DOI:10.1111/j.1600-0625.2006.00525.x
www.blackwellpublishing.com/EXD

Original Article

Development and characterization of a canine skin equivalent

Montserrat Serra[1], Pilar Brazís[1], Anna Puigdemont[2], Dolors Fondevila[3], Víctor Romano[4], Celina Torre[4] and Lluís Ferrer[3]

[1]UNIVET, SL, Universitat Autònoma de Barcelona, Barcelona, Spain;
[2]Departament de Farmacologia, Terapèutica i Toxicologia, Facultat de Veterinària, Universitat Autònoma de Barcelona, Barcelona, Spain;
[3]Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, Barcelona, Spain;
[4]Affinity Pet-care, Sant Cugat, Barcelona, Spain
Correspondence: Dr Montserrat Serra, UNIVET, SL, Edifici M, Facultat de Medicina, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Barcelona, Spain, Tel.: (+34) 93 581 33 61, Fax: (+34) 93 581 46 40, e-mail: montserrat.serra.muxi@uab.es

Accepted for publication 17 October 2006

Abstract: The development of a complex cellular model, which incorporates the basic cell components of the dog skin, would be a useful tool to investigate the biology and pathology of canine skin and also to replace animal testing partially. The aim of the present study was to develop and characterize a canine skin equivalent. Epidermal keratinocytes and dermal fibroblasts were freshly isolated from skin biopsies from healthy dogs. Fibroblasts were embedded into a bio-matrix from collagen type I matrix protein; this built the scaffold where the keratinocytes were seeded, at air exposed conditions. At 3, 7, 15 and 21 days of culture in special growth media, skin equivalents were analysed by histological, immunohistochemical and electron microscopical techniques. At 15 days, keratinocytes underwent differentiation to a multilayer epidermis with *stratum basal*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. Expression of epidermal cytokeratins in keratinocytes was detected by immunhistochemistry, and followed the same pattern than in the normal canine epidermis. Fibroblasts from the skin equivalent expressed vimentin as dermal fibroblasts do. A basement membrane (BM) was observed underneath the epidermis; ultrastructurally, it was similar to the normal canine BM and collagen IV and laminin 5 were detected immunohistochemically as major components of this structure. Skin equivalents developed from canine cutaneous cells presented a similar morphological structure than healthy canine skin. Moreover, the immunohistochemical analysis revealed the expression of the major markers of the epidermis (keratins), dermis (vimentin) and BM (collagen type IV, laminin 5).

Key words: canine – organotypic skin culture – skin equivalent

Please cite this paper as: Development and characterization of a canine skin equivalent. Experimental Dermatology 2007; 16: 135–142.

Introduction

Classically, research in veterinary dermatology had been supported mainly by in vivo studies (clinical trials, clinicopathologic studies, pharmacologic studies). Cellular models (primary cultures or stable cell lines) have been developed to reduce the research in vivo. However, these cellular models are still away from the normal canine skin and present obvious limitations. The basic structure of normal canine skin consists of an external layer, the epidermis, and of an internal layer, the dermis, which have an epithelial and mesenchymal origin, respectively (1–3). The interface between the epidermis and dermis is formed by a functional basement membrane (BM) zone made up of matrix proteins (4). Interactions between the different cell populations, for instance, which are of an extraordinary importance in the biology of the skin, cannot be investigated adequately in conventional cell cultures or cell lines.

Therefore, the development of a more complex model such as a canine organotypic skin culture, which incorporates the basic cell components of the dog skin, would be a useful tool to investigate the biology and pathology of canine skin and also would represent an important alternative to replace partially or reduce animal testing.

A first approach to the development of an 'artificial canine skin' is the development of organotypic cocultures of the two major cell populations of the skin: keratinocytes and fibroblasts. These primitive skin equivalents have been successfully developed from murine and human skin and have demonstrated to be very useful in dermatological research (5, 6). Organotypic skin equivalents can be used as tools for studies in skin biology and physiology, toxicity testing, as skin substitutes for wound closure, and in general for clinical, biological and pharmacologic applications (7, 8).

Organotypic skin equivalents are three-dimensional systems that are engineered by seeding keratinocytes onto a

dermal equivalent composed of fibroblasts embedded into a gel bio-matrix made of collagen and other matrix proteins (6, 9–14).

The dermal substrate will diffuse nutrients from the medium towards the epidermal compartment and it will support keratinocyte proliferation and differentiation to a multilayer epidermis with *stratum basale, stratum spinosum, stratum granulosum* and *stratum corneum* (5, 15–17). As a result of the interaction between keratinocytes and fibroblasts, a functional BM made up of matrix proteins is formed between the dermal and epidermal parts of the skin model. Under these conditions, a skin equivalent is formed and it shows similarity with the native tissue from which it was derived (2, 18).

Recently, one canine model was established to characterize the recessive keratinization defect of Norfolk terrier dogs (19) and another artificial skin was reconstructed in vitro to characterize a canine model of dystrophic bullous epidermolysis (20). However, these canine skin equivalent models have been established to characterize specific diseases, and they have not been evaluated for their similarity to normal canine skin.

In view of this, the aim of the present study was to develop and characterize a canine skin equivalent model resembling, as much as possible, the normal canine skin as well as to investigate some histological and cytological parameters concerning tissue preservation and differentiation.

Materials and methods

Skin samples

Samples of the skin of the abdominal area were obtained from surgical procedures (spaying and mastectomies) performed at different veterinary centres or from dogs euthanized in a public pound (Granollers, Spain).

