Infection stages of the dermatophyte pathogen *Trichophyton*: microscopic characterization and proteolytic enzymes

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Dermatophytes are pathogenic fungi that infect human skin, nails and hair and cause dermatophytosis. *Trichophyton mentagrophytes* is one of the most widespread species that belong to this group. Infection of the skin tissues include several stages, i.e., adhesion to the surface of the skin, invasion into the sublayers by the penetration of fungal elements and secretion of enzymes that degrade the skin components. In this study we have followed the morphology of the fungal elements, such as arthroconidia and hyphae, during the adhesion and invasion stages. Skin explants were inoculated with the dermatophyte and observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Skin explants were also inoculated with a transgenic isolate of *T. mentagrophytes* expressing the green fluorescent protein (GFP). The infected sublayers were investigated by confocal scanning laser microscopy (CSLM). As an adaptation to the tissue environment, the dermatophyte produced long fibrils when it is on the open surface of the stratum corneum, while short and thin fibrils are produced inside the dense sublayers. The short and long projections might have a role in adhesion. Invasion may be produced by mechanical and biochemical means. Invasion of the tissue showed hyphal branching and growth in multiple directions. The proteolytic profile was assayed by substrate gel and proteolytic activity. Two serine proteases of similar molecular weight were secreted during growth on the epidermal matrix components keratin and elastin. The dermatophyte may use the proteolytic enzymes to invade the surface and also the deep layer of the skin in immunocompromised patients. Dermatophytes, which are well adapted infectious agents, seem to use their mechanical and biochemical capabilities to invade the skin tissue effectively.

**Keywords** *Trichophyton*, infection, proteases, microscopy

**Introduction**

Skin infections due to dermatophytes are distributed worldwide. Dermatophytes invade the stratum corneum or keratinized structures derived from the epidermis, causing skin lesions, as well as hair and nail infections [1–4]. Dermatophytic fungi have been shown to have keratinolytic and other proteolytic and lipolytic activities [5,6]. Serine proteases which are involved in extracellular protein catabolism have been found to be produced by dermatophytes and their release has been suggested to play a major role in the invasion of the skin [7]. The hydrolysis of keratin by proteinases is an important aspect of fungal pathogenesis, providing a source of nutrition on the outer skin layer, which normally should be a barrier to pathogens.
Some studies concerning the infectivity and pathogenicity of dermatophytes used animal models for investigating these processes [8]. Other researchers have used non-invasive methods, obtaining sheets of stratum corneum stripped by adhesive tapes [9] or obtaining separate corneocyte cells to study dermatophyte infections [10]. Recently, a novel ex vivo model for the study of dermatophytes adherence to and invasion of the stratum corneum was developed by our laboratory [1]. The model included all the skin tissue components. Dermatophytes are not part of the normal skin flora, thus initial contact between the fungal element and the stratum corneum layer is an important event in the establishment and initiation of the infection of the stratum corneum [11]. Duek et al. [1] found that adherence between the spores and the stratum corneum took place after 12 h and by 24 h, germination had commenced to be followed three days later, by the penetration of mycelium through the stratum corneum layer.

In this study, the host-parasite relationship was studied by: (i) following the morphology of the infecting fungal elements during adhesion to the surface and the sublayers of the skin tissue by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively, (ii) following with a transgenic Trichophyton mentagrophytes isolate that expresses the green fluorescent protein (GFP) [12], the morphology of its infecting elements and the invasion path during penetration of the skin sublayers under scanning confocal laser microscopy (SCLM), and (iii) the characterization of the proteolytic profile of the secreted proteases from T. mentagrophytes grown on the extracellular matrix proteins keratin and elastin as sole substrates.

Materials and methods

Strain and culture conditions

A clinical isolate of T. mentagrophytes var. mentagrophytes was obtained from Rambam Hospital, Haifa and maintained at 30°C on Sabouraud dextrose agar (SDA, Difco) containing 0.05 mg/l chloramphenicol. Fungal microconidia were isolated as described previously [1] and incubated on mineral medium broth (MM) [12], in presence of 0.5% keratin (Sigma) or 0.25% elastin (Sigma) at 30°C for 14 days with shaking at 200 rpm. Arthroconidia were prepared as described previously [13]. In brief, arthroconidia were incubated on SDA in an atmosphere of 10% CO₂, 90% air at 30°C. The cells were harvested by the same procedure as microconidia [1] and diluted with phosphate buffer saline (PBS) to obtain a pure arthroconidial suspension. The arthroconidia were used to inoculate human skin explants which had been obtained as a by-product from plastic surgery and maintained in sterilized skin graft fluid (SGF) [1], in order to follow the morphology of the fungal elements while adhering to and invading the skin tissue.

