Secreted subtilisins of *Microsporium canis* are involved in adherence of arthroconidia to feline corneocytes

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Microsporium canis is a pathogenic fungus that causes a superficial cutaneous infection called dermatophytosis, mainly in cats and humans. The mechanisms involved in adherence of *M. canis* to epidermis have never been investigated. Here, a model was developed to study the adherence of *M. canis* to feline corneocytes through the use of a reconstructed interfollicular feline epidermis (RFE). In this model, adherence of arthroconidia to RFE was found to be time-dependent, starting at 2 h post-inoculation and still increasing at 6 h. Chymostatin, a serine protease inhibitor, inhibited *M. canis* adherence to RFE by 53 %. Moreover, two mAbs against the keratinolytic protease subtilisin 3 (Sub3) inhibited *M. canis* adherence to RFE by 23 %, suggesting that subtilisins, and Sub3 in particular, are involved in the adherence process.

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INTRODUCTION

Pathogenic dermatophytes are fungi that have the ability to invade keratinized structures, such as the superficial cornified skin layers, hairs and nails, causing a superficial cutaneous infection called dermatophytosis. *Microsporium canis* is the main agent of dermatophytosis in cats, its natural host (Scott *et al.*, 1995), and is responsible for a frequent zoonosis that has increased in several European countries (Lunder & Lunder, 1992; Arrese *et al.*, 2003). To date, little is known about the physiopathology of the infection. The first pathogenic event consists of adherence of arthroconidia to the stratum corneum, followed by their germination and invasion of the keratinized epidermis (Zurita & Hay, 1987). The mechanisms involved in adherence of *M. canis* to epidermis have never been investigated. Most studies aiming to identify *M. canis* virulence factors have focused on proteases, including keratinases. A family of three subtilisins (Descamps *et al.*, 2002), a family of five metalloproteases (Brouta *et al.*, 2002; Jousson *et al.*, 2004) and two dipeptidyl peptidases (GenBank accession nos. DQ286524 and DQ286525) have been identified, but so far no definitive role in fungal virulence has been assigned to these enzymes, although the *in vivo* secretion of several of them has been demonstrated

(Mignon *et al.*, 1998, 2005; Descamps *et al.*, 2002). The role of secreted proteases in virulence has, however, been demonstrated in *Candida albicans*. This yeast produces a family of ten secreted aspartic proteases (Saps), which are involved in adherence and invasion of skin and mucosal surfaces (Ollert *et al.*, 1993; Monod & Borg-von Zepelin, 2002; Naglik *et al.*, 2003). The role of Sap1, Sap2 and Sap3 in the adherence process has been demonstrated in several experiments using a specific aspartic protease inhibitor (Ollert *et al.*, 1993), Sap1–3 mutant strains (Watts *et al.*, 1998) and antibodies against Sap2, which are able to block adherence of *C. albicans* to epithelial cells (De Bernardis *et al.*, 2007).

In order to gain a better understanding of the molecular mechanisms involved in *M. canis* adherence to feline epidermis, we developed an adherence model of *M. canis* arthroconidia to feline corneocytes, using a reconstructed interfollicular feline epidermis (RFE) (Tabart *et al.*, 2007). This model was then used to assess the role of subtilisins, including the highly keratinolytic protease Sub3, in fungal adherence.

METHODS

Arthroconidia production. *M. canis* strain IHEM 21239 isolated from a naturally infected cat was used for all experiments. Arthroconidia were produced as described previously (Tabart *et al.*,

Abbreviations: AAPF-pNa, *N*-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide; RFE, reconstructed interfollicular feline epidermis.

2007). Briefly, *M. canis* was grown on 2 % yeast extract/1 % peptone agar (VWR) in an atmosphere containing 12 % CO₂ at 30 °C for 15 days.

Detection of expression of genes encoding subtilisins in *M. canis* arthroconidia using nested RT-PCR. Arthroconidia were ground with a pestle and mortar under liquid nitrogen, and RNA was extracted using an RNeasy plant mini kit (Qiagen). On-column DNase digestion was performed using an RNase-free DNase set (Qiagen). RNA was eluted in 60 µl water, and 5 µl of the obtained solution was used as a template for RT-PCR (45 cycles) using an Access RT-PCR System (Promega) and primer pairs specific for the *M. canis* genes *SUB1*, *SUB2* or *SUB3*, and for the actin gene as a control, as described previously (Descamps *et al.*, 2002). In order to confirm the amplification of mRNA rather than contaminating genomic DNA, a control was performed by omission of the reverse transcriptase step in a parallel reaction. The crude reaction product (5 µl) was then used as a template in a standard PCR (35 cycles) using a PCR core system I kit (Promega) and internal primers specific for *M. canis* actin, *SUB1*, *SUB2* or *SUB3* (Descamps *et al.*, 2002). The expected amplicon lengths for these genes were 282, 388, 1010 and 480 bp for *M. canis*, respectively.