Keratinocytes isolation and culture

Skin samples were well shaven and cleaned with 70% EtOH/Betadine before starting with cell isolation. Fat tissue and blood vessels were removed from the skin and then samples were washed with PBS, cut into small fragments (0.5 cm^2) and they were digested with dispase II solution (Boehringer Mannheim®, Indianapolis, IN, USA) for 16 h at 4°C. Next day, after incubation for 30 min at 37°C in the same solution, the dermis was separated from the epidermis. Epidermal fragments were washed with $PBS^{-/-}$ and digested with a solution of 0.05% trypsin-0.02% EDTA during 20 min at 37°C, while shaking. Cells were filtered twice with cell strainers (100 and 40 μm pore size, respectively) and centrifuged at 1200 rpm for 5 min. Then 1×10^6 cells/cm^2 were plated and grown in a humidified atmosphere at 37°C with 5% CO_2 for 1 week in DMEM/F12 (3:1) with 10% FCS (Gibco, Rockville, MD, USA), 10^{-6} M hydrocortisone, 10^{-6} M isoproterenol and 10^{-7} M insulin (Sigma, St. Louis, MO, USA).

Fibroblast isolation and culture

Cultures of normal canine dermal fibroblasts were also established from canine skin samples. Briefly, washed skin samples were chopped into 1 mm^3 fragments and incubated for 140 min in 15 ml of DMEM per gram of skin containing 30 mg bacterial collagenase (Gibco), 18 mg hyaluronidase, 12 mg pronase, 1.5 mg DNAse, supplemented with bovine albumin (all of them from Sigma) and antibiotics. After digestion, cutaneous cells were washed with PBS and grown in a humidified atmosphere at 37°C with 5% CO_2 for 2 days. Medium was changed twice a week and cells were used at passages 2–5.

Preparation of collagen gel bio-matrix

Collagen gel bio-matrix was developed by adding mature fibroblasts to a collagen rat tail type I collagen solution. Briefly, the different components to obtain the collagen solution were mixed in a cold sterile glass bottle: 1/10 ml of total collagen solution of HBSS 10X (Gibco), 1.5 mg/ml of rat tail type I collagen solution (Sigma) in 0.1% acetic acid, NaOH 1 N to obtain a pH of 7.2–7.3 and finally fibroblasts diluted in FCS (1/10 ml of total collagen solution) were added to the mix while gently shaking.

Different fibroblast concentrations (4×10^4–8×10^4 fibroblasts/ml collagen solution) were tested. After shaking the collagen-fibroblasts solution, 3 ml was put into each Transwell chamber (24 mm diameter, 3 μm pore size polycarbonate membrane; Corning, New York, NY, USA). Plates were placed into the incubator for 10 min, to polymerize, and then 2 ml of DMEM–5% FCS were added at the bottom of each well. Collagen gel bio-matrix samples were grown in a humidified atmosphere at 37°C with 5% CO_2 during 7 days and the medium was changed twice a week.

Skin equivalents obtaining

To obtain the skin equivalents it was necessary to seed keratinocytes onto the collagen gel bio-matrix. Different keratinocyte concentrations (2.5×10^5–1×10^6 keratinocytes/50 μl medium) were tested for its addition to the collagen gel bio-matrix. Subsequently, the cultures were fed with the same keratinocyte medium but containing 1% serum, 1×10^{-2} M L-serine, 1 μM DL-α-tocopherol-acetate and a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid and 2.4×10^{-5}M bovine serum albumin. At the time of lifting the cultures at the air–liquid (A/L) interface, a new medium without serum, with 30 μM linoleic acid and 50 μg/ml ascorbic acid was used. The cultures were grown for additional 7, 14 or

21 days, at the A/L interface. The culture medium was renewed three times a week. All supplements were purchased at Sigma-Aldrich.

Histological analysis of skin equivalents

Skin equivalents samples were fixed in 10% formalin and embedded in paraffin wax at days 4, 7, 14 and 21 of culture. Sections (4 μm) were cut and stained by routine methods with haematoxylin and eosin (H&E) for histological evaluation and also were used for immunohistochemical analysis.

Mcmannus' Periodic Acid Schiff's (PAS) staining was used to demonstrate the BM presence in our skin equivalents models. Slides were treated with 5% periodic acid for 10 min. After washing for 10 min with running water slides were treated with the Schiff reagent for 30 min. After another wash, slides were counterstained with Mayer's haematoxylin.

Fontana–Masson silver method of staining was performed to identify melanin and differentiate the different layers and cells of the skin. Slides were treated with 10% silver nitrate for 1 h at 56°C. After washing, samples were treated for 10 min with 0.2% gold chloride. Samples were washed again and treated with 5% sodium tiosulfate. Finally, after another wash, slides were counterstained with nuclear fast red staining.

Immunohistochemical analysis of skin equivalents

For all the immunohistochemical analysis, inhibition of endogenous peroxidase with 3% H_2O_2 (33%) was performed and the non-specific sites were blocked with 2% bovine serum albumin in TBS for 1 h at room temperature before addition of primary antibody.

After incubation with the corresponding primary antibody, samples were incubated with the secondary antibody as follows: a biotin-labelled goat anti-mouse IgG for cytokeratins, keratin 10 and vimentin staining; a biotin-labelled rabbit anti-goat IgG for collagen IV staining and a biotin-labelled goat anti-rabbit IgG for laminin 5 staining. All the secondary antibodies were used at a 1:200 dilution in TBS for 1 h at room temperature and were from Dako (Glostrup, Denmark).

