Expression and potential function of cathelicidin antimicrobial peptides in dermatophytosis and tinea versicolor

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Objectives: This study was designed to characterize the role of the human cathelicidin LL-37 in fungal skin infections such as dermatophytosis and tinea versicolor.

Methods: The in vitro antimicrobial activity of synthetic antimicrobial peptides including the human cathelicidin LL-37 against Malassezia furfur and several dermatophytes was determined. Immunostaining was performed to determine expression of cathelicidin in skin biopsies from patients with tinea pedis, tinea corporis and tinea versicolor. Cathelicidin peptide expression was evaluated by western blotting and mRNA expression was studied in keratinocytes exposed to M. furfur or Trichophyton rubrum.

Results: LL-37 inhibits the growth of fungi with an MIC of 20–30 μM for M. furfur and 12.5 μM for Trichophyton mentagrophytes and T. rubrum. LL-37 also shows fungicidal activity with a minimum fungicidal concentration (MFC) of 12.5 and 25 μM for T. mentagrophytes and T. rubrum, respectively. An increase in cathelicidin expression was observed in human skin tissue infected with fungi compared with healthy skin. Western blotting of skin scrapings demonstrated that human cathelicidin is processed from its precursor into an active peptide in both healthy and infected plantar skin.

Conclusions: These findings support a hypothesis that antimicrobial peptides such as cathelicidins can play a role in skin defence against dermatophytes and M. furfur.

Keywords: LL-37, CRAMP, antifungal peptides

Introduction

Cationic antimicrobial peptides play important roles in the immune defence systems of many organisms from insects to humans. In human skin, two main groups have been most extensively studied: β-defensins and cathelicidins. In particular, mammalian skin and other epithelial surfaces are major producers of, and are influenced by, the expression of functional cathelicidins. The unique member of the cathelicidin gene family present in humans, is induced in keratinocytes during inflammatory skin disorders such as psoriasis. Recently, it has been demonstrated that cathelicidins are produced by the eccrine apparatus and secreted into human sweat. After secretion onto the skin surface, cathelicidin is processed by a serine protease-dependent mechanism into multiple antimicrobial peptides, such as RK-31 or KS-30, that are distinct from the best known human cathelicidin peptide, LL-37, found in neutrophils. These smaller forms of cathelicidins are more potent antimicrobials than LL-37.

The location and regulation of expression of cationic peptides such as cathelicidins has suggested that they are important as a first line of immune defence against skin pathogens. Most work with cathelicidins has focused on their ability to kill bacterial pathogens. For example, the mouse cathelicidin nCRAMP has been shown to have an important role in defence against invasive bacterial infection by Group A streptococci. Furthermore, cathelicidin expression has been associated with resistance to infection by vaccinia virus. Less information is available on the role antimicrobial peptides may play in fungal infections. To date, no information is available on the role of cationic peptides such as cathelicidins in defence against cutaneous dermatophyte infections or infection by Malassezia furfur.

The production of antimicrobial molecules by epithelia as well as granulocytes places them in a unique position to defend against typically non-invasive organisms such as dermatophytes. Trichophyton, Epidermphyton and Microsporum species are the causative agents of dermatophytosis. These keratinophilic...
filamentous fungi parasitize the keratinized tissues of the epidermis, hair and nails, often with minimal inflammatory response.11 Despite their common occurrence and recurrence in individuals with apparently normal immune systems, the defect or defects in these patients that render them susceptible to infection are unknown.

A distinct fungal infection of skin, tinea versicolor, has similarities to the dermatophytes in terms of the superficial nature of the colonization and the tendency to select specific individuals for recurrent infection. Also known as pityriasis versicolor, the distinctive clinical manifestations of discoloured lesions on the chest, trunk, face or abdomen typically occur without triggering a host inflammatory response. In this disorder the causative organism is *M. furfur*, part of the normal human cutaneous flora and implicated in the pathogenesis of several cutaneous diseases (pityriasis versicolor, seborrhoeic dermatitis, *Malassezia* folliculitis and atopic dermatitis).12–14 These yeasts are dimorphic, existing in both yeast and mycelial phases. In pityriasis versicolor the yeast changes from the blastospore form to the mycelial form. Systems that seem to impart susceptibility in some individuals are unknown.

