

Three-Dimensional Culture of Keratinocytes and the Formation of Basement Membrane for Canine Footpad Substitute

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(Received 20 July 2006/Accepted 22 February 2007)

ABSTRACT. A pad equivalent for a dog was prepared as a substitute for the loss of footpad. In addition to the time course of formation on epidermal morphogenesis, we investigated expressions of α_6 integrin subunit as adhesive molecule, and laminin and type IV and VII collagens as extracellular matrices of basement membrane components. Epithelium of the pad equivalent was thick enough to be easily confirmed at 5 days at the air-liquid interface, but many creases appeared on it at 7 days, and it shrank at 10 and 14 days. Keratinocytes were increased in 4 to 5 cell layers at 1 day at the air-liquid interface, differentiating into basal cell layer. Granular and corneal cell layers were confirmed until 5 days, and maintained their shape at least until 14 days. Alpha 6 integrin was expressed at almost the same fluorescent intensity as native pad tissue at 1 day at the dermal-epidermal junction. Laminin and type IV collagen were intermittently expressed at 5 and 10 days, respectively, at the dermal-epidermal junction, and at 14 days the fluorescence showed almost the same intensity as native pad tissue. The expression of type VII collagen was discontinuous at 2 days at the dermal-epidermal junction, but remained as it was at 14 days. The present findings suggested that although the formation of anchoring fibrils in basement membrane was incomplete, the pad equivalent in the dog was reconstructed similar to a native pad by epidermal morphogenesis.

KEY WORDS: air-liquid interface, canine, epidermis, footpad, keratinocyte.

J. Vet. Med. Sci. 69(6): 611–617, 2007

The footpad of dogs is indispensable for a weight-bearing surface just as the heel is in humans. Footpad tissue forms a thick corneal cell layer, functioning to absorb the normal force received from the ground and to resist the frictional force of ambulation [5, 16]. It is often injured by trauma such as laceration and abrasion that require surgical treatment [33]. It is known that the optimal tissue reconstructing the injured pad is only the footpad tissue from other intact sites since haired skin can not bear such stress generated during ambulation [16]. When each of the metacarpal or metatarsal pads is severely injured, segmental pad grafting or pedicle pad replacement using the second or the fifth digital pad can be performed [9, 24, 32]. In addition, if more extensive damage involving complete loss of all the footpads of a limb occurs, segmental pad grafting or delayed reimplantation using bipedicle direct flap has been reported to succeed [8, 25]. However, these surgical methods of grafting require a large amount of footpad tissue with limited availability from donor sites. To resolve this problem, it is desirable to develop a footpad substitute grown *in vitro* using autologous pad tissue collected.

Recent advances in regenerative medicine have made cultured epithelial sheet grafting available for patients suffering from large skin defects such as thermal burns, resulting in a low take rate less than 50% [28]. On the other hand, a skin equivalent Bell *et al.* [6] devised in 1983 has been clinically utilized as a skin substitute for acute and chronic wound closure [13, 22, 28]. It is comparatively easy to handle and has the advantage of a higher take rate than cultured epithelial sheet. It consists of a dermal equivalent made up of fibroblasts in a collagen matrix and an epidermis that is

differentiated from keratinocytes seeded on the dermal equivalent [3, 4, 7, 15, 26]. To prepare skin equivalents, dermal fibroblasts are needed not only in the proliferation and the differentiation of keratinocytes [10, 35], but also in the formation of basement membrane components [2, 11, 14, 18, 20]. Basement membrane at the dermal-epidermal junction is mainly constituted with laminin, and type IV and VII collagens, adhering to basal cell *via* hemidesmosome composed of $\alpha_6\beta_4$ integrin, and to dermis *via* anchoring fibrils composed of type VII collagen [20]. Epithelium of skin equivalent forms a multilayered structure which is similar to that of normal skin [11, 12, 15, 34]. In addition, skin equivalent also forms basement membrane including the expression of $\alpha_6\beta_4$ integrin, laminin, and type IV and VII collagens [2, 4, 12, 20, 29].