Avidin–biotin and diaminobenzidine (DAB) were used as the detection system. Sections of normal canine epidermis served as a positive control and antiserum that did not react with the canine control tissue served as a negative control.

Cytokeratins staining

Keratinocytes from the canine skin equivalents were analysed for their specific expression of different cytokeratins (5, 6, 8, 17–19) by means of immunohistochemical analysis. Slides were pretreated with 0.1% trypsin (20 min, room temperature) before addition of primary antibody. Samples were incubated with the monoclonal antibody to pan-cytokeratin at 1:25 (vol/vol) in TBS-2% BSA (clone MNF116; Dako, Carpinteria, CA, USA) for 16 h at 4°C.

Immunohistochemistry for *cytokeratin 10 (K10)* was also performed on formalin-fixed sections with RKSE60 (Chemicon, Temecula, CA, USA). To improve antigen retrieval for K10, sections were heated in a citrate buffer (pH 6) in a water bath at 97°C for 20 min. Then, samples were incubated with the monoclonal antibody to cytokeratin at 1:17 (vol/vol) in TBS-2% BSA for 16 h at 4°C.

Vimentin staining

Fibroblasts from the canine skin equivalents were analysed for their specific vimentin expression by means of immunohistochemistry. Citrate buffer pretreatment (20 min, 98°C) was performed before addition of primary antibody. Samples were incubated with the monoclonal antibody to vimentin at 1:50 (vol/vol) in TBS-2% BSA (clone V9; Dako, Carpinteria, CA, USA) for 1 h at room temperature.

Collagen IV staining

To visualize the BM formation a goat polyclonal anti-collagen IV (Southern Biotechnology, Birmingham, AL, USA) was used. A protease type XIV pretreatment (8 min, 37°C) was performed to the slides before addition of primary antibody. Sections were incubated with the polyclonal antibody to collagen IV at 1:150 (vol/vol) in TBS-2% BSA for 16 h at 4°C.

Laminin 5 staining

Also, to visualize the BM formation, a rabbit polyclonal anti-laminin 5 (Dako, Denmark) was used. Slides were pretreated with protease type XIV (8 min, 37°C) before addition of primary antibody. Sections were incubated with the polyclonal antibody to laminin 5 at 1:800 (vol/vol) in TBS-2% BSA for 16 h at 4°C.

Transmission electron microscopy analysis

Samples of canine skin equivalents were processed for electron microscopy to evaluate the development of the dermal epidermal junction, as described earlier (21). Briefly, the samples were immersed in a solution containing 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4. They were then post-fixed in 1% osmium tetroxide. After post-fixation, the specimens were dehydrated in graded ethanol up to 100% and embedded in Spurr's low viscosity resin. Ultrathin sections were obtained with the ultramicrotome Leica EM UC6 (Leica Microsystems GmbH, Wetzlar, Germany), disposed onto 300 mesh cupper grids and stained with 2% uranyl acetate and Reynolds solution. Specimens were observed in a Hitachi H-7000 electron microscope (Hitachi, Schaumburg, IL, USA).

Results

Keratinocytes and fibroblasts isolation, culture and characterization

Dispase II treatment (epidermal removal) allowed the isolation of the keratinocytes from different canine skin samples. Cell populations showed viability ratios between 90% and 95% at the primary culture (Fig. 1). Keratinocyte cultures were divided for the first time after 10 days of culture. The subcultures grew slower and with variable viability ratio (85–90%). Therefore, to establish pure and viable keratinocytes cultures to introduce to the skin equivalents, primary cultures were used in all the experiments.

The enzymatic digestion of the skin was the chosen method to obtain dermal fibroblasts. This technique allowed the establishment of several pure fibroblasts lines, all of them with viability ratios between 95% and 98%. The different cell lines established from canine skin were divided for the first time after 7–10 days of culture and their subcultures presented a high viability ratio (94–98%) as well (Fig. 1).

Canine skin equivalents: histological, immunohistochemical and ultrastructural characterization

The specific collagen gel bio-matrix was successfully developed by incorporating of 4×10^4 fibroblasts/ml in 1.5 mg/ml rat tail type I collagen solution. Analysis of dermal and epidermal cells was performed every week during a 3-week period. In general, the developed collagen gel bio-matrix samples were able to grow in these conditions up to 3 weeks.

Skin equivalents were successfully developed by incorporating 5×10^5 keratinocytes/collagen gel bio-matrix. At day 4 of culture cells proliferate but there was no evidence of a structured multilayer epidermis, whereas it was already observed at 7 days of culture. At day 14 of culture, the structure of skin equivalents was much more defined and, despite they were grown for up to 3 weeks, the optimal growth period was observed after 2 weeks.

The epidermis harvested on day 14 was four cell layers thick with distinct basal, spinous and granular cell layers and a compact stratum corneum (Fig. 2). Fig. 2a–f shows convincingly the progress of keratinization. There were already clear signs of beginning stratification at day 4, showing denser alignment of basal cells, flattening of cells like in stratum spinosum, and hyaline granules in SG-cells with dead cells on the top. This seems to occur pretty early presumably due to high cell density or attachment rate. The presence of melanocytes every 10–15 keratinocytes approximately was also detected, as it is found in the normal skin.