The purpose of the present study was to assess the response and antifungal activity of the cathelicidins against skin pathogens such as dermatophytes and *M. furfur*. Our observations suggest that antimicrobial peptides such as cathelicidins may play a role in the normal defence of the skin against these organisms.

**Materials and methods**

**Synthesis of peptides**

Synthetic peptides were prepared by Synpep Corporation (Dublin, CA), purified through a HPLC column and their identity confirmed by mass spectrometry. Peptide stocks were dissolved in sterile double-distilled water at a 320 μM concentration, lyophilized and kept at –80°C until use.

**Microbial strains and isolates**

The yeast *M. furfur* ATCC 46267 and several dermatophytes including *Arthrodema oate* CECT 2797 (teleomorph of *Microsporum canis*), *Trichophyton mentagrophytes* var. goetzii CECT 2957, *T. mentagrophytes* var. interdigitale CECT 2958, *Trichophyton rubrum* CECT 2794 and a clinical isolate of the fungus *T. rubrum* were used in this study. *M. furfur* was cultured in modified Dixon (mDixon) medium prepared in the laboratory from 4% malt extract (Fluka Biochemika, Steinheim, Germany), 0.6% Bacto Peptone (Becton, Dickinson and Company, MD, USA), 1% glucose (Sigma-Aldrich Co., St Louis, MO, USA) and 1% Tween 80 (Sigma-Aldrich Co.) at 37°C with vigorous shaking for 1–2 days. A suspension of 1–5 × 10⁶ cfu/mL was prepared in 1 mM sodium phosphate buffer pH 7.0 (NaPB) and used in the antifungal assays as a 10× stock solution. The dermatophytes were cultured on potato dextrose agar (PDA; Difco, Detroit, USA) plates for 10–14 days at 25°C. Conidia were collected from the agar and transferred to sterile double-distilled water. After filtration the suspension was titrated with a haemocytometer, adjusted to a concentration of 1–3 × 10⁵ conidia/mL in 1 mM NaPB and used in the antifungal assays as a 10× stock solution.

**In vitro antimicrobial activity assays**

Microorganisms were grown *in vitro* in sterile 96-well microtitre plates (Corning Inc., Corning, NY, USA) in a final volume of 100 μL. The assay mixture contained 10 μL of a 10× suspension of each microorganism (final concentration 1–5 × 10⁸), 20 μL of culture medium [mDixon or potato dextrose broth (PDB) for yeast or fungi, respectively], 10 μL of a 10× stock solution of each synthetic peptide and 60 μL of 1 mM NaPB. Each sample also contained 16 μg/mL chloramphenicol per well to avoid bacterial contamination.

For *M. furfur*, the microtitre plates were incubated at 30°C for 1 day. Then aliquots of each pathogen/peptide combination were plated in agar plates and incubated at 37°C for 3 days in order to count the colonies. The colonies of each sample incubated with peptide were compared with the colonies of sample without peptide and with the initial number of colonies that we used in the experiment. The MIC was defined as the minimal concentration to produce at least 95% inhibition of fungus growth and the minimum fungicidal concentration (MFC) was defined as the minimal concentration to produce at least 99% killing.

For the dermatophytes, microtitre plates were incubated at 25°C for 5 days and the MIC was defined as the lowest peptide concentration that prevented visual growth at the end of the experiment. After 3 days of incubation, aliquots of each pathogen/peptide combination were plated in agar plates and incubated at 25°C for 5 days in order to determine the MFC as the lowest peptide concentration at which no growth was seen in PDA plates.