The present study deals with the preparation of a pad equivalent in the dog as a pad substitute, while investigating the time course of the epidermal formation and the expression of α_6 integrin, laminin, and type IV and VII collagens in basement membrane zone components.

MATERIALS AND METHODS

Animals: Four clinically healthy female beagle dogs (2 to 3 years old, weighing 7 to 10 kg) were used in this study. These dogs were treated in accordance with the guideline approved by the Animal Use Committee of Gifu University.

Harvest and isolation of fibroblasts: Each dog was sedated with medetomidine before maintaining anesthesia with oxygen under isoflurane inhalation. One of the carpal pads was cleansed and disinfected after clipping hair around

it. A full-thickness pad section was obtained by use of $\Phi 6$ mm biopsy trephine (Biopsy punch, Kai Industries Co., Ltd., Gifu, Japan) and was transferred into a dish with Dulbecco's Ca(-)Mg(-) phosphate-buffered saline (PBS(-)). The wound was closed by suture before covering with dressing materials. The pad section was cleansed in a dish containing a 2% povidone-iodine solution (Meiji Seika, Co., Tokyo, Japan) from which the dermal connective tissue was trimmed off. The pad sample was cut into 8 pieces so as to leave the dermal side face down in a dish for 30 min. After that, 5 ml of Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (FBS, lot No. 2B0018, JRH Biosciences, Lenexa, KS, U.S.A.) was gently poured into the dish to be incubated at 37°C in a humid 5% CO₂ atmosphere. The medium was changed every 3 days. When colonies reached 70 to 80% confluence in 10 days after confirming migration of fibroblasts from the pad pieces, the fibroblasts were passaged. The fifth to tenth passage was used in this study.

Harvest and isolation of keratinocytes: Each dog identical with the one harvested for the fibroblasts was anesthetized under the same conditions described above. Both metacarpal and metatarsal pads were cleansed and disinfected after clipping hair around those sites. Six full-thickness pad sections were obtained by use of $\Phi 6$ mm biopsy trephine and were transferred into a dish with PBS(-). The wounds were closed by suture before covering with dressing materials. After the removal of dermal connective tissue and 5 to 6 rinsings in PBS(-), all the pad sections were cut into 4 pieces, respectively. They were incubated at 4°C for 12 to 16 hr in a dish containing 25 mg/ml dispase (Godo Shuzo Co., Tokyo) prepared in PBS(-). The epidermis was then separated from the dermis and placed in a flask containing 0.125% trypsin/0.5 mM EDTA solution (Gibco, Grand Island, NY, U.S.A.). After gently stirring for 20 min, 10 ml of 60% FBS/PBS (-) was added followed by centrifugation at 200 g for 5 min. The supernatant was discarded, and the pellet was washed one more time with PBS(-). After determination of the cell viability and the number by trypan blue staining, the cells were resuspended in William's medium E supplemented with 10% Nu-serum IV (Becton & Dickinson Lab., Franklin Lakes, NJ, U.S.A.), 10⁻¹⁰ M cholera toxin (Sigma), and 2 mM L-glutamine (Gibco). Then, 1 to 2 × 10⁶ cells in 5 ml were plated on a 60 mm collagen dish and incubated at 37°C in a humid 5% CO₂ atmosphere. The medium was changed initially at 4 days and then every 2 days. Passage two was used in this study.