The development of a clear basal membrane in the skin equivalents was not observed through the PAS nor the Fontana–Masson staining (data not shown). However, to assess whether the BM was formed under the culture conditions used, the presence and localization of major BM components, such as collagen IV and laminin 5, were

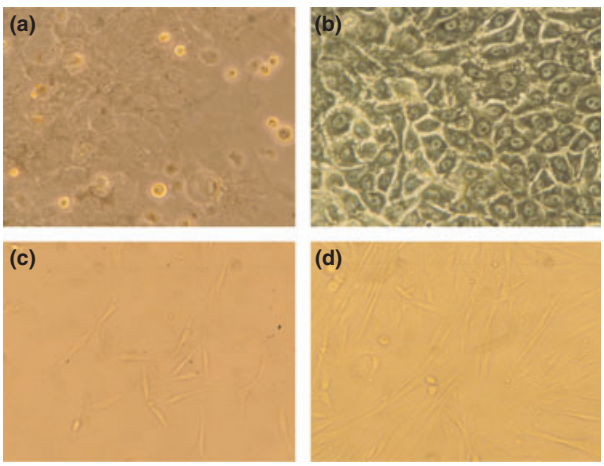

Figure 1. Isolated keratinocytes and fibroblasts from canine skin biopsies. (a,b) Isolated canine keratinocytes; (a) 4 days culture; (b) 9 days of culture. (c,d) Isolated canine skin fibroblasts; (c) 3 days of culture; (d) 10 days of culture. (a–d) 400×.

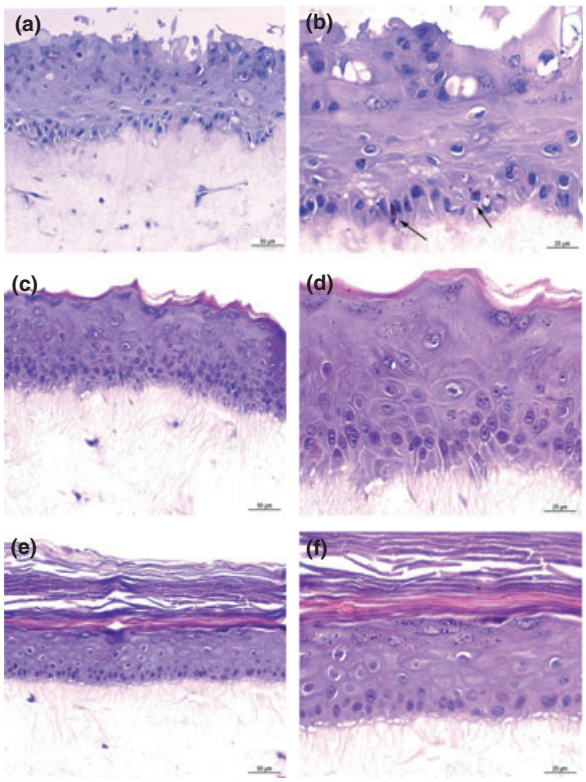

Figure 2. Morphological analysis of skin equivalents at different times of culture. (a,b) day 4 of culture; melanin is shown (arrows) between the basal keratinocytes; (c,d) day 7 of culture; (e,f) day 14 of culture. (a,c,d) 200×, scale bar: 50 μm; (b,e,f) 400×, scale bar: 25 μm.

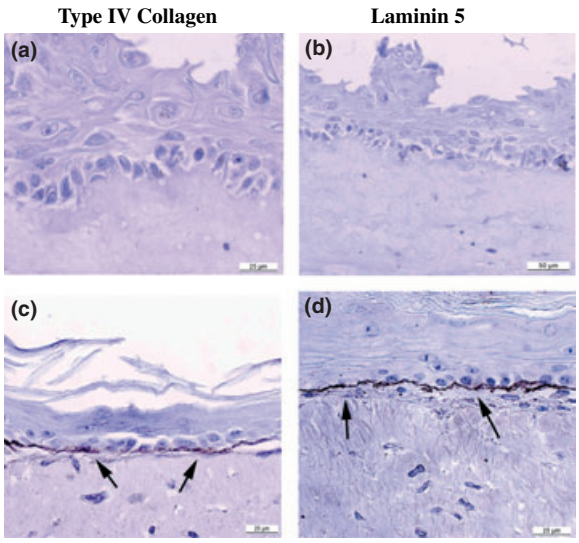

Figure 3. Basement membrane components analysis: type IV collagen and laminin 5 expression. (a,b) day 4 of culture. There was a lack of expression of both proteins; (c,d) day 14 of culture. Both collagen IV and laminin 5 expression were located at the dermal-epidermal junction (arrows). (a,c,d) 400×, scale bar: 25 μm. (b) 200×, scale bar: 50 μm.

