all patients studied with only slight variations. Immunohistochemistry of differentiation-associated proteins in lesional compared with uninvolved skin of the same patient is shown in Figure 2.

In healthy epidermis, involucrin expression was confined to the upper spinous layers and the stratum granulosum. Faint staining of the spinous layers was only occasionally observed. The band of epidermal involucrin staining significantly broadened in tinea corporis and was found in all suprabasal layers. Spots of staining were recognized even in the basal cell layer. This result showed a premature expression of involucrin in tinea corporis.

The zone stained for loricrin broadened in infected skin compared with normal skin. Loricrin staining in healthy skin was seen as a sharp band confined to the stratum granulosum. In tinea corporis, faint broadening of the stained zone occurred most probably owing to disease-related thickening of the granular cell layer (hypergranulosis) in mycotic skin. However, loricrin staining extended to the upper spinous layers in an irregular pattern, showing broad gaps of expression in lesional skin.

In healthy skin, filaggrin staining was found in the upper granular layer and transition zone to the lower horny layers of the skin. In lesional skin, the thickness of the stained band increased. This was again due to the increased thickness of the granular layer alongside an overall increase in epidermal thickness. Although the stained band broadened, this band contained gaps that resulted in decreased total staining for filaggrin.

Remarkably, we found very strong expression of hBD-2 in suprabasal epidermal layers with increasing staining from the lower to mid-spinous layers into the granular layer of lesional skin. In contrast, there was no staining for hBD-2 in healthy skin. In double-staining analysis using PAS in combination with a hBD-2 antibody, simultaneous staining for fungal hyphae in the stratum corneum and strong staining for the defensins in spinous and granular layers were noted (Figure 3). Interestingly, staining intensity was somewhat less in areas with high fungal load than in areas with low fungal load.

Immunostaining for basal K5 was primarily restricted to the basal epidermal layer in healthy skin. In lesional skin, extension to suprabasal epidermal layers occurred in a homogeneous distribution, but staining intensity was significantly reduced. Proliferation-associated K6 was not expressed in healthy control skin. In contrast, pronounced staining in the suprabasal layers appeared in lesional mycotic-infected skin. Suprabasal differentiation-related K10 expression was found in the entire suprabasal compartment in healthy controls. Staining for K10 was concentrated in the
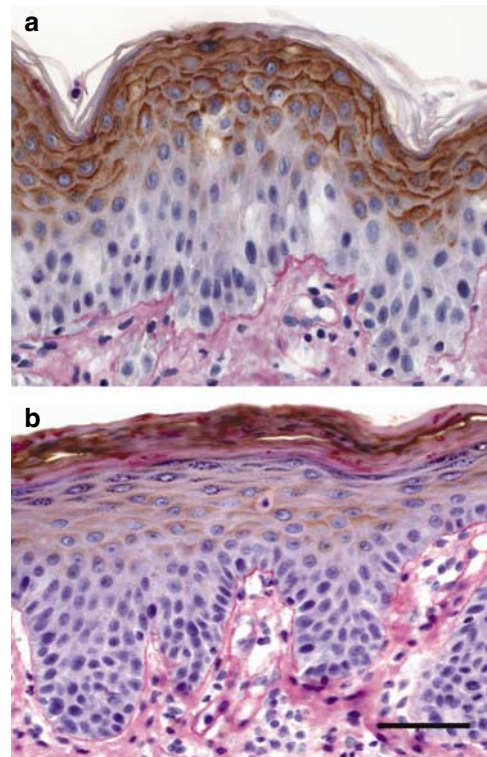
Figure 3. **Colocalization of hyphae and hBD-2 expression.** (**a**) Double staining of hBD-2 and PAS shows a strong expression of hBD-2 in tinea however, (**b**) with a slight reduction in areas underneath a high fungal hyphae load. Bar = 50 μm.

granular and upper spinous layers in lesional skin, but was reduced or absent in the lower spinous layers of the epidermis. Immunostaining for basal keratin K14 was localized in the basal and the lower spinous layers in control skin. Staining of the entire nucleated epidermis occurred with the highest staining intensity in lesional skin still in the basal layer. For proliferation-associated keratin K16, there was faint staining in the basal layer in healthy skin. In tinea, intense staining occurred in all suprabasal layers. Inflammation-associated K17 was not expressed in healthy skin. In lesional skin, staining was found in suprabasal layers with increasing intensity toward the stratum granulosum.

Weak, although significant, activity of hBD-2 against *T. rubrum*

A turbidimetric assay showed that the recombinant and the natural hBD-2 in two concentrations expressed a weak, although significant, antifungal activity against *T. rubrum* (Figure 4) ($P < 0.05$, $n = 4$). In contrast, fluconazole used as control was highly active.