Organotypic cultures: Organotypic cultures of keratinocytes on a collagen gel involved with fibroblasts were established in accordance with the method of Jakic-Razumovic *et al.* [17]. Briefly, the dermal equivalent collagen gel was prepared as follows. Seven volumes of type I collagen solution (Cellmatrix Type I-A, Nitta Gelatin Inc., Osaka, Japan) were mixed with one volume of E tissue medium (DMEM : Ham's F-12=3:1), one volume of a reconstructive buffer (2.2 g NaHCO₃ and 4.77 g HEPES in 100 ml 0.05 N NaOH, Nitta Gelatin Inc.) and one volume of

1 to 2 × 10⁶ cells/ml fibroblasts (5th to 10th passage) in FBS on ice. Dermal equivalent solution was poured into a culture insert dish with a nitrocellulose membrane base (Cell culture insert 6 wells, Becton & Dickinson Lab.). It was placed at 37°C in a humid 5% CO₂ atmosphere for 1 hr to induce gel formation in the insert. Then the gelatinized dermal equivalent was overlaid with 0.5 × 10⁶ keratinocytes in 1 ml DMEM supplemented with 10% FBS, 10⁻⁸ M retinoid acid (Sigma), 0.4 µg/ml hydrocortisone, 0.1 nM cholera toxin, 5 µg/ml transferrin (Sigma), 2 nM 3,3'-5 tri-iodothyronine (Sigma), 10 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin (WAKO, Osaka), incubating at 37°C in a humid 5% CO₂ atmosphere for 5 to 6 days. After that, the medium was changed into DMEM supplemented with 10% FBS and 0.4 µg/ml hydrocortisone every day. When the keratinocytes were 80 to 90% confluent on the surface of the collagen gel, the medium was removed to wash the organotypic cultures with PBS(-). Then an insert dish was placed in an outer dish, in which filter paper was laid at the base, and the medium was poured into the outer dish so as not to submerge the collagen gel. The organotypic cultures were incubated at 37°C in a humid 5% CO₂ atmosphere for 14 days.

Gross and histological examination: Epidermal morphological findings were observed at 0, 1, 2, 3, 4, 5, 6, 7, 10, and 14 days after culturing at the air-liquid interface. Each organotypic culture was fixed in 10% formalin and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin and eosin.

Immunofluorescence analysis: The expressions of cell adhesion molecule (α_6 integrin subunit) and basement membrane components (laminin, type IV, and VII collagens) were observed at 0, 1, 2, 3, 4, 5, 6, 7, 10, and 14 days after culturing at the air-liquid interface by indirect immunofluorescence (IIF). Each culture was embedded in a fixative (OCT compound, TISSUE-TEK; Elkhart, IN, U.S.A.). Five-micrometer-thick frozen sections, which were obtained at -20°C, were air-dried and then fixed in acetone at 4°C for 10 min. The primary antibodies used in this study were: rat anti-human CD49f [integrin α_6] monoclonal antibody (Chemicon, Temecula, CA, U.S.A.) at a dilution of 1:100; rabbit anti-laminin polyclonal antibody (Chemicon) at a dilution of 1:100; polyclonal antibody to collagen type IV (Quartett Immunodiagnostika und Biotechnologie GmbH, Berlin, Germany) at a dilution of 1:10; and monoclonal anti-collagen type VII (Sigma) at a dilution of 1:100. After incubation with primary antibodies at 4°C overnight, sections were washed with PBS(-) and then incubated with FITC-conjugated goat anti-rat IgG (Zymed Laboratories Inc., San Francisco, CA, U.S.A.), goat anti-rabbit IgG (Zymed) and goat anti-mouse IgG (Zymed) for 1 hr at 4°C. The sections were again washed in PBS(-) and mounted with 10% glycerin before examining under the fluorescence microscope (Carl Zeiss, Oberkochen, Germany). As negative control, PBS(-) was used instead of each primary antibody.