Skin inoculation

The arthroconidia suspension was spread equally over the surface of each skin piece, as previously described [1]. The samples were incubated for 6, 12, and 24 h (short times) and 7 or 14 days (long times) at 30°C. The medium (SGF) was changed daily. In addition, the human skin explants were incubated with a transgenic isolate of T. mentagrophytes that expresses GFP [12] and incubated for 21 days under the same conditions as mentioned above, to follow the invasion and the structure of the pathogenic fungi in the skin tissue.

For SEM and TEM observations, the same inoculation procedures were performed but with the wild type dermatophyte.

Electron and confocal microscopy

For SEM and TEM studies, the specimens were prepared as previously described [1]. In brief, skin specimens were fixed overnight in 2% glutaraldehyde, post fixed with 1% OsO₄ for 2 h and dehydrated in ascending concentrations of alcohol. For TEM observations the samples were embedded, after fixation in Epon for 48 h at 56°C, cut into thin sections, placed on copper grids and stained with uranyl acetate and lead citrate.

The samples were viewed with a Leo 982 digital scanning electron microscope at an acceleration of 6 kV and a JOEL JEM SX transmission electron microscope. The invasion of the GFP-expressing line into the skin tissue explants was visualized by Nikon E600 fluorescence microscope and radiance 2000 confocal laser scanning microscope as described previously [12]. Samples of infected skin were scanned at a depth of 12 layers with intervals of 0.82 μm. The images were analyzed by Image pro plus version 5.0.2.9 software. Non infected and infected skin explants with wild type T. mentagrophytes, served as controls.

Purification of the protease filtrate and proteolytic assays

The culture medium was separated from keratin or elastin remnants by filtration through a 10,000 Da molecular cutoff 0.22 μm filter (Millipore) and concentrated by filtration using Centriprep with a
molecular cutoff 10,000 Da (Millipore). The protein concentration was determined using the Bradford method [14]. Standard assay of proteolytic activity was carried out with azocoll (Sigma) as substrate in 100 mM Tris-HCl [pH 7.5] and 1 mM CaCl2. Aliquots of 50 μl were incubated with 3 mg of substrate at 37°C for 2 h under continuous shaking. The degradation of the substrate was estimated by measuring the absorbance at 520 nm after centrifugation. One unit of azocollitic activity releases 0.1 A520 units min⁻¹ under the assay conditions.

Keratinolytic activity was measured using keratin azure (Sigma). Samples of 100 μl were incubated with keratin azure (4 mg) at 37°C for 24 h in the same Tris buffer as mentioned above. One unit of keratinolytic activity was determined to be the increase of 0.03 A595 units h⁻¹ under the assay conditions.

Elastin degradation was analyzed by using elastin congo red (Sigma) as the substrate. Samples of 100 μl were incubated with the substrate (5 mg) at 37°C for 2–3 h in Tris buffer as noted above. The reaction was stopped by removing the substratum through the use of centrifugation. A change of 0.9 A595 units h⁻¹ corresponded to 1 unit of elastolytic activity.

Substrate gel analysis

General protease activity was assayed in non-denaturing polyacrylamide gels copolymerized with substrate as described previously [15], but with a few modifications. The resolving gel (10% polyacrylamide) was polymerized in the presence of 8 mg/ml gelatin (Sigma). After electrophoresis, the gels were washed twice in renaturation buffer (TBSx1, 2.5% Triton 100) for 20 min each. The gels were then incubated for 24 h in zymography buffer (50 mM Trizma base [pH 8.0], 10 mM CaCl2, 0.02% Na-azide). They were then stained with solution containing 95% ethanol, 150 ml/l formaldehyde, 0.1% Coomassie brilliant blue R-250 for 30 min and destained in a solution of 5% methanol and 7.5% Acetic acid until the proteolytic bands could be visualized. In some experiments the samples were preincubated with protease inhibitors for the inhibition assay before electrophoresis, i.e., the inhibitors: PMSF 1 mM, AEBSF 2 mM, O-phenanthroline 1 mM and EDTA 5 mM were preincubated with enzyme for 20 min at ambient temperature.

