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In vivo experimental model of human gingival mucosa using immunodeficient mice

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Background and Objective: To establish an *in vivo* experimental model for examining human periodontal tissue, the present study examined several transplant techniques that maintain the structure and characteristics of human gingival mucosa.

Material and Methods: Human oral mucosal tissue samples were collected from the gingiva ($n = 11$), palate ($n = 1$), and tongue ($n = 3$). These mucosal grafts were transplanted onto BALB/c *nu*/*scid* mice with double-mutant immunodeficiency. Murine skin, twice the size of the graft, was cut open in an '⊐'-shape. Next, the connective tissue side of the graft was placed onto the murine connective tissue. Immunohistochemical analysis was also performed, using polyclonal rabbit antibody to involucrin, monoclonal antibody to vimentin, monoclonal antibody to CD34, and monoclonal antibody to Ki-67, to determine whether the characteristics of human oral mucosa were maintained.

Results: When the connective tissue side of the graft was placed on the murine fascial membrane, the histological structure of the graft was maintained for 60 d. These grafts were examined for human characteristics using human-specific antibodies. Immunohistochemically, the expression patterns of involucrin, vimentin, and Ki-67 indicated that transplanted mucosa revealed normal human characteristics, including differentiation and proliferation up to 80 d. CD34 was not detected in the graft endothelial cells.

Conclusion: The present study revealed that the novel technique of transplantation of human gingival mucosa in *nu*/*scid* mice may serve as an *in vivo* experimental model of periodontal disease.

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Animal models of dogs and rodents or culture cells have contributed to the generation of new knowledge in biological sciences, including periodontology. Periodontitis has been induced by placing a bacterial plaque-retentive silk ligature in the gingival sulcus around the molar teeth of rodents (1). In addition, alveolar bone loss has been induced by injection of *Porphyromonas gingivalis* (2). While animal models have provided a large range of data, it is sometimes difficult to determine whether the findings are applicable to humans. Although human culture cells were found to be excellent models for reconstructing human characteristics, information about the host response was not prominent (3). Recently, it has been considered that because individuals are not equally susceptible to the destructive effects of periodontal infections, periodontal disease not only may be caused by bacterial infection but also may be associated with host susceptibility (4,5). Variability in host responses among individuals contributes significantly to the expression of periodontal diseases (5). Thus, research into the host response in humans is considered to be important in the analysis of periodontal disease. However, an *in vivo* experimental model using human gingival tissue has not yet been developed.

Immunodeficient mice have been developed to accept the transplantation of human tissues, and such animals, so-called 'hu-mice', have been widely used as human *in vivo* experimental models (6,7). As immunodeficient mice, nude mice and scid mice are most often used. Nude mice lack B cells and have compromised cellular immunity (8), whereas scid mice lack T and B cells and have severe immunodeficiency without cellular and humoral immunity (9,10). However, the amount of serum immunoglobulin increases with aging, which is referred to as the 'leaky phenomenon' (11). The *nu*/*scid* mice used in the present study are a cross between nude and scid mice (8) and do not exhibit the leaky phenomenon (8). In other words, their condition of severe immunodeficiency is maintained for long periods of time (8). In the transplantation of human skin tissue, *nu*/*scid* mice are more suitable than nude or scid mice because the rejection rate for human skin grafts is small (12). Therefore, *nu*/*scid* mice may be better suited for transplanting mucosal grafts compared with other immunodeficient mice.

Studies using the transplantation of human skin tissue onto the skin of *nu*/*scid* mice have been conducted to investigate the carcinogenesis of human skin using carcinogens (13), the carcinogenesis of transplanted precancerous skin lesions (14), and the analysis of psoriatic lesions (15). Because the results obtained in immunodeficient models with transplanted human tissue more accurately reflect the reactions in the human body, they are suitable for drug development and other clinical applications. For example, the reactivity of human lymphocytes stimulated by bacteria has been investigated using scid mice (16). However, little research using immunodeficient mice has been conducted in the analysis of periodontal disease. In a study of mucosa, Holmstrup *et al.* (17) transplanted oral mucosal tissue onto a subcutaneous layer of the nude mice. Their methods were flawed for the following reasons: (i) no human characteristic was examined and (ii) after the transplantation of oral mucosa onto mice, the graft tissue is greatly changed morphologically. Therefore, it is necessary to develop a transplantation method that can maintain the structure and characteristics of human gingival tissue.