RESULTS

Gross and histological appearances of pad equivalent: Figure 1 shows the time course of gross appearance of the pad equivalent after culturing at the air-liquid interface. The epithelium of the pad equivalent presents a smooth surface, but is difficult to confirm at 0 day (Fig. 1-A). The epidermal surface of the pad equivalent is dried at 1 day and becomes

thick at 5 days (Fig. 1-B, C). After creasing at 7 days (Fig. 1-D), the epidermal equivalent on collagen gel shrank so as to be readily confirmed at 10 and 14 days (Fig. 1-E, F). Figure 2 shows the time course of histological appearance of the pad equivalent after culturing at the air-liquid interface. Keratinocytes are piled up into only 2 to 3 cell layers on the dermal equivalent at 0 day (Fig. 2-A). At 1 day, epithelium becomes stratified into 4 to 5 cell layers, in which the basal

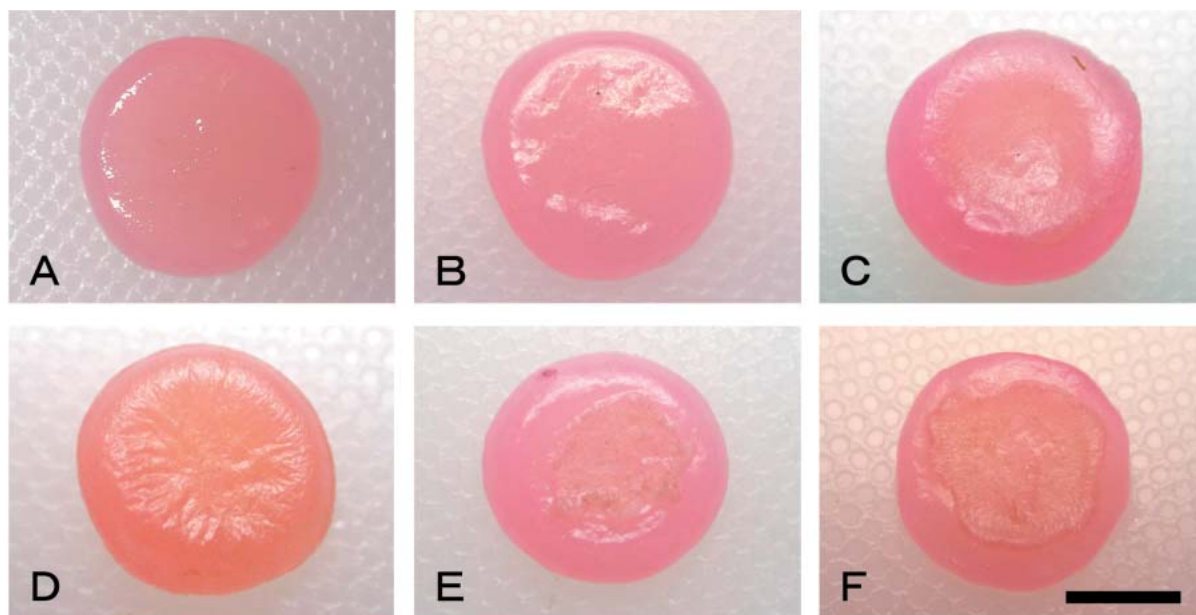


Fig. 1. Photographs of pad equivalents culturing at air-liquid interface for (A) 0, (B) 1, (C) 5, (D) 7, (E) 10, and (F) 14 days, respectively. Bar=5 mm.