**Tissue sampling and immunohistochemistry**

All human tissues were collected with the approval of the UCSD Human Research Protections Program. We used archival tissue paraffin sections from five different adult patients (two tinea corporis, two tinea versicolor and one unknown tinea), Clinical data were unremarkable in all patients with the exception of one patient with tinea corporis who was HIV+. Also a fresh-frozen section from biopsy of one patient with tinea pedis was immediately embedded in optimal cutting temperature compound (Sakura Finetechical Co., Tokyo, Japan), frozen into liquid nitrogen and kept at –80°C until use.

For paraffin sections, endogenous peroxidase activity was blocked with 30 min incubation in 0.3% H₂O₂, blocked with 2% goat serum in phosphate-buffered saline (PBS) for 30–60 min at room temperature and incubated with rabbit anti-LL-37 polyclonal antibody [1 : 300 in PBS containing 3% BSA (Sigma, St Louis, MO, USA)] for 2 h at room temperature. As a negative control, the polyclonal antibody was replaced by normal rabbit pre-immune IgG diluted with PBS containing 3% BSA at the same protein concentration as the primary antibody. The signal was detected with a Vectorstain ABC Elite Rabbit kit (Vector Laboratories, Burlingame, CA, USA) and processed by means of an ABC kit (Vector Laboratories). Finally, sections were incubated in 0.02% diaminobenzidine with 0.05% H₂O₂ in PBS for 5 min and counterstained with Mayer’s haematoxylin for 30 s.

Fresh-frozen sections were cut at 10 μm and fixed with 10% buffered formalin for 15 min at room temperature and immersed in PBS for 10 min. For these sections, the staining protocol was identical but a dilution of 1 : 500 (instead of 1 : 300) of the primary antibody was used.

**Protein extraction, immunoprecipitation and western blotting**

Tina pedis infected and healthy skin scrapings were dissolved in 1 mL of 1% Triton X-100 in PBS buffer containing protease inhibitor (Complete Mini, EDTA-free, Roche, Mannheim, Germany), then vortexed, incubated for 1–2 h on ice, homogenized with a Power Gen 125 homogenizer (Fisher Scientific, Pittsburgh, PA, USA) and
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centrifuged for 10 min at 16,000 g. Samples were incubated with 10 μL of 1.25 mg/mL chicken anti-LL-37 polyclonal antibody for 4 h at 4°C. Then, 50 μL of protein G-agarose was added and samples were incubated overnight at 4°C. After centrifugation for 2 min at 4500 rpm at 4°C, the pellet was washed in PBS three times and prepared in 30 μL of SDS loading buffer containing a cocktail of protease inhibitors. All samples were heated for 10 min at 100°C and analysed by western blotting. Proteins were separated using 16% Tris/tricine peptide gels (GeneMate Express Gels, ISC BioExpress, Kaysville, UT, USA) and transferred to a PVDF membrane (0.45 μm) Immobilon-P (Millipore, Bedford, MA, USA) at 50 V for 3 h. For positive control, 80 ng of LL-37 synthetic peptide was applied. The membrane was treated with blocking solution [3% non-fat dry milk, 0.5% BSA, 0.87% NaCl and 0.3% Tween 20 in TSB] overnight at 4°C then detected with rabbit anti-LL-37 polyclonal antibody (1:5000 dilution of stock 0.73 mg/mL in blocking solution) for 15 min, the signal was detected using a goat anti-rabbit IgG conjugated with horseradish peroxidase (Dako A/S, Denmark) and developed using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA, USA).