To establish an *in vivo* experimental model of human gingival tissues, the present study examined several transplantation techniques that maintain the structure and characteristics of human gingival tissue using *nu*/*scid* mice.

Material and methods

Animals

Eight-week-old male BALB/c *nu*/*scid* mice of double-mutant immunodeficiency were obtained from the Central Institute for Experimental Animals (8). Mice were kept in an isolator and had free access to autoclaved food and boiled water.

Human tissue

Healthy oral mucosal tissue samples from 15 consenting individuals (10 men and five women) were obtained between 1997 and 1998 at the Department of Oral Surgery, Tokai University, given with informed consent. These individuals had an average age of 54 years. Keratinized mucosal tissue was collected from the gingiva ($n = 11$), palate ($n = 1$), and tongue ($n = 3$). Each sample was divided into two sections. One section was transplanted and the other section was used for histopathological analysis.

Transplantation methods

After dividing each mucosal sample into two, the graft section was promptly rinsed with water and then stored in gauze soaked in physiological saline containing antibiotics. The graft sizes ranged from 5×4 mm to 10×5 mm. After each mouse was anesthetized with Nembutal, the skin was sterilized with alcohol. The entire transplantation procedure was carried out on a clean bench. The grafts were transplanted using one of the following three methods (shown in Fig. 1).

Direct transplantation onto murine skin (method I) —Murine skin was cut to match the size of the graft and the graft was sutured onto the murine skin in two places. Tegaderm® dressing (3M Health, Tokyo, Japan) was used to cover the surgical area.

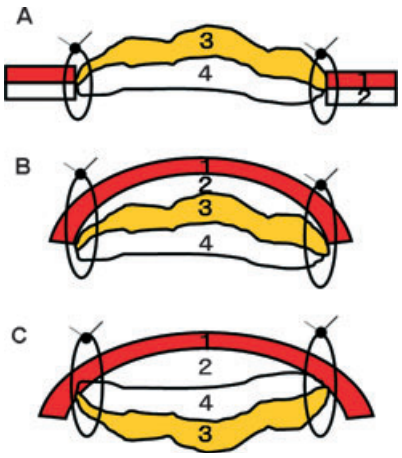

Fig. 1. Schematic diagrams for the three transplantation methods. (A) Method I. (B) Method II. (C) Method III. 1. Murine epithelial tissue. 2. Murine subepithelial tissue. 3. Human oral mucosal epithelial tissue. 4. Human oral mucosal subepithelial connective tissue.

Subcutaneous transplantation (methods II and III) —Murine skin, twice the size of the graft, was cut open in an '⊐'-shape (formation of a flap). Next, the graft was placed onto the murine subepithelial tissue and sutured onto the murine skin in two places. In method II, the epithelial side of the graft was placed onto the murine connective tissue, and in method III, the connective tissue side of the graft was placed onto the murine connective tissue. Finally, the flap was returned to its original position and sutured. The surgical wound was sterilized and then covered with a Tegaderm® dressing.

Method I was employed in two mice (one gingiva and one tongue), method II in three mice (two gingiva and one tongue), and method III in 10 mice (eight gingiva, one palate, and one tongue). In method I, the transplanted graft was analyzed after 30 d in both mice. In method II, the transplanted graft was analyzed after 30 d in two mice (two gingiva) and after 80 d in one mouse (one tongue). In method III, the transplanted graft was

analyzed after 30 d in one mouse (one gingiva), after 60 d in three mice (three gingiva), after 70 d in three mice (two gingiva and one tongue), and after 80 d in three mice (two gingiva and one palate).

The experimental protocol used in this study was reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental College and was carried out in adherence with the Guidelines for Animal Experimentation of Kanagawa Dental College.

Histological and immunohistochemical analysis

Each oral mucosal graft was fixed in 4% paraformaldehyde for 24 h for histological analysis, after which they were embedded in paraffin wax. Hematoxylin and eosin staining was performed.