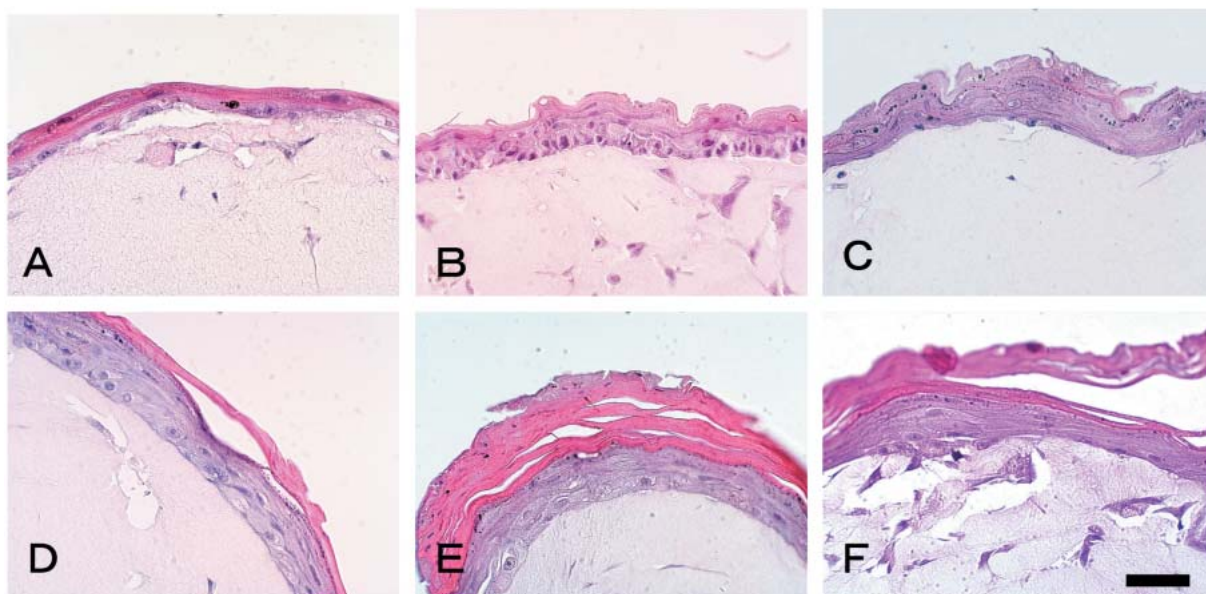


Fig. 2. Photomicrographs of pad equivalents culturing at the air-liquid interface for (A) 0, (B) 1, (C) 3, (D) 4, (E) 5, and (F) 14 days, respectively. Hematoxylin and eosin stain. Bar=50 μ m.

cell layer is confirmed (Fig. 2-B). At 3 days, epithelium became stratified into 5 to 6 cell layers, in which basal and granular cell layers were confirmed (Fig. 2-C). The corneal cell layer appeared at 4 days, and became thick at 5 days (Fig. 2-D, E). After that, the corneal cell layer was maintained until 14 days without exfoliation (Fig. 2-F).

Immunofluorescence analysis on basement membrane formation of pad equivalent: Time courses of the expressions on α_6 integrin subunit, laminin, type IV and VII collagens after culturing at the air-liquid interface are shown in Figs. 3 to 6. The expression of α_6 integrin subunit is continuously observed at the dermal-epidermal junction in native pad tissue (Fig. 3-A). On the pad equivalent, α_6 integrin subunit is intermittently expressed even at 0 day at the dermal-epidermal junction (Fig. 3-B). The fluorescence of α_6 integrin subunit is continuously observed from 1 day through 14 days shows as much intensity as native pad tissue (Fig. 3-C). The expression of laminin is continuously observed at the dermal-epidermal junction in native pad tissue (Fig. 4-A). On the pad equivalent, laminin is discontinuously expressed from 5 through 10 days at the dermal-epidermal junction (Fig. 4-B). After that, the fluorescence of laminin is continuously observed at 14 days with as much intensity as native pad tissue (Fig. 4-C). The expression of type IV collagen is continuously observed at the dermal-epidermal junction in native pad tissue (Fig. 5-A). On the pad equivalent, the fluorescence of type IV collagen started to be discontinuously observed at 10 days at the dermal-epidermal junction (Fig. 5-B). At 14 days, the fluorescence of type IV collagen continuously shows as much intensity as native pad tissue (Fig. 5-C). The expression of type VII collagen is continuously observed at the dermal-epidermal junction in native pad tissue (Fig. 6-A). On the pad equivalent, type VII collagen fluorescence is intermittently observed at 2 days at the dermal-epidermal junction (Fig. 6-B). The intensity of the fluorescence is increased from 6 through 10 days (not shown). The fluorescence is still discontinuously observed at the dermal-epidermal junction at 14 days, but the intensity is inferior to that of native pad tissue (Fig. 6-C).

