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Reconstructed interfollicular feline epidermis as a model for *Microsporum canis* dermatophytosis

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Microsporum canis is a pathogenic fungus that causes a superficial cutaneous infection called dermatophytosis. The complexity of mechanisms involved in dermatophytic infections makes relevant *in vivo* studies particularly difficult to perform. The aim of this study was to develop a new *in vitro* model of *M. canis* dermatophytosis using feline fetal keratinocytes in reconstructed interfollicular epidermis, and to investigate its relevance in studying the host–pathogen relationship. Histological analysis of reconstructed interfollicular feline epidermis (RFE) revealed a fully differentiated epidermis. A proliferation assay showed replicating cells only in the basal layer, indicating that RFE is a well-stratified living tissue, leading to the formation of a horny layer. Histopathological analysis of RFE infected by *M. canis* arthroconidia revealed that the fungus invades the stratum corneum and produces SUB3, a keratinase implicated in the infectious process. In view of these results, an *M. canis* dermatophytosis model on RFE seems to be a useful tool to investigate mechanisms involved in natural *M. canis* feline infections.

INTRODUCTION

Microsporum canis causes a superficial cutaneous infection, mainly in cats, its natural and reservoir host, but also in dogs, other pets and humans. As for other dermatophytoses, the pathogenesis of *M. canis* infection remains poorly understood, mainly because relevant *in vivo* studies are particularly difficult to perform. Additionally, to date, there is no *in vitro* model of feline dermatophytosis to investigate the role of putative *M. canis* virulence factors and to study the keratinocyte response to infection. Nevertheless, *in vitro* models were shown to be useful to study the pathogenesis of some non-*M. canis* dermatophytoses. In this context, *Trichophyton rubrum*, *Trichophyton interdigitale* and *Trichophyton quinckeanum* were shown to adhere to stripped stratum corneum within 4 h (Zurita & Hay, 1987). Interleukin-8 and tumour necrosis factor alpha were also shown to be produced in keratinocyte monolayers inoculated with *Trichophyton mentagrophytes* (Nakamura *et al.*, 2002). In a more elaborate model of *T. mentagrophytes* infection using reconstructed human epidermis (RHE), the infection process was shown to be similar to that observed *in vivo* (Rashid *et al.*, 1995). Consequently, in the present study, we developed for the first time both the culture of normal feline keratinocytes and a model of interfollicular reconstructed feline epidermis (RFE). Additionally, we demonstrate the relevance of RFE to the *in vivo M. canis* infection process.

Abbreviations: BrdU, bromodeoxyuridine; RFE, reconstructed feline epidermis; RHE, reconstructed human epidermis; SUB3, subtilisin-like protease 3.

METHODS

Fungal growth and arthroconidia production. The *M. canis* strain IHEM 21239 isolated from a naturally infected cat was used for all experiments. After isolation, the fungus was grown on Sabouraud's dextrose agar (Gibco, Life Technologies). Then, arthroconidia were produced essentially as previously described by Gupta *et al.* (2003). Briefly, arthroconidia were obtained from 15-day-old cultures on 2 % yeast extract/1 % peptone agar (VWR Scientific Products) in an atmosphere containing 12 % CO_2, at 30 °C. Surface mycelium and conidia were scraped with a scalpel blade and transferred to PBS (pH 7.2). After gentle agitation for 1 h, the fungal suspension was filtered through three Miracloth layers (22–25 μm pore size; Calbiochem) to remove hyphae and centrifuged at 3000 ***g*** for 5 min. The pellet containing arthroconidia was washed three times in PBS, resuspended in PBS and stored at 4 °C until use. Arthroconidia concentration was determined by serial dilutions on Sabouraud's dextrose agar and adjusted to 1×10^7 cells ml^{-1}. Preliminary studies showed that the mortality rate was non-significant until more than 3 months after arthroconidia isolation. In all cases, arthroconidia were used within 1 month.

Isolation of feline keratinocytes and fibroblasts. The cells were obtained from feline fetuses over 30 days of age, in cooperation with a veterinarian surgeon and with the agreement of the cat's owner.