Furthermore, immunohistochemical analysis was performed using a Histofine® staining kit (Nichirei, Tokyo, Japan), according to the instruction manual. All primary antibodies recognized human-specific markers. Sections were incubated with polyclonal rabbit antibody to involucrin (1 : 100; Harbor Bio-Products, Norwood, MA, USA), monoclonal antihuman vimentin (V9; Nichirei), monoclonal antihuman CD34 (NU-4A1; Nichirei), or monoclonal antihuman Ki-67 (MIB-1, 1 : 50; DAKO, Glostrup, Denmark) for 1 h at room temperature. As a negative control, phosphate-buffered saline was used instead of primary antibody.

Results

Method I was employed to transplant grafts onto two mice. In both cases, the graft was reduced in size and appeared dry to the naked eye (Fig. 2A). In addition, as a result of shrinkage, the mucosal graft became detached from the murine skin in some areas (arrow). Histological analysis showed marked infiltration of inflammatory cells, mostly consisting of neutrophils. Where the human oral mucosal graft was transplanted, keratin plugs had formed (Fig. 2B, +). The graft from the tongue mucosa showed marked keratinization (data not shown). Furthermore, the human oral mucosal tissue was continuous with the murine skin, and involucrin was only detected in the epithelial tissue of human oral mucosa (data not shown). These findings show that while human oral mucosal tissue can be transplanted onto murine skin, the structure of human oral mucosa cannot be maintained.

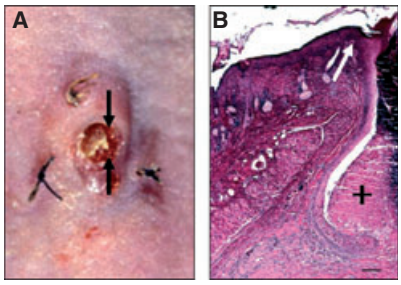

Fig. 2. Method I. (A) Macroscopic image of the oral mucosal graft. The graft has shrunk in size and is dry. The human oral mucosal graft is also detached from the murine skin (arrow). (B) Histological images of the graft. The arrow indicates the transition between murine skin (left) and human oral mucosa (right). Inflammatory cell infiltration is seen in the subepithelial connective tissue. Keratin plugs are seen in the outside of the gingival epithelium of the graft (+) (hematoxylin and eosin stain; bar, 200 μm).

Method II was employed to transplant grafts onto three mice. At 30 d after transplantation, several cysts had formed in the mouse subepithelial connective tissue (Fig. 3A). Around the cysts, mild neutrophil infiltration was confirmed (Fig. 3A). Marked keratinization of cystic wall epithelia was confirmed, including in the case of the tongue tissue (data not shown). At 80 d after transplantation, cystic wall epithelial degeneration and cystic wall destruction were confirmed (Fig. 3B). Furthermore, interstitial scarring was observed around the cysts. Therefore, with method II, grafts underwent cystic transformation and cysts tended to disappear after 80 d.

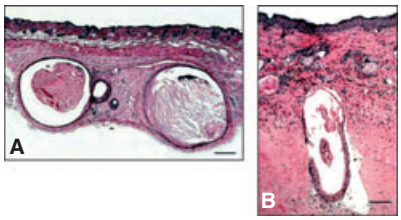

Fig. 3. Method II. (A) Pathological image of the graft (30 d after transplantation). Keratocysts are seen in murine subepithelial tissue (hematoxylin and eosin stain; bar, 250 μm). (B) Pathological image of the graft (80 d after transplantation). Shrunken cysts with scarring are observed (hematoxylin and eosin stain; bar, 200 μm).