DISCUSSION

The epithelium of the pad equivalent, which was spread over collagen gel, was dried on the surface at 1 day at the air-liquid interface, and at 5 days it became thick enough to be readily confirmed. The skin equivalent based on this study has almost the same thickness as native skin, and is easier to handle than the epidermal sheet in the grafting [28, 30]. Moreover, the thick corneal cell layer of the skin equivalent functioned as a barrier against the external stresses on the graft, and that gives rise to a high take rate [28]. As for reconstruction of a pad whose structure is similar to skin, a thick corneal cell layer is supposed to be favorable for the subsequent grafting. The pad equivalent prepared in this study was thick enough to be clearly observed at 5 days, considering that it was applicable to the grafting. But there were many creases on the epithelium, which shrank at 10

and 14 days. Moreover, the epidermal part of the pad equivalent was mobile at 14 days, indicating that the adhesion at the dermal-epidermal junction was insufficient.

It is known that keratinocytes seeded on a collagen gel containing fibroblasts only proliferate to be piled up in submerged culture, while they are lifted up to the air-liquid interface to be multilayered and differentiated [3, 21]. In the present study, keratinocytes on a collagen gel proliferated and became confluent in submerged culture before differentiating at the air-liquid interface. Consequently, keratinocytes only piled up in 2 to 3 layers at 0 day at the air-liquid interface, and at 1 day they increased 4 to 5 layers so as to differentiate into the basal cell layer. The epithelium then became 5 to 6 layers including the granular cell layer at 3 days. The corneal cell layer, which appeared at 4 days, became thick at 5 days, maintaining the morphology until at least 14 days. Shirakata *et al.* [30] prepared a human skin equivalent, in which the number of layers of keratinocytes were 1 to 2 prior to culturing at the air-liquid interface, then differentiating into 5 to 8 layers until 7 days, and maintaining the shape at least until 14 days. In addition, since the structure of the epithelium of canine skin equivalent Jakic-Razumovic *et al.* [17] prepared was similar to that of normal skin in 8 days, it was considered that the epithelium of the pad equivalent was generally developed at the air-liquid interface in about one week. But they indicate at the same time that the skin equivalent does not form rete ridges as well as the pad equivalent prepared in this study [17]. Moreover, Limat *et al.* [19] also reported that the epithelium of the human palmar skin equivalent developed a thick corneal cell layer, although not forming rete ridges without the grafting onto nude mouse. Therefore, in the present study, it was suggested that the structure of epithelium of the pad equivalent in the dog was generally developed, but it was difficult to prepare the pad equivalent *in vitro* comparable to a native pad.

Since basement membrane, which is at the dermal-epidermal junction, not only mediates the adhesion to each of them, but also influences the proliferation and the differentiation of keratinocytes [1], the time course of the expressions on α_6 integrin subunit as adhesive molecule, and laminin, and type IV and VII collagens as extracellular matrices were investigated during the formation of pad equivalent. Alpha 6 integrin subunit and laminin intermittently expressed at 0 and 5 days at the dermal-epidermal junction, respectively. Since α_6 integrin subunit mediates signal transduction from dermal extracellular matrix to keratinocytes to promote the reepithelialization such as the proliferation of keratinocytes and epidermal differentiation [23], the early expression of α_6 integrin subunit in this study could be supposed to be related to subsequent epidermal formation. In addition, laminin 5 bound to α_6 integrin subunit expressed at 2 days at the dermal-epidermal junction on human skin equivalent as reported by Amano *et al.* [2]. The expression of laminin in this study was observed at 5 days when the epidermal morphogenesis had almost been completed, but the reason was not known. It followed that type