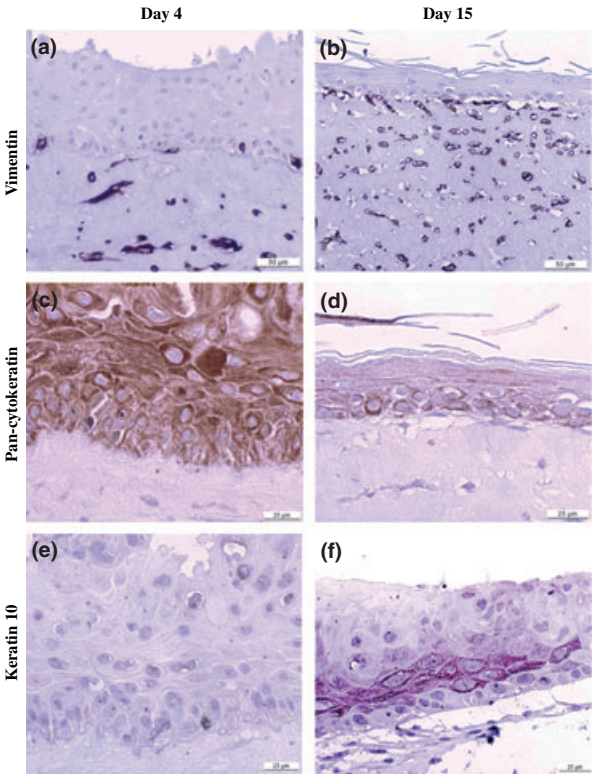

Figure 4. Vimentin and keratins expression in canine skin equivalents. (a,c,e) Day 4 of culture; (b,d,f) Day 15 of culture; (a,b) Vimentin immunohistochemistry; (c,d) Pan-cytokeratin immunohistochemistry; (e,f) Cytokeratin 10 immunohistochemistry; (a,b) 200×, scale bar: 50 μm; (c–f): 400×, scale bar: 25 μm.

examined. As detected by immunhistochemistry, the expression of both collagen IV and laminin 5 was confined to the epidermal–matrix junction from day 14, whereas there was no expression at day 4 (Fig. 3).

Canine skin equivalents were also studied with a specific antibody for vimentin (Fig. 4a,b), which is highly specific for cells of mesenchymal origin, like fibroblasts. Vimentin-immunoreactivity was detected in the fibroblasts of the skin equivalents dermis (collagen gel bio-matrix), which demonstrated that these fibroblasts had the same vimentin expression than the normal dermal fibroblasts have, whereas the lack of reactivity with the vimentin antibody by keratinocytes agreed with non-expression of vimentin by the normal epidermis.

The identification of keratinocytes of the skin equivalents was performed by the immunodetection of cytokeratins, which are specific markers for the epithelial differentiation. Cytoplasmic staining of keratins was already demonstrated at 4 days of culture by the pan-cytokeratin antibody (recognizing K5, 6, 8, 17 and 19), confirming the epidermis cells were pure keratinocytes (Fig. 4c,d). The lack of reactivity with the pan-cytokeratin antibody by the collagen gel bio-matrix agreed with non-expression of cytokeratins by the normal dermis. On the other hand, suprabasal cell layers of the skin equivalents expressed cytokeratin 10, a marker for terminal differentiation, were only seen at day 15 (Fig. 4e,f).

The ultrastructure of the skin equivalent was examined by transmission electron microscopy. Desmosomes and hemidesmosomes were already observed at the basal membranes of keratinocytes at 7 days of culture, with associated tonofilaments extending into the cytoplasm (Fig. 5). Basal cells opposite to the basal lamina appeared to have re-established the normal basal keratinocyte relationship with the basal lamina and exhibited a smooth inferior plasma membrane surface with variable numbers of hemidesmosomes spaced along its length at 2 weeks of culture. Ultrastructural analysis also revealed the presence of keratohyalin granules, desmosomes between the cells and a slight discontinuous lamina densa.

Discussion

In the present study a canine skin equivalent with well structured epidermal and dermal compartments, was successfully developed and characterized.

Several human and murine skin equivalent models have been developed up to the moment, but only few canine models have been described until now (19, 20, 22). Considering the lack of a well-characterized canine skin equivalent, our model was specifically designed to be used in investigations in the field of veterinary medicine.