Adherence assays. Adherence assays were performed on an RFE comprising a fully differentiated epidermis built up with feline keratinocytes seeded on a dermal equivalent, as described previously (Tabart *et al.*, 2007). Briefly, feline fetal fibroblasts and keratinocytes were cultured as monolayers in fibroblast growth medium (FGM) and keratinocyte growth medium (KGM; BioWhittaker), respectively, and used at second passage. Fibroblasts were adjusted to 1×10^3 cells ml⁻¹ in FGM containing 2 mg rat tail collagen ml⁻¹ (Roche Applied Science). The cellular suspension was poured onto a cell insert (Anopore, 0.63 cm diameter, 0.2 µm diameter pore size; VWR) supplied with a stainless steel ring, and the cell inserts were immersed in FGM for 24 h. The medium was then replaced with KGM, whilst proliferating keratinocytes were seeded onto the dermal equivalent in cell inserts at 5×10^5 cells cm⁻². After immersion for 24 h in KGM, the cell inserts were lifted to the air-liquid interface, and the medium was replaced with RFE culture medium (Tabart *et al.*, 2007) and changed every other day for 14 days. As the RFE was lifted to the interface, exchanges between reconstructed epidermis and the culture medium occurred only through the dermal equivalent. Twenty-four hours before RFE inoculation, penicillin, streptomycin and Fungizone were removed from the RFE culture medium.

For adherence assays, a 20 µl arthroconidial suspension (5×10^6 cells ml⁻¹ in PBS) was spread over the surface of the RFE and co-cultures were incubated in a humidified atmosphere containing 5 % CO₂ for 0, 2, 4 or 6 h at 37 °C. After washing in PBS, conidia that were adherent to RFE were labelled using Calcofluor White (20 µg in water; fluorescent brightener 28; Sigma) (Ruchel & Schaffrinski, 1999) and counted under a fluorescence light microscope (440 nm; Nikon; Fig. 1). The experiment was performed in triplicate and the incubation time was unknown to the observer.

Production of mAbs. Recombinant 31.5 kDa keratinolytic subtilisin (rSub3) was purified by cation-exchange chromatography from the supernatant of a transformed *Pichia pastoris* colony (Descamps *et al.*, 2003) and dialysed against PBS. BALB/c (Sp2/0) mice were immunized subcutaneously on days 0, 21, 42 and 59 using 50 µg rSub3 mixed with Quil A adjuvant (Isconova). On day 61, mice were euthanized. Spleen cells (3.5×10^6 cells ml⁻¹) were suspended in RPMI 1640 (Cambrex) and mixed with myeloma cells (SP2/0-Ag 14, ATCC CRL 8287, 1.7×10^6 cells ml⁻¹) in the presence of PEG (Sigma). Cells were then centrifuged for 5 min at 1000 g, suspended in a selective medium (RPMI 1640 containing 10 % fetal calf serum and hypoxanthine, aminopterin and thymidine) and distributed into

Fig. 1. Adherence model for *M. canis* IHEM 21239 on RFE. Adherent arthroconidia (indicated by arrows) were stained using Calcofluor White and visualized under a fluorescence light microscope (440 nm). Bar, 5 µm.

96-well microplates in the presence of mouse peritoneal cells as a feeder layer. Secretion of rSub3 antibodies was assessed by ELISA. After cloning by limiting dilution, mAbs were produced in flasks and purified by affinity chromatography using protein A (Sigma) and evaluated for their ability to inhibit the activity of rSub3 towards the synthetic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF-*p*Na; Sigma) as described previously (Mignon *et al.*, 1998). Two mAbs were selected (6B8 and 4F10) and Western blotting was performed to demonstrate their specific binding to Sub3. In addition to rSub3, a crude exo-antigen containing Sub3 and many other secreted proteases of *M. canis* was used. This exo-antigen was prepared as described previously (Mignon *et al.*, 1998). Briefly, *M. canis* strain IHEM 21239 was grown in a liquid medium containing cat keratin, and the culture supernatant was harvested after 15 days and referred to as crude exo-antigen. rSub3 was used as a positive control in a Western blot. Following separation of the proteins by 12 % SDS-PAGE, the gel was electroblotted for 1 h at 45 mA onto PVDF membrane in Tris/glycine buffer with 20 % methanol, and then treated with a mixture of the two mAbs and horseradish peroxidase-conjugated rabbit anti-mouse Ig (Dako). The blots were treated with the chemiluminescent detection reagent ECL Plus (Amersham Pharmacia) and were exposed to film.