Except when mentioned, solutions and vessels were kept on ice during the entire procedure. The fetal skin was cut into small pieces. After removal of subcutaneous fat, the samples were incubated overnight at 4 °C floating on William's Medium E supplemented with 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1}, 2.5 μg fungizone ml^{-1} and 5 mg dispase ml^{-1} (all from Gibco). The epidermis was then separated from the dermis with a scalpel blade, rapidly chopped and trypsinized for 30 min at 37 °C in 0.12 % trypsin/0.02 % EDTA (Gibco) in PBS. The cell suspension was filtered through a cell strainer, 70 μm pore size (VWR), and centrifuged for 5 min at 900 ***g*** at 4 °C. The cellular pellet was resuspended in keratinocyte growth medium (KGM) composed of keratinocyte basal medium supplemented with Singlequots (Biowhittaker). The cells were then incubated at 37 °C for 24 h in a humidified atmosphere containing 5 % CO_2. De-epidermized dermis was cut into small pieces and incubated for 7 days in fibroblast growth medium (FGM) containing Dulbecco's Modified Eagle's Medium, 10 % fetal bovine serum, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 2.5 μg fungizone ml^{-1} (all from Gibco). Medium was changed every other day.

Cell type characterization. Cultured keratinocytes and fibroblasts were immunolabelled using a rabbit polyclonal anti-human pan-cytokeratin and a mouse polyclonal anti-human vimentin antibody, respectively (Sigma Aldrich), essentially as described by Chen *et al.* (2002). Briefly, cells grown for 48 h on coverslips were washed with PBS and fixed for 10 min in methanol. Endogenous peroxidases were inhibited by incubating the cells for 20 min in 3 % H_2O_2 in PBS. Coverslips were rinsed with PBS and sequentially incubated for 1 h with non-immune goat serum (Dako) and anti-pan-cytokeratin or anti-vimentin antibody. After washing with PBS, a horseradish peroxidase-coupled mouse anti-rabbit or rabbit anti-mouse antibody (Dako) was applied for 30 min. After washing with PBS, amino ethyl carbazole (Dako) was used as a chromogen and slides were counterstained for 5 min with Mayer's haematoxylin.

Dermal equivalent. Fibroblasts were used at passage 3–5 for all experiments. Cells were harvested by trypsinization for 5 min at 37 °C [0.025 % trypsin/0.01 % EDTA (Gibco) in PBS] and adjusted to 1×10^3 cells ml^{-1} in FGM supplemented with 200 μg NaOH ml^{-1}, 2.1 mg $NaHCO_3$ ml^{-1}, 50 μg ascorbic acid ml^{-1} and 2 mg rat tail collagen ml^{-1} (Roche Applied Science). The cellular suspension was poured on a cell insert (Anopore 0.63 cm diameter, 0.2 μm diameter pore size; VWR) supplied with a stainless steel ring and incubated for 30 min at 37 °C, in a humidified atmosphere containing 5 % CO_2. After collagen polymerization the cell inserts were immersed in FGM and incubated under the same conditions for an additional 24 h. The medium was then replaced with KGM.

Reconstructed interfollicular feline epidermis. RFE was developed essentially as described for RHE (Poumay *et al.*, 2004). Proliferating keratinocytes from primary cultures in KGM were trypsinized and seeded on dermal equivalent in cell inserts at 5×10^5 cells cm^{-2}. After immersion for 24 h in KGM, cell inserts were lifted using a stainless steel grid support to form an air–liquid interface. The medium was replaced by RFE culture medium [a 1 : 1 mix of KGM and Dulbecco's Modified Eagle's Medium : Ham's F-12 (3 : 1)] supplemented with 2 mM glutamine, 100 μM non-essential amino acids, 1 mM pyruvate, 60 U penicillin ml^{-1}, 60 μg streptomycin ml^{-1}, 2.5 μg fungizone ml^{-1} (all from Gibco), 10 mM HEPES (Biowhittaker), 1×10^{-10} M cholera toxin, 5 μg insulin ml^{-1}, 0.4 μg hydrocortisone ml^{-1}, 20 μg adenine ml^{-1}, 5 μg transferrin ml^{-1}, 1.5 ng tri-iodo-L-thyronine ml^{-1}, 2 ng epidermal growth factor ml^{-1}, 2.85 mM calcium and 100 μg ascorbic acid ml^{-1} (all from Sigma). Medium was changed every other day for 14 days.