Impaired lamellar body extrusion and disturbed formation of lipid bilayers in tinea

Dermatophyte hyphae are identified within the corneocytes but also in the extracellular spaces of the stratum corneum. Ultrastructural analysis revealed disturbed lamellar body extrusion at the stratum granulosum/stratum corneum interface and disturbed formation of extracellular lipid bilayers.
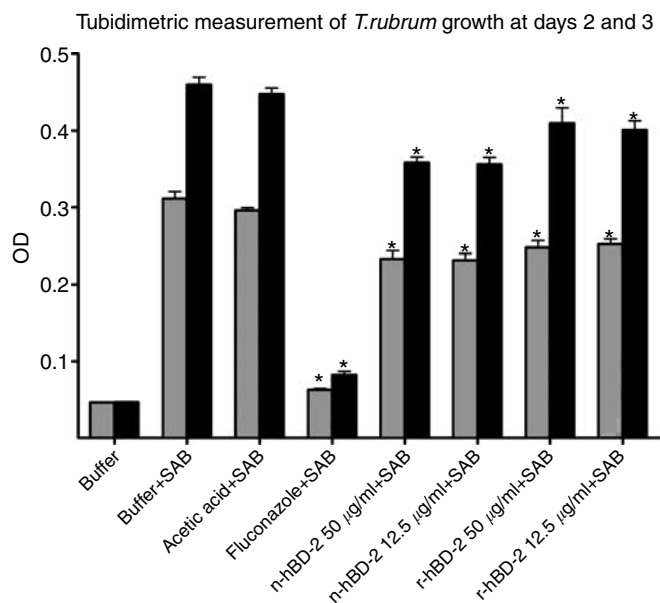
Tubidimetric measurement of *T.rubrum* growth at days 2 and 3

Figure 4. **Turbidimetric analysis.** Turbidimetric assay revealed that natural (n-hBD-2) or recombinant (r-hBD-2) hBD-2 shows weak but statistically significant (*$P< 0.05$, $n=4$) antimicrobial activity against *T. rubrum* after 2 days (gray) or 3 days (black) of incubation. In contrast, fluconazole that served as control was highly active (*$P< 0.05$, $n=4$) compared with phosphate buffer.

Bulged extracellular areas with clots of uneven material were found in the stratum corneum of lesional skin (Figure 5).

DISCUSSION

As *T. rubrum* is by far the most frequent and important cause of tinea corporis in adults in Germany (Brasch, 1990), we focused our investigations on that single fungal species. A diversity of mechanisms is considered relevant for its interaction with an infected host. Similar to other dermatophytes, *T. rubrum* can release a broad panel of enzymes including keratinases, aminopeptidase, and lipases that may damage corneocytes and lead to extracellular growth of hyphae (Das and Banerjee, 1977; Brasch *et al.*, 1991; Da Silva *et al.*, 2005). Neutrophils are attracted by activation of complement (Dahl, 1986) and probably by lipid-like factors as well (Kahlke *et al.*, 1996; Schröder *et al.*, 2002).

In this study, we found a significantly impaired permeability barrier in tinea corporis. TEWL, as a marker of barrier function, increased more than threefold in lesional compared with nonlesional skin from the same patients and the same body area. Electron microscopy revealed disturbed lamellar body extrusion and disturbed stratum corneum lamellar bilayer organization, indicating a disturbed skin barrier structure. We also showed that the fungal hyphae disrupted the normal stratum corneum structure (Figure 5a). The extracellular space in the stratum corneum shows clotted material (Figure 5c), most likely the result of impaired lamellar body content and extrusion (Figure 5b). This finding is specific for tinea and has not been described before in other diseases including atopic dermatitis, which also shows
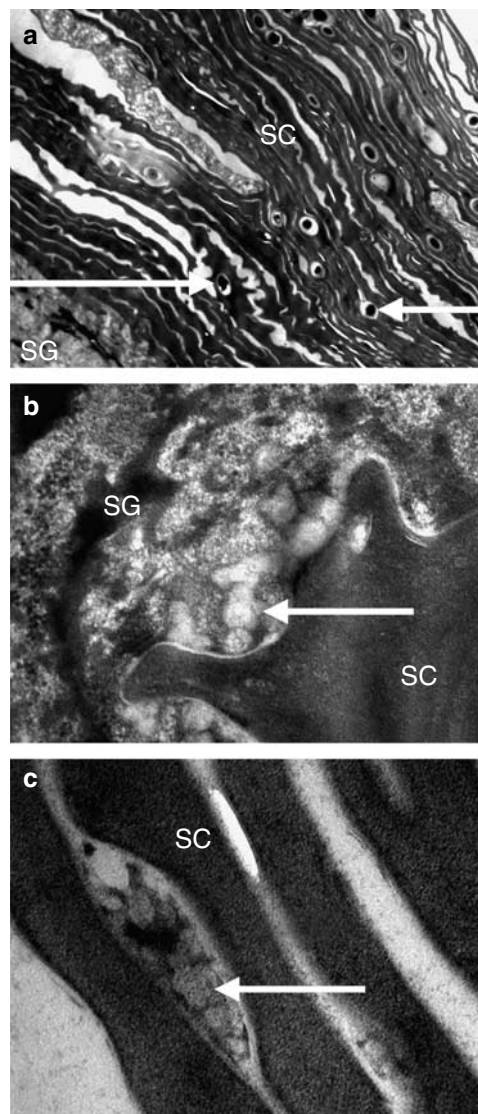