Results

Alteration of T. mentagrophytes morphology during the adhesion to the stratum corneum surface and sublayers

Scanning electron microscopy observations of the infected skin tissue surface with microconidia [1] and arthroconidia revealed short fibrils stretching out from the fungal elements and connecting them to the skin tissue surface. Sometimes there was more than one strand [1] which produced a network of fibrils (Fig. 1A and B). In a dense area of arthroconidia, fibrils were seen to connect between adjacent arthroconidia (Fig. 1C). The arthroconidia elements were seen to have short bulges on the surface, and some of them started to expand after the first contact with the skin tissue surface. Transmission electron microscopy observations of infected sublayers of the stratum corneum showed
penetration and formation of arthroconidia. In the deeper layers, changes were observed on the surfaces of the fungal elements and thin appendices were detected in the entire areas surrounding the fungal element. These may possibly be used by the fungal element to adhere to the tissue layers (Fig. 2A and 2B). At the contact site of the fungal element, the thin fibrils began to vanish and the fungal element started to flatten near the contact area with the skin (Fig. 2C).

T. mentagrophytes morphology during the invasion into the deep layers of the stratum corneum

Observations by confocal scanning laser microscopy (CSLM) revealed that the elongated hyphae invade the deep layers of stratum corneum in no specific direction. The same infected skin explants were also observed by light microscopy. CSLM provided optical sectioning, revealing fluorescence from different hyphae and regions of the hyphae, as the plane of the section went deeper into the infected skin tissue sections. The orientation of the hyphae varied, showing that the elongation path is not restricted to a single plane or direction, but rather follows a meandering path. The mycelium also produces branching hyphae as it infects the deep layers (Fig. 3). No other fungal elements were observed.

Secretion of proteases in the presence of keratin and elastin

The secreted protein fractions were isolated from T. mentagrophytes cultures grown on keratin or elastin as the sole carbon and nitrogen sources. The fractions were analyzed by electrophoresis on activity gels and three discrete protease bands were detected on these gels (Fig. 4 – arrows). Two bands (1 and 3, Fig. 4) were induced by either keratin or elastin, and a third (band 2, Fig. 4) by keratin only. Proteases corresponding to bands 1 and 3 were inhibited by the serine protease inhibitors PMSF and AEBSF. Neither of them was inhibited by metalloprotease inhibitors such as O-phenanthroline and EDTA. The molecular masses of the two serine proteases were about 20 kDa and 80 kDa (Fig. 4).

Induction of similar specific proteolytic activity by keratin and elastin

The secreted protein fractions were assayed for total proteolytic activity indicated by azocoll degradation, as well as for specific proteolytic activities against keratin (keratinolytic activity) and elastin (elastinolytic activity). The activities were measured by using a specific dye releasing substratum. Both keratinolytic and elastolytic activities were induced by growth on either keratin (Fig. 5A) or elastin (Fig. 5B) as the sole carbon and nitrogen substrates. The keratinolytic activity levels of the secreted fractions were similar on either keratin or elastin as sole sources of carbon and nitrogen. Elastolytic activity levels were also similar on both substrates; the general proteolytic activity was measured by azocoll degradation which was used as a control.
Discussion

Adherence of microorganisms to host tissues is an important step in the establishment of disease. *Candida albicans* has been shown to adhere to mucosal cells and corneocytes [16]. Unlike candidosis, dermatophytosis is acquired from an exogenous source as they are not part of the normal flora. Thus, initial contact between arthroconidia and stratum corneum is an important event in the initiation and establishment of the infection of the stratum corneum [12]. At the surface of the stratum corneum, adhesion occurred by the establishment of short and long fibrils (Fig. 1), which appear to anchor and connect the arthroconidia to the tissue surface and prevent the disconnection of the exposed fungal element under the rough conditions on the skin surface. The use of fibrils for adhesion was also observed in other pathogenic fungi such as *C. albicans* that infects corneocytes; the fibrils are called adhesins [16]. However, the infection of the inner layer was not the same as on the surface and instead of long fibrils, short and thin fibrils covering the entire surface of the tissue were observed (Fig. 2).