Cell culture and treatment with fungi

Normal human keratinocytes (NHK) isolated from foreskin were cultured in EpiLife medium (serum-free keratinocyte medium; Cascade Biologics, Portland, OR, USA) supplemented with 0.06 mM calcium, EGDS (EpiLife defined growth supplement) and penicillin/streptomycin. Cell culture was maintained by adding fresh complete medium every 2–3 days and cell number was determined to be between 20 and 30% of 94°C and 12.5 μM for T. mentagrophytes and T. rubrum (Table 1). However, an inhibitory effect was not observed on the growth of

Reverse-transcription (RT) reaction

Total RNA from keratinocytes was isolated using a Qiagen RNEasy Mini Kit (Qiagen, Valencia, CA, USA) and retrotranscribed by reverse transcriptase using a Retroscript kit (Ambion, Austin, TX, USA). Briefly, first strand synthesis combined 2 μg of RNA with 0.4 mM dNTPs and 4 μM random decamers in 16 μL at 80°C for 10 min. The reaction was iec and 2 μL of 10× RT–PCR buffer, 1 μL of RNase inhibitor and 1 μL of mMLV RT added and incubated at 42°C for 1 h, then denatured at 92°C for 10 min.

Real-time PCR

Quantitative PCR was performed using an Applied Biosystems 7000 Sequence Detection System (Foster City, CA, USA). Primers were designed for the target genes LL-37, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. The sequences of the cathelicidin primers are F-CCAGGACGACAGCAGTCA and R-CTTCCACCAGCCGTCCCTTC (product size 59 bp). The sequences of the GAPDH primers are F-CTTAGACCCTCTGCCCAAG and R-TGGTCATGAGTCTCCACAG (product size 62 bp). An aliquot of 5 μL of RT reaction was added to 12.5 μL of TaqMan® PCR Master Mix (Applied Biosystems), 0.25 μL of 20 μM GAPDH primers, 0.5 μL of 2 μM GAPDHALF probe, 0.5 μL of 50 μM LL-37 primers, 1 μL of 5 μM LL-37-FAM probe and 4.75 μL of RNase/DNase free H2O. The thermal profile was 50°C for 2 min, 95°C for 10 min and 40 cycles of 94°C for 15 s then 60°C for 1 min. Results were analysed using the Comparative Ct Method (User Bulletin 82, Applied Biosystems).

Briefly, Ct values were normalized to GAPDH used as endogenous control and then normalized to a sample of non-treated keratinocytes chosen as a calibrator.

Results

In vitro antifungal activity of cathelicidins

The antimicrobial activity of the cathelicidin peptides LL-37, CRAMP and PR-39 was tested against M. furfur and compared with other antimicrobial peptides, such as magainin, cecropin P1 and dermcidin (Figure 1a). We observed a similar effect for all the cathelicidins tested with complete inhibition of growth at 25 μM. The comparison with the other antimicrobial peptides showed that magainin 2 had the highest antimicrobial activity against M. furfur. Cecropin P1 had moderate activity against the yeast at 25 μM. However, dermcidin, a peptide present in sweat, did not show activity against M. furfur.

Treatment with different concentrations of the peptides LL-37 (MW 4532) and CRAMP (MW 4332) resulted in delay or complete inhibition of the growth of M. furfur and the dermatophytes T. mentagrophytes and T. rubrum. The MIC of both peptides was determined to be between 20 and 30 μM for M. furfur (Figure 1b) and 12.5 μM for T. mentagrophytes and T. rubrum (Table 1).