Histological evaluation. After 14 days of growth at the air–liquid interface, RFE was histologically evaluated and compared to normal feline epidermis. Briefly, RFE was fixed for 24 h in 4 % buffered formaldehyde and embedded in paraffin. Tissue sections (7 μm thick) perpendicular to the RFE were stained with haematoxylin and eosin.

Proliferation assay. The culture medium of RFE placed at the air–liquid interface for 11 and 13 days was supplemented with 10 mM bromodeoxyuridine (BrdU) for either 72 or 24 h, respectively. At day 14, RFE was fixed in 4 % formaldehyde and processed as described above. BrdU incorporation was immunohistochemically evaluated using the BrdU In-situ Detection kit according to the manufacturer's instructions (BD Biosciences).

Histopathological and immunohistochemical analyses. RFE was seeded with 1×10^5 *M. canis* arthroconidia, incubated for 5 days at 37 °C with 5 % CO_2 in a humidified atmosphere, and processed for histopathology as described above, except that haematoxylin and eosin were replaced by periodic acid–Schiff. Immunodetection of the *M. canis* keratinolytic protease SUB3 (Mignon *et al.*, 1998; Descamps *et al.*, 2002) was performed in infected RFE sections essentially as previously described (Mignon *et al.*, 1998). Briefly, slides were sequentially incubated with a non-immune goat serum (Dako) containing 3 % BSA (Sigma), a rabbit anti-SUB3 polyclonal antibody, and then with a fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin antibody (Dako). Control consisted of anti-SUB3 primary antibody omission.

RESULTS AND DISCUSSION

KGM and FGM select feline keratinocytes and fibroblasts

The nature of cultured cells was determined by immunochemistry using an anti-pan-cytokeratin or an anti-vimentin antibody. As shown in Fig. 1(a), cells cultured in KGM were polygonal, with a cytoskeleton labelled with anti-pan-cytokeratin but not with anti-vimentin (Fig. 1b) antibody. In contrast, cells cultured in FGM were fibroblastic with a cytoskeleton labelled with anti-vimentin (Fig. 1d) but not with anti-pan-cytokeratin (Fig. 1c) antibody. This demonstrates that FGM and KGM are suitable for selecting feline fibroblasts and keratinocytes, which are subsequently used to develop dermal equivalent and RFE, respectively.

RFE is fully differentiated

Staining of RFE with haematoxylin and eosin (Fig. 2a, b) revealed a structure composed of four typical successive layers (basal, spinous, granular and cornified) as commonly observed *in vivo*. It is noticeable that the cornified layer is very thick. Additionally, the high reproducibility of RFE was independent of the origin of feline fetuses. Thus this is the first description of a fully differentiated RFE. The use of both gel collagen and feline fibroblasts was critical for epidermal differentiation. The relative disorganization of the basal cells could be related to the absence of basement membrane, and the high number of keratinocytes seeded on the collagen gel, a prerequisite to obtain a rapid confluence of cells. The proliferation of basal keratinocytes during the differentiation process of RFE was evaluated using their capacity to integrate BrdU. In RFE incubated