Method III was employed to transplant grafts onto 10 mice. After 30 d, the grafts had maintained the structure observed before transplantation (data not shown). This condition was maintained well for up to 60 d (Fig. 4B). Epithelial tissue exhibited no abnormal keratinization, cellular morphology or structure, and the epithelial structure after transplantation was the same as that before transplantation (Fig. 4-A,B). Subepithelial connective tissue showed no abnormalities in the alignment and thickness of collagen fibers, and the structure of subepithelial connective tissue after transplantation was the same as that before transplantation. Furthermore, the graft diameter remained approximately the same after transplantation. However, the grafts underwent complete cystic transformation within 70–80 d (data not shown). Involucrin was detected in the spinal layer, but not in the basal layer, in the epithelial tissue of the grafts at 60 d (Fig. 4C). Expression of vimentin was detected in fibroblasts of the subepithelial connective tissue (Fig. 4D). MIB-1-positive cells were seen in the basal and parabasal layers (Fig. 4E). No apparent expression of CD34 was observed in the human endothelial cells (Fig. 4F, arrow). These expression patterns were also observed in transplanted epithelial tissue of the cyst wall at 70 and 80 d. In addition, these immunohistochemical profiles were consistent with expression patterns in the original oral mucosa before transplantation, except for that of CD34. These findings clarify that, with method III, the histological structure of the grafts was maintained for up to 60 d. Grafts underwent cystic transformation after 70 d and cystic formation

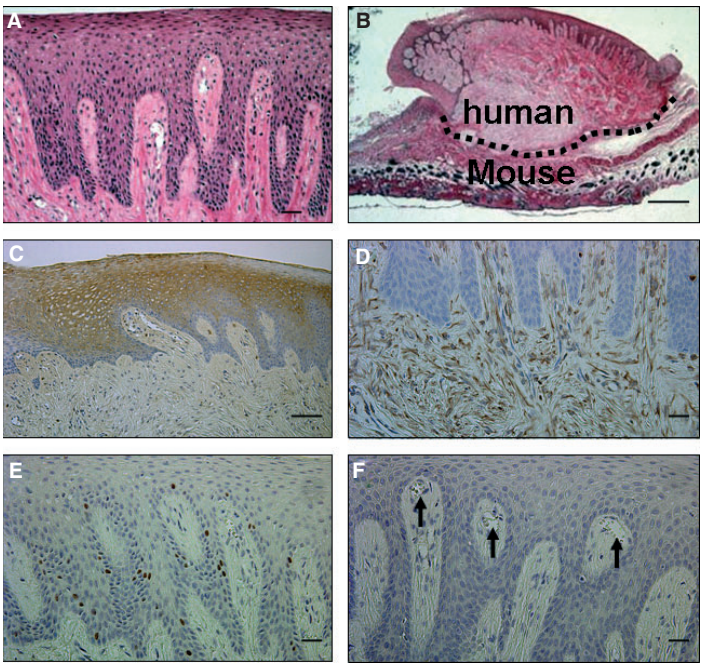

Fig. 4. Method III. (A) Pathological image of the original gingival mucosa before transplantation. No histological abnormalities are seen in the gingival mucosal tissue (bar, 30 μm). (B) Pathological image of the graft 60 d after transplantation.The histological features of the graft are the same as those of the original mucosa (bar, 500 μm). (C) Immunostaining for involucrin. Positive reaction for involucrin is detected in the spinal layer of squamous epithelium (bar, 50 μm). (D) Immunostaining for vimentin. Expression of vimentin is observed in the spindle-shaped cells of subepithelial connective tissue (bar, 30 μm). (E) Localization of MIB-1-positive cells. MIB-1-positive cells are seen in the basal and parabasal layers of graft epithelium (bar, 30 μm). (F) Immunostaining for CD34. No apparent expression of CD34 is noted in endothelial cells (arrows, bar, 30 μm).

was complete in 80 d. Immunohistochemical analysis showed that the human characteristics of the grafts were maintained even after 80 d, although normal gingival structure was not maintained.

Discussion

In method I, the graft was sutured directly to the mouse skin. As a result, the surface of the grafts appeared remarkably dry and reduced. In addition, the grafts were infected in all cases. Because the characteristics of the oral mucosa and the skin are greatly different regarding the type of keratin protein, the skin is stronger when dry (18). Human skin has been reported to be successfully transplanted onto the skin of *nu/scid* mice (12,19). However, this transplantation method was unsuitable for transplanting the oral mucosa. Therefore, because a moist condition was necessary for transplanting the oral mucosa, we transplanted oral mucosa into the mouse subcutaneous layer.