Figure 1. In vitro antifungal activity of antimicrobial peptides against M. furfur in 20% mDixon medium (pH 5.5). (a) Yeast was incubated in the absence of peptide (black bar) or in the presence of 10 μM (white bars) and 25 μM (striped bars) LL-37, mCRAMP, magainin 2, cecropin P1, PR-39 and dermcidin. After 24 h, yeast was plated onto mDixon agar plates to count cells. Data represent the mean ± SD of triplicate determinations from one experiment. (b) Yeast was incubated at different concentrations of LL-37 (diamonds) and mCRAMP (squares) and after 24 h, yeast was plated onto mDixon agar plates to count cells. Percentage of growth was calculated as (cfu/mL of sample with peptide)/cfu/mL of sample without peptide) × 100. Data represent the mean ± SD from at least two experiments and triplicate determinations from each experiment.
A. otae at the highest concentration of LL-37 tested (i.e. 100 μM) (Table 1). A slight fungicidal effect of cathelicidins against M. furfur was observed, and CRAMP was more fungicidal than LL-37 (the percentage of cell surviving was 73.8 – 7.1 or 3.3 – 0.6 in the presence of 25 μM LL-37 or CRAMP, respectively). In the case of the dermatophytes, we defined the MFC value as the minimal concentration that prevented visible growth of the fungus, and this was determined to be 12.5 μM for T. mentagrophytes var. goetii CECT 2957, 12.5 μM for T. mentagrophytes var. interdigitale CECT 2958, 12.5 μM for T. rubrum CECT 2794 and 25 μM for T. rubrum clinical isolate (Figure 2 and Table 1).

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<th>Fungi</th>
<th>MIC (μM)</th>
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<tr>
<td>Arthroderma otae CECT 2797</td>
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<td>Trichophyton mentagrophytes var. goetii CECT 2957</td>
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Figure 2. In vitro fungicidal activity of LL-37 against dermatophytes. A. otae CECT 2797 (a), T. mentagrophytes CECT 2957 (b), T. mentagrophytes CECT 2958 (c) and T. rubrum CECT 2794 (d) were incubated in the absence of peptide (control) or in the presence of 6, 12 or 25 μM LL-37 in 20% PDB. After 3 days of incubation, fungi were grown in PDA plates for 5 days.

A. otae at the highest concentration of LL-37 tested (i.e. 100 μM) (Table 1). A slight fungicidal effect of cathelicidins against M. furfur was observed, and CRAMP was more fungicidal than LL-37 (the percentage of cell surviving was 73.8 ± 7.1 or 3.3 ± 0.6 in the presence of 25 μM LL-37 or CRAMP, respectively). In the case of the dermatophytes, we defined the MFC value as the minimal concentration that prevented visible growth of the fungus, and this was determined to be 12.5 μM for T. mentagrophytes and 25 μM for T. rubrum (Figure 2 and Table 1).

Cathelicidin expression and localization in human tinea-infected skin

To further explore cathelicidin expression and localization, biopsies of tinea pedis were examined for cathelicidin protein expression by immunohistochemistry. Abundant cathelicidin was detected in epidermis with a strong staining found in the superficial layer and in the stratum corneum (Figure 3a). To study the differential expression between healthy and infected skin, immunostaining of paraffin sections was carried out. As seen in Figure 3(b), there was an increase in cathelicidin protein expression in epidermis of skin from patients with tinea corporis.
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Figure 4. (a) LL-37 detection by immunoprecipitation and western blotting using LL-37 antibody. Immunoprecipitated fractions from normal (lane 1) and tinea-infected skin (lane 2) were analysed by western blotting. LL-37 synthetic peptide (80 ng) was used as a positive control for the western blotting (lane 3). The arrow indicates that the mature form of the peptide is present in extracts. The asterisks show immunoglobulin band staining from immunoprecipitation and are unrelated to sample. The two bands in lane 3 are due to aggregation of the synthetic peptide control. (b) LL-37 mRNA is induced in normal human keratinocytes (NHK) infected with fungi. NHK in culture were infected with M. furfur and T. rubrum and the relative expression of LL-37 mRNA was quantified by real-time PCR. Data were normalized to GAPDH and compared with samples from untreated keratinocytes. The relative expressions of mRNA LL-37 in NHK infected with both fungi are statistically different at the 99% confidence interval (Student’s t-test).

(panel iii) or tinea versicolor (panel v), compared with healthy skin (panel i) (see asterisks in Figure 3b, panels iii and v). Moreover, cathelicidin expression co-localized with the fungus in the stratum corneum (see arrow in Figure 3b, panel iii).