**Fig. 3** Images of skin tissue sections infected by the *Trichophyton mentagrophytes* mycelium. (A) Light microscopy observations of infected (A) and non-infected (B) skin tissues, the arrow points to the spreading mycelium (×500). (B) Confocal images of human skin explants infected with transformant expressing the green fluorescent protein (GFP). The arrows follow invasion of the hyphae into the stratum corneum. Successive frames are shown at increasing depth.

**Fig. 4** Activity gel assay of secreted proteases filtered from cultures of *Trichophyton mentagrophytes* on keratin (A) or elastin (B) as the sole carbon and nitrogen source. The proteases were incubated with the indicated protease inhibitors before the assay. U, untreated fraction; serine protease inhibitors; PM- PMSF, AE-AEBSF, metalloprotease inhibitors; ED-EDTA, PH, O-phenanthroline; MR, size marker.

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fungal element were observed (Fig. 2). The fungal elements were attached to the stratum corneum sublayers by these fibrils, and after adhesion these thin layers started to vanish at the contact side. The internal tissue layers are very dense and the fungal elements may need different adherence strategies in these areas as compared to the open surface of the stratum corneum. Another property of adhesion in the inner layers is that the fungal element becomes flattened (Fig. 2). We believe that this conversion serves to increase the contact site with the tissue sublayers. The surfaces of the arthroconidia formed on the skin layers were not smooth and contained bulges. The latter seem to be a kind of contact structure and began to expand as the arthroconidia adhered to the surface. These fibrils were not seen on non-adhering arthroconidia (data not shown). Surface carbohydrate-specific adhesins on microconidial surfaces are proposed to play an important role in the adhesion and invasion of the fungus during the infectious process of dermatophytosis [17]. The *C. albicans* adhesins are also thought to be receptors which play a role in the ligand-host cell receptor interactions [18,19]. Although *T. mentagrophytes* and *C. albicans* belong to different families and their infection mechanisms are not identical, there seems to be a lot in common in their adherence methods.

In areas where the density of the arthroconidia is high, some fibrils were observed to connect with adjacent arthroconidia (Fig. 1). This connection may imply the building of a strong and stable arthroconidia complex on the skin tissue and might have biofilm-like properties. There is also a possibility that these fibrils may be a type of interaction which enables the adjacent arthroconidia cells to communicate.

Invasion of the fungal pathogen into the skin tissue was followed by fluorescence in the GFP-expressing line. CSLM provided multidimensional images of the spreading of *T. mentagrophytes* into the skin. The meandering paths of the hyphae, rather than progressing in the same plane or along a straight line, might aid their penetration into the deep tissue (Fig. 3).
kind of invasion was also seen in fungal pathogens of plants invading the dense tissue [20,21]. No fungal elements other than hyphae were seen, and the hyphae produced a complex of branches. It is believed that in addition to the mechanical penetration of the fungal elements, proteolytic enzymes degrade the skin tissue components. Incubating the dermatophyte with extracellular matrix proteins such as keratin, one of the major proteins of the epidermal layer and elastin, which is one of the important components of the dermis layer, revealed that both proteins, although belonging to different skin areas, may induce similar proteases (Fig. 4). Each one of the components triggers a similar specific proteolytic activity against keratin (keratinolytic activity) or elastin (elastinolytic activity) (Fig. 5). Thus, it seems that dermatophytes may produce and secrete proteases in response to epidermal extracellular matrix components such as keratin, during their invasion into the epidermal layer. These induced proteases may contribute to the potential of the dermatophytes to degrade components of deeper layers, such as elastin in the dermis, in immunocompromised or immunosuppressed patients [22]. Published results on the induction of the Trm4 protein on keratin and elastin as a sole substrates [23] support these conclusions. Upregulation and secretion of proteases of T. mentagrophytes may contribute to the potential of dermatophytes to invade into the host deep tissues. The molecular basis of the interaction between pathogenic fungi and their hosts is, in general, still obscure. Based on the data reported here, we propose a working hypothesis in which constitutive enzymes act on the skin tissue to release inducers, which in turn reprogram the transcriptional and protein secretion patterns of the pathogen (Fig. 6).

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References