Figure 5. **Electron microscopy study of the transition zone between stratum granulosum and stratum corneum.** Ultrastructural analysis in tinea caused by *T. rubrum* identified hyphae in the corneocytes (**a**, long arrow) and in the extracellular space (**a**, short arrow). (**b**) Disturbed lamellar body extrusion at the transition from the stratum granulosum (SG) to the stratum corneum (SC) and lack of lipid bilayer formation are signs of the skin barrier disturbance. (**c**) The extracellular space in the stratum corneum shows clotted material.

disturbed barrier function and disturbed epidermal differentiation (Fartasch *et al.*, 1992). Our electron microscopy studies point to the direct effects of fungal lipase on the disturbance of lipid extrusion and deposit of clotted material. However, we cannot rule out that the release of fungal keratinases, which leads to injury to the keratinocyte membrane, may cause inflammation followed by increased proliferation and disturbed epidermal differentiation. Possibly, both mechanisms may be active, although tinea corporis induced by *T. rubrum* usually exhibits only moderate inflammation. TEWL values in tinea were similar to the values in lesional skin of atopic dermatitis (Jensen *et al.*,

2004) showing similar degrees of barrier disruption despite different causes.

A disturbance in barrier function is accompanied or caused by changes in epidermal proliferation and differentiation. We have previously shown that epidermal DNA synthesis increases during permeability barrier repair (Proksch et al., 1991) and described a fivefold increase in epidermal proliferation in atopic dermatitis (Jensen et al., 2004). Here, we found a sevenfold increase in epidermal proliferation in tinea corporis. Similar results have been previously described in animal experiments (Tagami, 1985; Tagami et al., 1985) and in annular tinea (Berk et al., 1976). Proliferation removes dermatophytes that cannot be removed by phagocytosis or immunoresponse because the hyphae reside exclusively in the ''dead'' stratum corneum (Tagami et al., 1989; Dahl, 1994).

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 (Ekanayake-Mudiyanselage et al., 1998). It is not surprising that proliferation-associated cytokeratins were expressed in lesional tinea corporis: intense staining for K6 and K16 in tinea in the suprabasal layers similar to atopic dermatitis lesional skin occurred (Jensen et al., 2004). The inflammation-associated K17 was not expressed in healthy skin. In lesional skin, staining was found in the upper spinous and the granular layers. The staining was less pronounced in tinea compared with atopic dermatitis (Jensen et al., 2004). This correlates with the clinical and histological findings, as inflammation in tinea corporis caused by *T. rubrum* is usually not severe.

Keratinocytes synthesize specific cytoskeleton basal (K5 and K14) and suprabasal (K1 and K10) keratins, as well as cornified-envelope associated proteins, in particular involucrin, filaggrin, and loricrin (Fuchs and Green, 1980; Moll et al., 1982; Cline and Rice, 1983; Watt, 1983; Hohl, 1993; Ekanayake-Mudiyanselage et al., 1998). Basal keratin K5, known to be extended into the suprabasal layers in atopic dermatitis and dry skin (Engelke et al., 1997; Jensen et al., 2004), almost entirely disappeared in the basal, lower, and mid-spinous layers in tinea corporis. Moderate staining appeared only in the upper spinous and the granular layers. Staining for the second basal keratin K14 was no longer restricted to the basal and lower spinous layers as in normal skin, but was equally distributed with moderate staining intensity in the entire nucleated epidermis. The expression of suprabasal/differentiation-related K10 was significantly reduced. Staining for K10 in tinea was only found in the granular and upper spinous layers and was focally disrupted. The reduction in K10 staining was more pronounced in tinea than atopic dermatitis, showing severely impaired differentiation. Interestingly, filaggrin and involucrin were not significantly changed in pityriasis versicolor, another mycotic infection (Fang, 1994), where epidermal changes and scaling are much less clinically apparent than in tinea corporis.