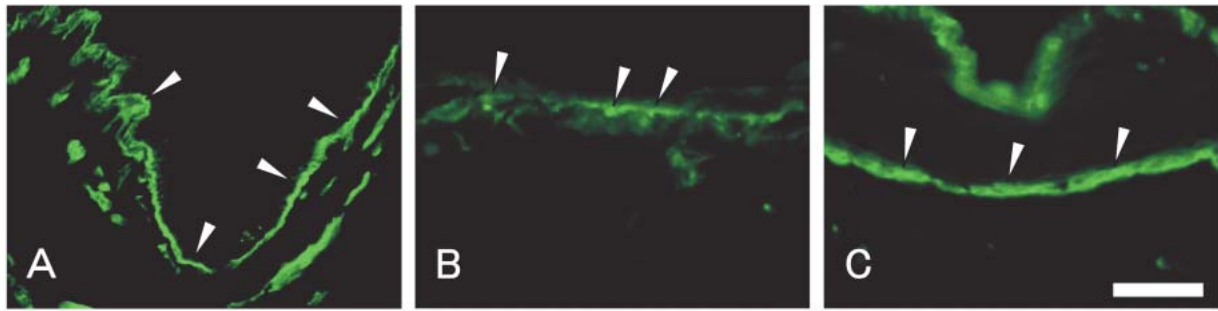


Fig. 3. Photomicrographs of immunofluorescence of α_6 integrin subunit in native pad tissue for (A) control, and in pad equivalents for (B) 0, and (C) 14 days, respectively. Arrowheads indicate α_6 integrin subunit. Bar=100 μm .

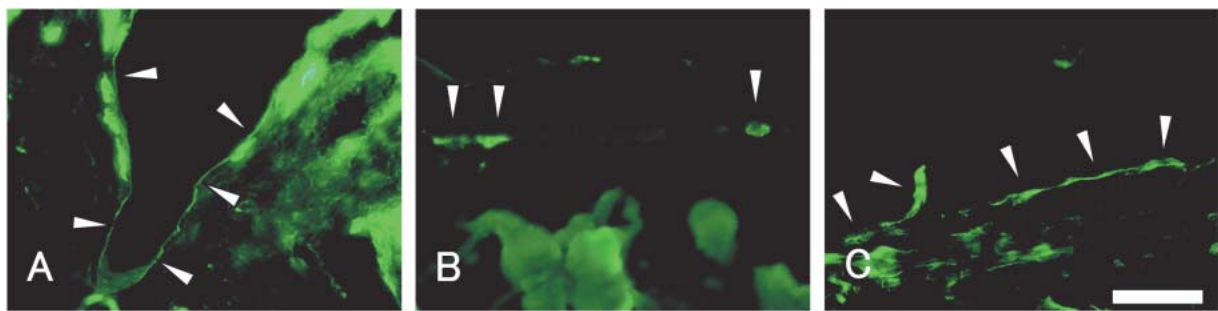


Fig. 4. Photomicrographs of immunofluorescence of laminin in native pad tissue for (A) control, and in pad equivalents for (B) 5, and (C) 14 days, respectively. Arrowheads indicate laminin. Bar=100 μm .

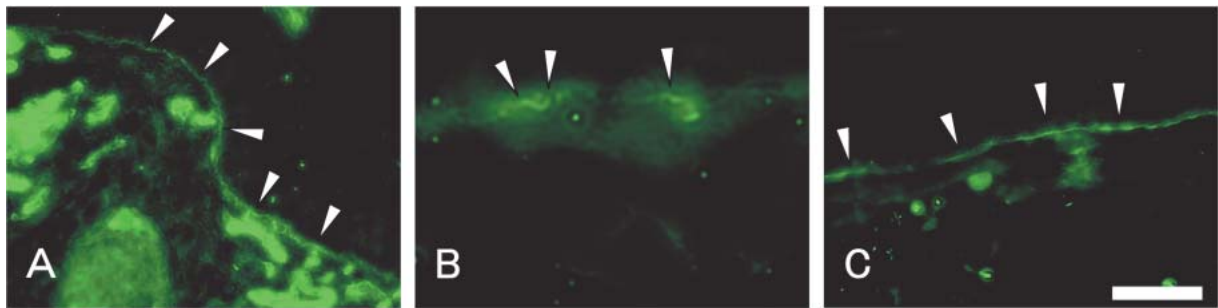


Fig. 5. Photomicrographs of immunofluorescence of type IV collagen in native pad tissue for (A) control, and in pad equivalents for (B) 10, and (C) 14 days, respectively. Arrowheads indicate type IV collagen. Bar=100 μm .