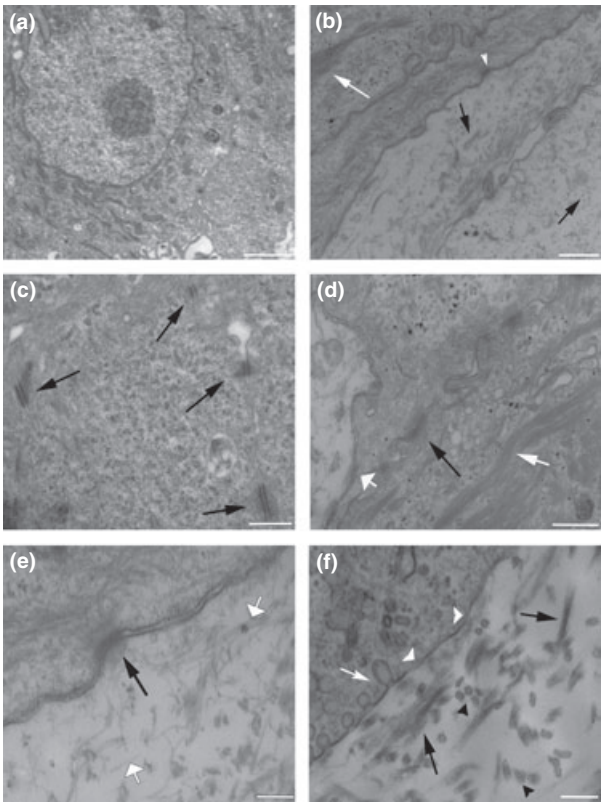

Figure 5. Ultrastructural characteristics of the canine skin equivalent model. (a) Basal keratinocyte from a 7-day skin equivalent is illustrated. (b) Dermal–epidermal junctional zone is disorganized in a 3 days skin equivalent, although some hemidesmosome is present (white arrow head). Also keratin fibrils (white arrow) and collagen fibrils (black arrows) can be seen. (c) Desmosomes (black arrows) between adjacent keratinocytes are already present in a 3-day skin equivalent. (d) Desmosomes (black arrow), keratin fibrils (white thin arrow), and hemidesmosomes (white thick arrow) are illustrated in basal keratinocytes from a 7-day skin equivalent. (e) At 15 days of culture a mostly continuous basal membrane and regularly structured hemidesmosomes (black arrow) are revealed. Collagen fibrils can be seen in cross and longitudinal section (white arrows). (f) At 21 days of culture basal membrane shows the formation of some caveoles (white arrow) and it starts showing some discontinuities (white arrow heads). Collagen fibrils can be seen in cross (black arrow head) and longitudinal section (black arrows). Scale bars: 2 μm (a), 1 μm (b), 500 nm (c,d,e) and 200 nm (f).

Keratinocytes from our skin equivalent underwent a differentiation to a multilayer epidermis composed of the four typical layers: *stratum basale, stratum spinosum, stratum granulosum* and *stratum corneum*, and epidermal cells were linked by desmosomes, as it was also shown at ultrastructural level. Epidermal normal thickness was already achieved at day 7 of culture. At this time, cornification was also evident. However, in another canine skin model developed with different growth culture conditions the well-formed stratum corneum was not present until day 21 (19).

Epidermal structure of our model was similar to that found in healthy skin, including a normal cornification process, a characteristic which is lacking in some other canine in vitro models. In the Norfolk terrier model, recently established to characterize the congenital keratinisation defect of this breed (19), organotypic cultures were grown in medium supplemented with EGF. This probably accounted for the hyperplasic and hyperkeratotic aspect of the epidermis. Moreover, these cultures showed a delayed differentiation, associated with an abnormal up-regulation of K16 expression. Besides these effects, other authors have reported a decreased expression of K10 in cell cultures supplemented with EGF (23, 24). In the present model, skin equivalent was developed in the absence of EGF, considered by Parish et al. 2005 (25) as the optimal culture condition for skin models, which allowed the obtaining of a more physiological structure. These specific culture conditions promoted the keratinocyte early differentiation, as shown by the expression of the early differentiation marker keratin 10 in all suprabasal cell layers, again like the normal canine epidermis.

The development of a dermo-epidermal junction in vitro and the proper differentiation of the epidermal layers are essential aspects required for further applications of skin equivalents.

In healthy skin, epidermal differentiation is linked to the underlying connective tissue. Thus, the composition and structural organization of the BM are crucial (26, 27), to preserve and regulate epidermal homeostasis (11, 28, 29), and it is also important for graft adhesion and stability (30). This highly specialized structure not only serves as a selective barrier but also provides a firm epidermal anchorage to the dermis at the same time. The BM structure represents a supramolecular assembly of several macromolecules including type IV collagen, laminins, perlecan and nidogen (26, 31).

In our model, epidermal cell–cell junctions, like desmosomes, were seen already at 3 days, whereas dermal–epidermal junction structures (stretches of lamina densa) became evident at 7 days of culture by transmission electron microscope. The presence of hemidesmosomes was also observed at the basal membranes of keratinocytes, with associated tonofilaments extending into the cytoplasm. However, a well-developed lamina densa was rarely observed by electron microscopy even in 14-day skin equivalents. Discontinuity in some areas of the lamina densa was observed, maybe due to the sample processing itself or because of the early development stage. Moreover, PAS staining did not reveal BM structure at 7 days of culture (data not shown), probably due to the lack of maturation of this structure. Discontinuity of BM has been also described in several human skin equivalent models (32, 33).

For the first time it was described an ultrastructurally defined dermal–epidermal junction in canine skin equiva-

lents, including anchoring fibrils for strong tissue connection, at 21 days of culture. It has been described that a fully mature BM ultrastructure appeared only after 3 weeks of cultivation of human SE (34, 35).

Immunohistochemical analysis of BM proteins was also performed. Protein markers of lamina lucida (laminin 5) and lamina densa (collagen IV) were specifically localized to the dermal–epidermal junction. Approximately 80–90% of the collagen in the healthy skin is type I collagen, whereas type IV collagen is confined to the basal lamina of the dermal–epidermal junction (36), vessels and epidermal appendages. It has been described that BM formation depends not only on the presence of the macromolecular components, such as collagen type IV and laminin, but also on the culture conditions (32). In our cultures, to facilitate collagen (proline) hydroxylation growth medium was supplemented with both vitamins C and E, which had been demonstrated not only to have skin barrier-stabilizing properties but also to protect against DNA damage, as well as promoted early development of BM in human skin equivalents (25, 37–39). The lack of vitamins C and E supplementation in the skin equivalents growth media caused a poor development of our canine skin equivalents (data not shown).

Arachidonic, palmitic and linoleic free fatty acids, in conjunction with the antioxidant α-tocopherol acetate, were also added to nutrient media of canine skin equivalents to provide precursors of epidermal barrier lipids and promote increased epithelial morphogenesis of the skin equivalents (40). The formation of stratum corneum barrier lipids in human cultured skin substitutes is enhanced by the presence of ascorbic acid and linoleic acid in the incubation medium, and, therefore, they play a key role for obtaining a permeability barrier close to normal skin (17, 35). Recently, a new combination of five nutrients (pantothenate, choline, nicotinamide, histidine and inositol) has been shown to up-regulate epidermal lipid synthesis and its function in canine epidermal skin barrier (41).