Involucrin staining was reduced in tinea corporis compared with normal skin. Although the stained area broadened in diseased skin, the intensity of the staining was significantly reduced. Similar findings have been described in atopic lesional skin (Jensen et al., 2004). Involucrin serves as a substrate for the covalent attachment of ceramides to the cornified envelope (Wertz et al., 1989; Marekov and Steinert, 1998). Very long chain length ceramides are covalently bound to cornified envelopes of the corneocytes to form the backbone for the attachment of free ceramides, cholesterol, and fatty acids. A reduction in involucrin may result in reduced ceramide binding, which consequently reduces barrier function. In lesional skin of atopic dermatitis, together with a reduction of involucrin, the amount of protein-bound ω-hydroxyceramides was significantly reduced (Macheleidt et al., 2002; Jensen et al., 2004). Loricrin staining was confined to the granular layers in tinea corporis and in normal skin. The stained band also broadened in tinea because of broadening of the granular layer; however, staining intensity was reduced and even focally absent. In contrast, we found increased loricrin expression in atopic lesional skin, showing that there are differences in the differentiation process between eczema and tinea. The genes for filaggrin, involucrin, and loricrin are localized on the same gene *1q21*, also known as the epidermal differentiation complex (Yoneda et al., 1992). Therefore, coordinated regulation of the three cornified envelope proteins is most likely. Hohl (1993) described overexpression of loricrin and involucrin in orthokeratotic acanthosis, such as lichen planus. In unspecified dermatitis, however, he found reduced loricrin expression (Hohl, 1993). The reason for the differences in loricrin content between tinea, atopic dermatitis, and other skin diseases remains unknown.

Filaggrin is an S100 protein and a marker of epidermal differentiation. It has been suggested that filaggrin degradation products are most important for water binding (Scott and Harding, 1986). We recently described reduced filaggrin content in atopic dermatitis and related these changes to the well-known dry skin associated with that disease (Jensen et al., 2004). Very recently, mutations in the filaggrin gene were found in up to 50% of atopic dermatitis patients of European heritage (Palmer et al., 2006; Ruether et al., 2006). In tinea corporis, we also found focally broadened staining for filaggrin but reduced staining intensity and broad gaps showing reduced content. This correlates with a significant reduction of skin hydration values as we found here. Skin hydration was reduced to about the same values as in lesional atopic dermatitis (Jensen et al., 2004).

In this study, we found strong staining intensity of the antimicrobial protein hBD-2 in the spinous and granular layers of tinea corporis. Similarly, the expression of hBD-2 in candidal leukoplakia (Abiko et al., 2002; Meyer et al., 2004; Schneider et al., 2005) and in *Malassezia furfur* infection has been described (Donnarumma et al., 2004). However, using double-staining technique, we showed that expression appeared to be most pronounced in areas with only scattered fungal elements. 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 was recently found that hyphae invasion of *Candida albicans* inhibits the expression of human β-defensins in experimental oral candidiasis (Lu et al., 2006). We showed here that natural and the 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. *T. mentagrophytis* has been shown to induce tumor necrosis factor and IL-8 production in keratinocytes (Nakamura et al., 2002). Tumor necrosis factor and IL-1, in particular, are potent inducers of hBD-2 expression (Harder et al., 2000), showing that inflammation induced by the pathogen causes the increase in hBD-2 expression in tinea corporis (Miyata et al., 1996; Ohta et al., 1998). However, keratinocytes may express hBD-2 as a protective response against bacterial superinfection, because tinea can be colonized by bacteria (Pomeranz and Fairley, 1994; Masri-Fridling, 1996). hBD-2's antibacterial activity is already well known (Schröder and Harder, 2006). Thus, increased hBD-2 expression may be an attempt to strengthen antibacterial activity and substitute for the impaired physical barrier in tinea. In conclusion, we 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.

MATERIALS AND METHODS
Patients
Skin scrapings from 232 patients with tinea corporis were examined by KOH preparation and the fungal pathogens were cultured and identified by standard methods (Meinhof, 1990; Campbell et al.,1996; Kane et al., 1997). Skin biopsies for immunohistochemistry and proliferation assays were obtained from tinea corporis-infected and nonlesional skin from the backs or thighs of a representative group of adult patients. Individuals showing an untreated, superficial dermatophytosis proven by fungal culture techniques were included in the study. The local ethical commission approved the study protocol, prepared in accordance with the Declaration of Helsinki Principles, and all patients fully consented to participate in the study.