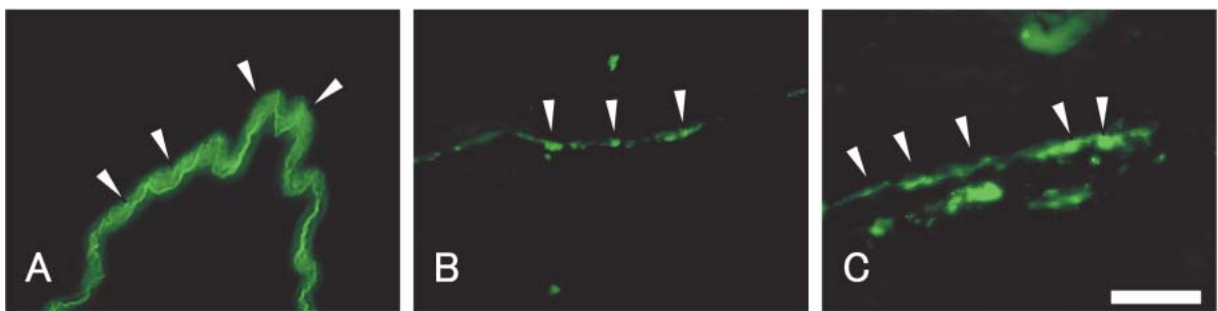


Fig. 6. Photomicrographs of immunofluorescence of type VII collagen in native pad tissue for (A) control, and in pad equivalents for (B) 2, and (C) 14 days, respectively. Arrowheads indicate type VII collagen. Bar=100 μm .

IV collagen, the main component of basement membrane, discontinuously expressed at 10 days at the dermal-epidermal junction, at which the time of expression almost coincided with the one reported by Amano *et al.* [2]. The expression of type VII collagen was intermittently observed at 2 days at the dermal-epidermal junction, and remained as it was until 14 days.

Amano *et al.* [2] also reported that type VII collagen was intermittently expressed at 5 days at the dermal-epidermal junction, but it was not continuously confirmed at 14 days. However, in the human skin equivalent, Smola *et al.* [31] reported that in spite of type VII collagen being expressed at 7 days and still being discontinuous at 14 days, it became continuous at 21 days. Moreover, ultrastructural observation revealed that basement membrane including anchoring fibrils mainly composed of type VII collagen was completely formed at 21 days. In this way, the formation of basement membrane including anchoring fibrils was required for at least 3 to 4 weeks [27, 31], suggesting that its formation, including anchoring fibrils, was incomplete at 14 days on the pad equivalent prepared in this study.

After the grafting, considering that the structural improvement of epithelium of skin equivalent is dependent on the formation of basement membrane [19], an additional 1- to 2-week culturing at the air-liquid interface might be expected to form a more secure basement membrane. But it is not practical for clinical use because of the long preparation time involved. On the other hand, as reported by Limat *et al.* [19], a human palmar skin equivalent was transplanted on nude mouse after culturing for 7 days at the air-liquid interface, so that the structure of the epithelium of the graft was improved with regard to the thickness of the corneal cell layer, and the basement membrane was formed 21 days after the grafting. With the pad equivalent prepared in this study, therefore, autologous grafting after 5 days when the corneal cell layer formed was supposed to promote the formation of basement membrane as well as the take of the graft.

In conclusion, it is suggested that the pad equivalent in the dog prepared in this study was reconstructed similar to a native pad by epidermal morphogenesis except for the incompleteness of basement membrane formation.

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