Canine skin equivalents were grown in vitro up to 3 weeks, with an optimal growth period of 2 weeks, when the epidermis was already composed of the distinct basal, spinous and granular cell layers and a compact stratum corneum.

The culture conditions for establishing a human reconstructed epidermis have been gradually modified over the years. The most significant changes have been the omission of serum, reduction of growth factor concentration and vitamin supplementation (17, 42). Under these modified conditions, a human skin equivalent that shows similarity with the native tissue from which it was derived is formed after 14 days of culture and can be grown for 6–7 weeks before senescence. At this time point, the keratinocyte proliferation ceases, which results in thinning of the viable epidermal layer and thickening of the stratum corneum (6).

In conclusion, this model is ideally suited to elucidate the regulatory mechanisms of cell–cell and cell–matrix interactions in canine basic skin research. Skin equivalents also offer an attractive approach for studies of epidermal and dermal characteristics after different conditions.

The applicability of the presently developed canine skin equivalent is mainly the investigation on skin biology, pathology, pharmacology and nutrition. It constitutes a useful approach for studies on the biology of the canine epidermis and dermis, specially considering that our model displays a normal appearing dermal–epidermal junction and a normal cornification process.

Despite some limitations, which are also present in the different human and mouse skin equivalent models, like the lacking of hair follicles, inflammatory cells, nerves and blood vessels (6, 33, 43), this model opens a wide field of application possibilities, from transplantation, over the wound healing, up to the employment as an in vitro test system. For all of these possible applications, a further functional characterization of this canine skin equivalent model would be necessary.

Acknowledgements

The authors thank the skin veterinary donors ('Centre d'Acollida d'Animals de Companyia del Vallès Oriental', 'Arvivet Veterinaris' and the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona) for their co-operation in providing skin biopsy samples, and Dr. A. El Ghalbzouri for his valuable help.

References

1 Wysocky A B. Skin anatomy, physiology, and pathophysiology. Nurs Clin North Am 1999: **34**: 777–797.
2 Loomis C A. Development and morphogenesis of the skin. Adv Dermatol 2001: **17**: 183–210.
3 Freedberg I M, Eisen A Z, Wolff K, Austen K F, Goldsmith L A, Katz S I. Fitzpatrick's Dermatology in general medicine. 6th edn, Vol. 1. USA: McGraw Hill, 2003.
4 Ghohestani R F, Li K, Rouselle P, Uitto J. Molecular organization of the cutaneous basement membrane zone. Clin Dermatol 2001: **19**: 551–562.
5 Bell E, Rosenberg M, Kemp P *et al.* Recipes for reconstituting skin. J Biomech Eng 1991: **113**: 113–119.
6 Ponec M. Skin constructs for replacement of skin tissues for in vitro testing. Adv Drug Deliv Rev 2002: **54**: S19–S30.
7 Damour O, Augustin C, Black A F. Applications of reconstructed skin models in pharmaco-toxicological trials. Med Biol Eng Comput 1998: **36**: 825–832 (Review).
8 Sorensen J C. Living skin equivalents and their application in wound healing. Clin Podiatr Med Surg 1998: **15**: 129–137 (Review).
9 Smola H, Thiekotter G, Fusenig N E. Mutual induction of growth factor gene expression by epidermal–dermal cell interaction. J Cell Biol 1993: **122**: 417–429.

10 Ponec M, Weerheim A, Kempenaar J, Mommaas A M, Nugteren D H. Lipid composition of cultured human keratinocytes in relation to their differentiation. J Lipid Res 1988: **29**: 929–961.

11 Maas-Szabowski N, Stark H J, Fusenig N E. Keratinocyte growth regulation in defined organotypic cultures through IL-1-induced keratinocyte growth factor expression in resting fibroblasts. J Invest Dermatol 2000: **114**: 1075–1084.

12 El Ghalbzouri A, Gibbs S, Lamme E, Van Blitterswijk C A, Ponec M. Effect of fibroblasts on epidermal regeneration. Br J Dermatol 2002a: **147**: 230–243.

13 El Ghalbzouri A, Lamme E, Ponec M. Crucial role of fibroblasts in regulating epidermal morphogenesis. Cell Tissue Res 2002b: **310**: 189–199.

14 Merne M, Syrjanen S. The mesenchymal substrate influences the epithelial phenotype in a three-dimensional cell culture. Arch Dermatol Res 2003: **295**: 190–198.

15 Asselineau D, Bernhard B, Bailly C, Darmon M. Epidermal morphogenesis and induction of the 67 kD keratin polypeptide by culture of human keratinocytes at the liquid–air interface. Exp Cell Res 1985: **159**: 536–539.

16 Boyce S T, Christianson D J, Hansbrough J F. Structure of a collagen-GAG dermal skin substitute optimized for cultured human epidermal keratinocytes. J Biomed Mater Res 1988: **22**: 939–957.

17 Ponec M, Weerheim A, Kempenaar J *et al.* The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. J Invest Dermatol 1997: **109**: 348–355.

18 Loomis J. Anatomy, histology and immunohistochemistry of normal human skin. Eur J Dermatol 2002: **12**: 390–399.

19 Barnhart K F, Credille K M, Ambrus A, Dunstan R W. Preservation of phenotype in an organotypic cell culture model of a recessive keratinisation defect of Norfolk terrier dogs. Exp Dermatol 2005: **14**: 481–490.