TEWL and stratum corneum hydration
We determined TEWL and stratum corneum conductance by biophysical methods to examine whether our patients exhibited changes in barrier function and stratum corneum hydration (Pinnagoda et al., 1990; Hashimoto-Kumasaka et al., 1993). Skin hydration and TEWL were measured noninvasively with a Tewameter TM210 and a Corneometer (both Courage & Khazaka, Cologne, Germany) in lesional and healthy skin as intraindividual control (Jensen et al., 2004).

Immunohistochemistry staining
Punch biopsies were fixed in formalin and embedded in paraffin. Hematoxylin and eosin and PAS stains were performed to confirm tinea infection. After deparaffination and rehydration, 5-μm-thick sections were incubated with 3% H_2O_2 for 5 minutes to block endogenous peroxidase activity and the rinsed sections were exposed to 5×5 minutes microwave irradiation (650 W) for antigen detection (Hazelbag et al., 1995). After blocking non-specific antibody binding by incubation with 20% normal pig serum (DAKO, Hamburg, Germany), the primary antibodies (anti-involucrin, anti-loricrin, anti-filaggrin, anti-hBD-2, or anti-keratin 5, 6, 10, 16, and 17) were applied (Rosenthal et al., 1992; Hohl, 1993). A goat anti-hBD-2 antibody was purchased from PeproTech (Rocky Hill, NJ). A strep–AB complex/HRP (DAKO, Hamburg, Germany) was used as a third antibody, followed by incubation with diaminobenzidine as peroxidase substrate. Some representative slides were subjected to double staining for fungi PAS and hBD-2 (immunostaining) to investigate colocalization.

Epidermal proliferation assay
As described for the previously mentioned histological slides, slides were generated and incubated with Ki-67 antibodies. The stained sections (one per slide) were examined microscopically ($160 \times$) by counting the labeled nuclei of interfollicular keratinocytes in seven microscopic fields per section. Labeling index in samples of patients with superficial dermatophytosis was defined by numbers of stained cells per millimeter epidermal basal membrane.

Turbidimetric assay
Turbidimetric antifungal susceptibility assay was performed analogously to Meletiadis et al. (2003). *T. rubrum* microconidia suspension was adjusted to two times 10^5 conidia/ml in 10 mM phosphate buffer (pH 7.3). Natural hBD-2 (n-hBD-2) extracted from psoriasis scales (Harder et al., 1997) and recombinant hBD-2 (r-hBD-2) (Sahly et al., 2006) were used in two dilutions, 25 and 100 μg/ml in 0.01% acetic acid. Phosphate buffer, 0.01% acetic acid, and 200 μg/ml fluconazole in phosphate buffer served as negative and positive control. Fifty microliters of conidia suspension were incubated with 50 μl dissolved agent (resulting in a concentration of hBD-2 of 12.5 and 50 μg/ml) or control for 2 hours at room temperature with continuous shaking in the wells of microtiter plates. Then, 100 μl Sabouraud broth was added to each well. In addition, to one well with conidia suspension, only 100 μl phosphate buffer was added. Photometric measurements (wavelength 620 nm) to determine turbidimetric growth curves were made at the start point (day 0) and at days 1, 2, and 3. Each test was carried out in duplicate.

Chemical fixation and ultramicrotomy for transmission electron microscopy
Skin samples were prefixed overnight in modified Karnovsky's medium at 4°C, washed twice with 0.2 M sodium cacodylate buffer for 10 minutes, and postfixed with 1% (wt/vol) OsO_4 or RuO_4 in 0.133 M sodium cacodylate buffer containing 0.5% (wt/vol) $K_4Fe(CN)_6$ at 4°C for 45 minutes (Elias and Friend, 1975). Specimens were dehydrated in ethanol and embedded in Epon 812 (Luft, 1961). Polymerization was carried out overnight at 60°C. Thin sections were cut with a Reichert Jung Ultracut E microtome with a diamond knife (Diatome, Biel, Switzerland), counterstained with uranyl acetate and lead citrate and examined in a CM 10 (Fei, Eindhoven, The Netherlands) (Reynolds, 1963). OsO_4 fixation was used to show lamellar body extrusion and RuO_4 was used to show stratum corneum lipid bilayers.

Statistical analysis

Statistical significance was determined using two-tailed Student's *t*-test. Each sample was measured in duplicate and the results are presented as the mean $\pm$ SEM.

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