Cathelicidin is processed to peptide form in human skin

Cathelicidin is synthesized as an inactive precursor protein known as hCAP18 and processed upon neutrophil granule fusion into an active peptide form LL-37. Normal adult skin from the trunk and extremities expresses minimal cathelicidin. To study the expression of the peptide form of cathelicidin in a site previously studied, and compare this with the response after dermatophyte infection, total protein from healthy and infected plantar skin was extracted and quantified using BCA assay. Equal volumes of the samples were immunoprecipitated from each extract using a LL-37 polyclonal antibody and the samples were analysed by western blotting (Figure 4a). Synthetic peptide LL-37 was used as a positive control. The synthetic peptide LL-37 appeared as two bands owing to the aggregation commonly seen with these charged amphipathic peptides. A band was present in healthy and infected skin after immunoprecipitation, showing the presence of processed cathelicidin in both healthy and infected skin of the sole.

Differential expression of mRNA cathelicidin in human keratinocytes incubated with T. rubrum and M. furfur

To determine whether fungal infection induced an increase in cathelicidin mRNA expression, human keratinocytes were treated for 24 h with M. furfur or for 40 h with T. rubrum and at a fungal cell/human keratinocyte ratio of 10:1. Total RNA was extracted immediately after the treatments and was quantitatively analysed by RT–PCR. Figure 4(b) shows that cathelicidin mRNA expression is induced to a small extent in human keratinocytes after infection with M. furfur and T. rubrum.

Discussion

This investigation demonstrated the activity of human and mouse cathelicidin peptides against important skin pathogens, M. furfur, T. mentagrophytes and T. rubrum. The form of these peptides found in human neutrophils (LL-37) or mouse granulocytes (CRAMP) inhibited the growth of these fungi with MIC values of 20–30 µM for M. furfur and 12.5 µM for Trichophyton spp. Surprisingly, the peptide dermcidin present in sweat did not show activity against the yeast M. furfur. Previous studies reported from our group have also shown that the synthetic peptide form of dermcidin is not active against other microorganisms, such as Candida albicans or Staphylococcus aureus.

Cathelicidins have been shown to be essential for defence against invasive bacterial infection by group A streptococci and vaccinia virus. Recently, the possible role of this family of peptides in candida skin infection has been addressed. In that study, mice deficient in cathelicidin were no more susceptible to infection by subcutaneous injection of C. albicans than control mice. This finding suggested an alternative role for cathelicidins in fungal infection compared with that found with group A streptococci. That is, the antifungal effect of cathelicidins is more likely to occur at the skin surface than in deep fungal infections where other immune effector mechanisms, such as the neutrophil oxidative burst, play a major role. The findings in the present study show that cathelicidins and other cationic antimicrobial peptides are active against M. furfur, T. mentagrophytes and T. rubrum, organisms that typically colonize only superficially on the skin, and provide additional evidence that the role of these peptides may be as a superficial barrier against pathogen proliferation.

While cathelicidins 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. In this study, the immunohistochemical experiment suggests that a small increase in cathelicidin expression occurs in skin infected by fungi. Analysis of mRNA in human keratinocyte cultures after exposure to M. furfur and T. rubrum was consistent with these observations of an increase in cathelicidin expression. Importantly, protein extracts analysed by western blotting from the unique epithelia of the plantar surface show that cathelicidin is normally present and is processed to an active peptide form. These observations suggest that the cathelicidins are involved in the normal epidermal response to
infection by these organisms, increasing when challenged to provide a more effective shield.

In summary, the current observations support the concept that cutaneous fungal infections by a wide variety of organisms may be influenced by the expression of antimicrobial peptides. The consequence of this hypothesis is that it suggests new avenues for therapy and new approaches to understanding individual susceptibility to these unique dermatologic diseases.

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Transparency declarations

None to declare.

References