20 Magnol J P, Pin D, Palazzi X, Lacour J P, Gache Y, Meneguzzi G. Characterization of a canine model of dystrophic bullous epidermolysis (DBE). Development of a gene therapy protocol. Bull Acad Natl Med 2005: **189**: 107–121.

21 van der Meulen J, van den Bergh B A, Mulder A A, Mommaas A M, Bouwstra J A, Koerten H K. The use of vibratome sections for the ruthenium tetroxide protocol: a key for optimal visualization of epidermal lipid bilayers of the entire human stratum corneum in transmission electron microscopy. J Microsc. 1996: **184**: 67–70.

22 Jakic-Razumovic J, Storb R, Sandmaier B M, Sale G E. An organotypic skin culture model in dogs. A model for transplantation immunopathology in canine systems. Transplantation 1994: **57**: 285–287.

23 Gibbs S, Silva Pinto A N, Murli S, Huber M, Hohl D, Ponec M. Epidermal growth factor and keratinocyte growth factor differentially regulate epidermal migration, growth, and differentiation. Wound Repair Regen 2000: **8**: 192–203.

24 Andreadis S T, Hamoen K E, Yarmush M L, Morgan J R. Keratinocyte growth factor induces hyperproliferation and delays differentiation in a skin equivalent model system. FASEB J 2001: **15**: 898–906.

25 Parish W E, Read J, Paterson S E. Changes in basal cell mitosis and transepidermal water loss in skin cultures treated with vitamins C and E. Exp Dermatol 2005: **14**: 684–691.

26 Breitkreutz D, Mirancea N, Schmidt C *et al.* Inhibition of basement membrane formation by a nidogen-binding laminin gamma1-chain fragment in human skin-organotypic cocultures. J Cell Sci 2004: **117**: 2611–2622.

27 Watt F M. Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J 2002: **21**: 3919–3926 (Review).

28 Fusenig N E, Limat A, Stark H J, Breitkreutz D. Modulation of the differentiated phenotype of keratinocytes of the hair follicle and from epidermis. J Dermatol Sci 1994: **7**: S142–S151 (Review).

29 Maas-Szabowski N, Shimotoyodome A, Fusenig N E. Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism. J Cell Sci 1999: **112**: 1843–1853.

30 Germain L, Guinard R, Rouabhia M, Auger F A. Early basement membrane formation following the grafting of cultured epidermal sheets detached with thermolysin or Dispase. Burns 1995: **21**: 175–180.

31 Timpl R. Macromolecular organization of basement membranes. Curr Opin Cell Biol 1996: **8**: 618–624 (Review).

32 Chamson A, Germain N, Claudy A, Perier C, Frey J. Study of basement membrane formation in dermal-epidermal recombinants in vitro. Arch Dermatol Res 1989: **281**: 267–272.

33 Mirastschijski U, Bugdahl R, Rollman O, Johansson B R, Agren M S. Epithelial regeneration from bioengineered skin explants in culture. Br J Dermatol 2006: **154**: 42–49.

34 Smola H, Stark H J, Thiekotter G, Mirancea N, Krieg T, Fusenig N E. Dynamics of basement membrane formation by keratinocyte–fibroblast interactions in organotypic skin culture. Exp Cell Res 1998: **239**: 399–410.

35 Stark H J, Baur M, Breitkreutz D, Mirancea N, Fusenig N E. Organotypic keratinocyte cocultures in defined medium with regular epidermal morphogenesis and differentiation. J Invest Dermatol 1999: **112**: 681–691.

36 Burgeson R E, Christiano A M. The dermo-epidermal junction. Curr Opin Cell Biol 1997: **9**: 651–658.

37 Boyce S T, Supp A P, Swope V B, Warden G D. Vitamin C regulates keratinocyte viability, epidermal barrier, and basement membrane in vitro, and reduces wound contraction after grafting of cultured skin substitutes. J Invest Dermatol 2002: **118**: 565–572.

38 Thiele J J, Hsieh S N, Ekanayake-Mudiyanselage S. Vitamin E: critical review of its current use in cosmetic and clinical dermatology. Dermatol Surg 2005: **31**: 805–813 (Review).

39 Placzek M, Gaube S, Kerkmann U *et al.* Ultraviolet B-induced DNA damage in human epidermis is modified by the antioxidants ascorbic acid and D-alpha-tocopherol. J Invest Dermatol 2005: **124**: 304–347.

40 Boyce S T, Williams M L. Lipid supplemented medium induces lamellar bodies and precursors of barrier lipids in cultured analogues of human skin. J Invest Dermatol 1993: **101**: 180–184.

41 Watson A L, Fray T R, Bailey J, Baker C B, Beyer S A, Markwell P J. Dietary constituents are able to play a beneficial role in canine epidermal barrier function. Exp Dermatol 2006: **15**: 74–81.

42 Gibbs S, Backendorf C, Ponec M. Regulation of keratinocyte proliferation and differentiation by all-trans-retinoic acid, 9-cis-retinoic acid and 1,25-dihydroxy vitamin D3. Arch Dermatol Res 1996: **288**: 729–738.

43 Casasco M, Carnaglia A I, Zerbinati N, Casasco A, Calligaro A. Morphological aspects of an artificial skin. Ital J Anat Embryol 2001: **106**: 239–249.