Inhibition of adherence. The serine protease inhibitor chymostatin (Sigma), a cocktail of protease inhibitors (protease inhibitor cocktail for use in tissue culture medium with a broad specificity for serine, cysteine and acid proteases and for aminopeptidases) (Sigma) supplemented with chymostatin, and a mixture of the two anti-Sub3 mAbs (1:1) were tested for their capacity to inhibit the adherence of *M. canis* arthroconidia to RFE. Chymostatin was dissolved in 10 mM DMSO and used at a final concentration of 100 µM in PBS. The cocktail of protease inhibitors stored in DMSO by the manufacturer was diluted 100-fold in PBS. The mAb mixture was used at a concentration of 500 µg ml⁻¹ in PBS. Adherence assays were performed as described above except that arthroconidia were exposed to the inhibitors for 1 h prior to the adherence assay. Chymostatin, the cocktail of protease inhibitors supplemented with chymostatin or the mAbs were also added to the RFE culture medium at the same final concentration as above. A control without inhibitors or mAbs was also used under the same conditions. An additional control with a non-specific mAb (mouse mAb AA5H to influenza virus nucleoprotein; Abcam) at the same concentration was also performed. Infected RFE were incubated for 4 h. The experiment was

performed in triplicate and the status of each RFE was unknown to the observer.

Expression of results and statistical analysis. Inhibition of *M. canis* adherence was expressed as the residual percentage of adherence to RFE in comparison with the controls. Significant differences between two sets of data were calculated using Student's *t*-test, with significance defined as $P < 0.005$.

RESULTS AND DISCUSSION

Detection of *SUB1* and *SUB3* mRNAs in arthroconidia

The *SUB1* and *SUB3* genes, but not *SUB2*, were found to be transcribed in arthroconidia (Fig. 2), strongly suggesting that the corresponding encoded proteases are produced in these spores. Arthroconidia were used because they constitute the infective stage of *M. canis* *in vivo*. They are shed from infected lesions and disseminated into the environment. Consequently, it can be postulated that the proteases Sub1 and Sub3 are involved in the early stages of infection, i.e. adherence to and/or invasion of corneocytes.

M. canis arthroconidia adhere to RFE up to 6 h post-inoculation

In the present model, adherence of arthroconidia to RFE was found to be time-dependent (Fig. 3), starting within 2 h and increasing up to 6 h post-inoculation (the last observation time). This is, to the best of our knowledge, the first report of a model to study *M. canis* adherence to epidermis. The kinetics of adherence was similar to that reported for other dermatophytes. Zurita & Hay (1987)

Fig. 3. Kinetics of *M. canis* IHEM 21239 adherence to RFE. Results are shown as the mean number ($\pm$ SD) of adherent arthroconidia.

observed that maximum adherence of *Trichophyton* sp. arthroconidia to human keratinocytes in suspension occurred within 3–4 h. Aljabre *et al.* (1992, 1993) used stripped sheets of stratum corneum or isolated keratinocytes to demonstrate that adherence of *Trichophyton mentagrophytes* arthroconidia was highest 6 h after contact and that germination of these spores began 4 h post-exposure. Adherence of *T. mentagrophytes* to human skin explants of full epidermis was maximal after 12 h (Duek *et al.*, 2004). In the present study, adherence of *M. canis* arthroconidia was studied using RFE. This model is likely to be very useful because both the cornified layer resulting from the epidermal differentiation process (Tabart *et al.*, 2007) and skin permeability of RFE were shown to be comparable to the *in vivo* situation.

M. canis adherence to RFE is inhibited by chymostatin and mAbs against Sub3

Chymostatin, a serine protease inhibitor that completely inhibits Sub3 activity (Mignon *et al.*, 1998), significantly inhibited the adherence of *M. canis* arthroconidia to RFE (53 % inhibition, $P < 0.005$, Fig. 4). In the yeast *C. albicans*, pepstatin A, an aspartic protease inhibitor, decreased *C. albicans* adherence to keratinocytes (45 % inhibition; Ollert *et al.*, 1993), and strongly reduced tissue damage caused to the vaginal and oral epithelia by *C. albicans* cells (Schaller *et al.*, 1999, 2003). A reduced adherence of Sap1-, Sap2- and Sap3-deficient strains to buccal epithelial cells has also been observed (Watts *et al.*, 1998). The inhibition of *M. canis* adherence to RFE by chymostatin clearly indicated that one or more subtilisins play an important role in *M. canis* adherence. The cocktail of protease inhibitors supplemented with chymostatin decreased the adherence of arthroconidia to RFE by 72 % (Fig. 4), suggesting that other proteases are also involved in the adherence process.