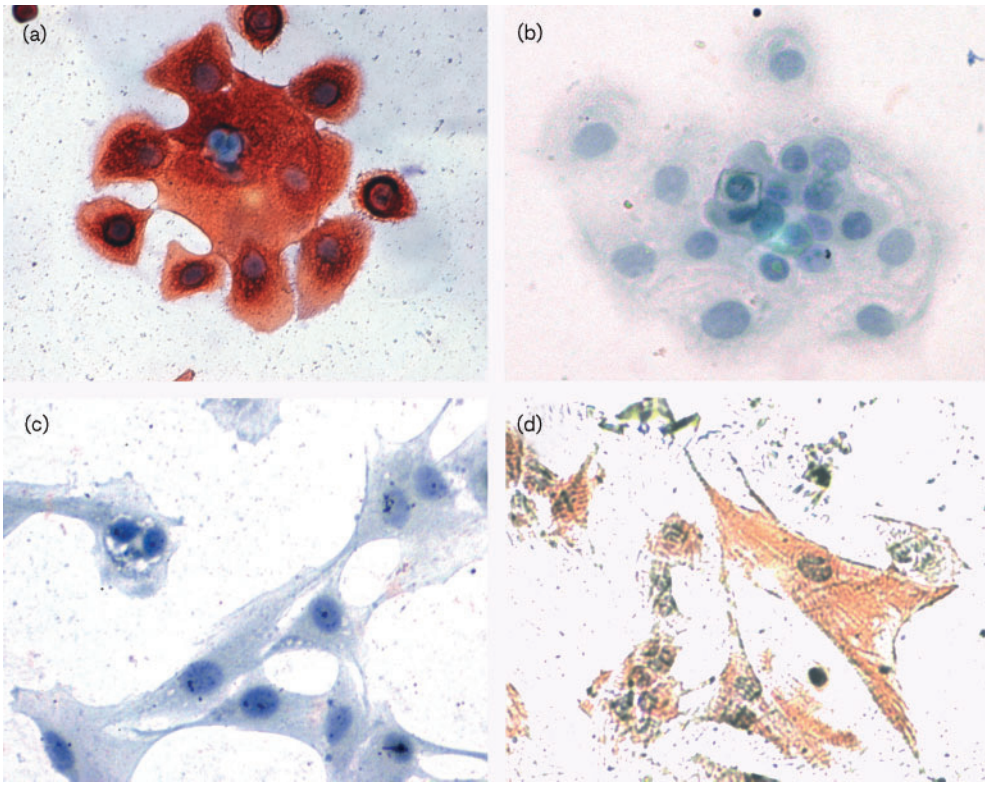

Fig. 1. Feline cells cultured in KGM and FGM are keratinocytes and fibroblasts, respectively. Non-confluent feline skin cells cultured in KGM or FGM were labelled with an anti-pan-cytokeratin or an anti-vimentin antibody followed by a secondary horseradish peroxidase-conjugated antibody. Over 95 % of the cells cultured in KGM were polygonal and exhibited a brown staining with pan-cytokeratin (a) whereas they were not stained for vimentin (b). Cells cultured in FGM were fibroblastic; none were stained for pan-cytokeratin (c) whereas over 95 % were stained for vimentin (d).

for 72 h from day 11, both the basal and spinous layers were stained (Fig. 2c). Basal keratinocytes were also stained in RFE incubated for 24 h from day 13 (Fig. 2d). These results, which were obtained in duplicate, show that division of keratinocytes is only observed in the basal layer. They also show that, in spite of a complete stratification, the keratinocyte division and maturation is continuous until the end of the differentiation process (day 14).

RFE can be infected by *M. canis*

Histologically, fungal hyphae were detected in the stratum corneum 5 days after RFE inoculation in more than five independent experiments, demonstrating the high reproducibility of this model (Fig. 2e, f). The fungus recovered from the infected stratum corneum was identified as *M. canis* after culture on Sabouraud's dextrose agar. Additionally, expression of SUB3, a keratinase implicated in the infectious process (Mignon *et al.*, 1998; Descamps *et al.*, 2002), was immunohistochemically detected in fungal hyphae (Fig. 2g, h) using an anti-SUB3 polyclonal antibody. These results demonstrate that *M. canis* not only grows on RFE but invades it, even in the absence of hair follicules, and produces SUB3 as previously demonstrated in naturally infected cats (Mignon *et al.*, 1998) and in experimentally infected guinea pigs (Mignon *et al.*, 1999).

In summary, for the first time a fully differentiated RFE was developed *in vitro*. This new serum-free model was demonstrated to be reproducible and relevant for studying *M. canis*–epidermis interactions. However, this model does not contain any hair follicles, sebaceous and sweat glands or leukocytes, which could limit its value for some applications. Similarly, the absence of cutaneous microflora could be a disadvantage in comparison with an *in vivo* model. In spite of these limitations, the RFE model displays many advantages. Firstly, in contrast with stripped stratum corneum and isolated hairs, RFE is a living tissue allowing the investigation of cellular responses against cutaneous pathogens. Secondly, the cornified layer resulting from the differentiation process in RFE mimics dermatophytic infection more realistically than keratinocyte monolayers. The potential involvement of *M. canis* proteases in both invasion of corneocytes and induction of epidermal cytokine production is currently under investigation. In the

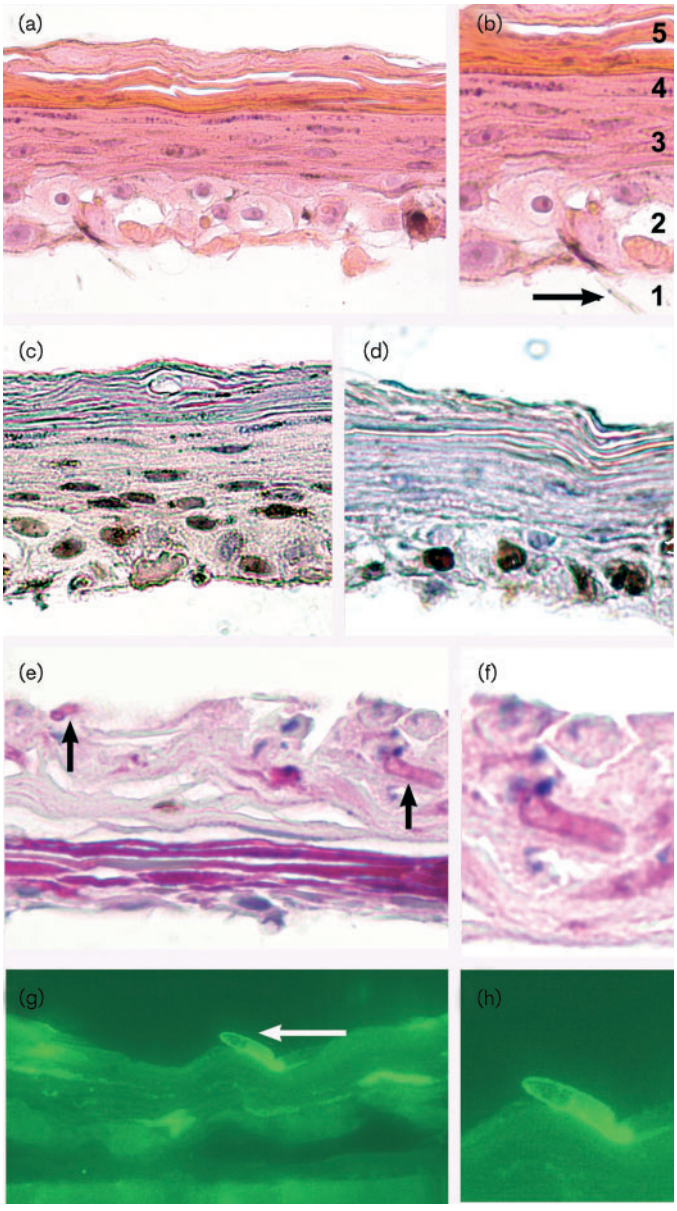

Fig. 2. RFE as a model for feline dermatophytosis caused by *M. canis*. (a, b) RFE stained with haematoxylin and eosin showed a limited number of fibroblasts in the collagen gel (1, black arrow), a basal layer consisting of cuboidal nucleated keratinocytes (2), a spinous layer, composed of differentiated large nucleated cells (3), a granular layer characterized by closely intricate flattened cells (4) and a superficial layer composed of anucleated cornified cells (5). RFE incubated for 72 h with BrdU from day 11 (c) or for 24 h from day 13 (d) exhibited a brown staining in the basal and spinous layers or only in the basal layer, respectively. (e, f) RFE cultured for 14 days at the air–liquid interface was inoculated with *M. canis* arthroconidia and cultured for an additional 5 days. Staining with periodic acid–Schiff showed *M. canis* hyphae (black arrows) in the stratum corneum. (g, h) RFE incubated 5 days after inoculation with *M. canis* was stained with a rabbit polyclonal anti-SUB3 antibody followed by a secondary fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin antibody. The white arrow shows positively stained *M. canis* hyphae. Control consisting of anti-SUB3 antibody omission resulted in an absence of staining.

near future, we plan to create an immortalized feline keratinocyte cell line, to abrogate the need for fresh fetal keratinocytes and allow large-scale studies centring on different feline skin pathogens.

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