Two mAbs against Sub3 (6B8 and 4F10) were produced and purified. Western blotting showed that these mAbs were specific to Sub3 and did not cross-react with other

Fig. 2. Transcription of *M. canis* IHEM 21239 *SUB1* and *SUB3* in arthroconidia. Lane 1, molecular mass markers (1 kb ladder; Fermentas), sizes (in bp) are indicated on the left; lanes 2, 3, 5 and 7, nested RT-PCR products obtained from arthroconidia, corresponding to internal cDNA fragments of the *M. canis* actin gene, *SUB1*, *SUB2* and *SUB3*, respectively; lanes 4, 6 and 8, control reactions (with the reverse transcriptase step omitted), corresponding to *M. canis* *SUB1*, *SUB2* and *SUB3*, respectively.

Fig. 4. Adherence of *M. canis* IHEM 21239 arthroconidia to RFE in the presence of chymostatin or chymostatin plus a cocktail of protease inhibitors. Chymostatin was used at a final concentration of 100 μ M and the cocktail of protease inhibitors at a 100-fold dilution. Adherence values in the presence of inhibitors (grey bars) are expressed as a percentage of the controls without inhibitor (100 %) (black bars) and are given as means $\pm$ SD.

secreted proteases (Fig. 5). The mAbs did not inhibit rSub3 activity towards AAPF-pNa (Fig. 6). However, they significantly inhibited adherence of arthroconidia to RFE by 23 % ($P < 0.005$; Fig. 7), indicating that inhibition could be due to steric hindrance rather than blockage of the active site. The non-specific control mAb did not inhibit adherence (Fig. 7), excluding non-specific steric effects of mAbs against Sub3. Cross-reactivity of the mAbs against Sub3 with Sub1 and Sub2 is unlikely, as Sub1 and Sub3 have only 41.2 % identity, and Sub2 and Sub3 have only 34.6 % identity (Descamps *et al.*, 2002). Overall, these results suggest that, among the subtilisins, Sub3 is

Fig. 5. Western blot showing the specificity of a mixture of mAbs 4F10 and 6B8 for Sub3. Lane 1, molecular mass markers (Precision Plus dual colour protein standards; Bio-Rad), sizes in kDa are indicated on the left; lane 2, rSub3; lane 3, exo-antigen.

Fig. 6. Activity of rSub3 towards AAPF-pNa in the presence of anti-Sub3 mAbs 6B8 and 4F10. Activity values are expressed as a percentage of the activity of rSub3 towards AAPF-pNa without mAbs (100 %). ●, 6B8; ▽, 4F10; □, 6B8+4F10.

implicated in the adherence mechanisms. The precise mechanisms by which secreted proteases contribute to the adherence process are not clear. Two hypotheses are currently favoured (Naglik *et al.*, 2003). Firstly, proteins could act as ligands for surface moieties on host cells, which does not necessarily require activity of these enzymes. In the pathogenic bacteria *Porphyromonas gingivalis* and *Streptococcus pneumoniae*, a membrane-associated dipeptidyl peptidase IV and a surface-associated serine protease named choline-binding protein G, respectively, have been implicated in adherence (Kumagai *et al.*,

Fig. 7. Adherence of *M. canis* IHEM 21239 arthroconidia to RFE in the presence of anti-Sub3 mAbs (6B8 and 4F10). The mAbs were used at a final concentration of 500 μ g ml $^{-1}$ (25 μ g each mAb per RFE). A non-specific control mAb was used at the same concentration. Adherence values are expressed as a percentage of the control test without mAbs (PBS only; 100 %) and are given as the mean $\pm$ SD.

2005; Mann *et al.*, 2006). Interestingly, the carboxyl-terminal extremity of Sub1 shows a remarkable abundance of proline-rich domains (Descamps *et al.*, 2002), suggesting that this protein could also be surface anchored. Secondly, secreted proteases may act as active enzymes to modify target ligands on the fungal surface or on epithelial cells, and this could lead to conformational changes, facilitating adherence of the fungus (Monod & Borg-von Zepelin, 2002). Our study on *M. canis* adherence mechanisms could lead to the implementation of new therapeutic tools aimed at preventing the adherence of arthroconidia to keratinocytes.

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