In method II, because it was known from previous reports (17,20) that oral mucosa inserted into the mice subepithelial layer formed epithelial cysts, the epithelial side of the grafts was sutured onto the murine fascial membrane. However, all grafts showed numerous cysts after 30 d. Because epithelial rupture causes epithelial fragmentation, which leads to cystic transformation, numerous cysts may form in the murine subepithelial connective tissue. This epithelial rupture is probably caused by the infiltration of murine subepithelial connective tissue cells into the transplanted epithelia. Therefore, it was clear that method II did not preserve the normal structure of gingival mucosa. Holmstrup *et al.* (17) inserted a filter to eliminate the effects of subepithelial connective tissue in the transplantation of oral mucosa. However, insertion of a foreign material could have increased the risk of infection and damaged the grafts. We decided not to adopt the filter insert method, although excluding the influence of subepithelial connective tissue was significant in determining the success of transplantation.

Although with methods I and II oral mucosa could not be successfully transplanted in the *nu/scid* mice, grafts were successfully transplanted onto all 10 mice with method III. When the connective tissue side of grafts was sutured onto the murine fascial membrane, cystic transformation was not seen for 60 d. In addition, the histological structure, including the polarity and keratinization of the grafts, was well maintained after transplantation. In other words, the best method for maintaining the structure of the gingiva is to suture the connective tissue side of the graft onto the murine fascial membrane. However, cystic transformation was confirmed after about 70 d and was complete in 80 d. Therefore, to maintain the structure of gingival tissue for longer periods of time, grafts need to be transplanted again after 60 d.

Reports of transplanting normal mucosa are few, although there are reports of attempting to transplant normal cells, such as blood cells (7,16) or skin (14,19), into immunodeficient mice. Holmstrup *et al.* (17) transplanted oral mucosal tissue into immunodeficient nude mice. However, one major disadvantage of their model was that because transplanted mucosal tissue underwent cystic transformation, the oral mucosal structure could not be maintained. In addition, because cystic transformation of grafts sometimes caused abnormal keratinization (17), their transplantation technique may have markedly altered the characteristics of human epithelia. In addition, Zhang *et al.* (21) transplanted human intestinal mucosa of colon and rectum into scid mice. Moreover, transplant mucosa was infected by *Shigella* 2 wk later, and the production of the cytokine was analyzed. However, there is doubt regarding the reliability of the

data because it was not confirmed whether the grafts maintained human characteristics. Because a graft originating from a human may be converted to have mouse characteristics, whether grafts maintain their normal human characteristics inside mice was determined immunohistochemically in this study. When human-specific antibodies were reacted with epithelial and subepithelial connective tissues, positive reactions were seen in both tissues 60 d after transplantation. Involucrin, an epithelial structural protein of 120 kDa, is associated with differentiation and maturation (22–24). Involcrin is observed, to a limited extent, in the upper part of the epithelium and is not detected in the basal layer by immunohistochemistry. Moreover, species specificity is high in the anti-involucrin immunoglobulin compared with the broad-spectrum antikeratin immunoglobulin. Involucrin is useful as a differentiation marker, indicating the polarity of squamous epithelium for humans. Expression of involucrin was observed in the spinal layer, but not in the basal layer in the epithelium of the graft. On the other hand, the expression of Ki-67 (as a proliferation marker) (25) was located in the basal and parabasal cell layers. Because normal differentiation and proliferation in the squamous epithelium of the graft was evident, it was suggested that the squamous epithelium of the graft retained human characteristics rather than mouse characteristics. Next, expression of vimentin was limited to spindle cells of the subepithelial layer. Vimentin, of 58 kDa, is an intermediate filament widely distributed as a cytoskeleton of nonepithelial cells (26,27). It is commonly used as a marker for nonepithelial cells. In method III, the mouse connective tissue and the human connective tissue were touching. In addition, vimentin is the most primitive intermediate filament (26,27). These facts suggest the possibility that a human organization may be converted in the mouse organization. However, human vimentin was detected only in the spindle-type cells of subepithelial connective tissue. It was suggested that subepithelial spindle-type cells of the graft preserved human characteristics rather than the characteristics of mice. Finally, antihuman CD34 immunoglobulin specifically detects expression in the endothelial cells. However, endothelial cells in the human area did not react with CD34 human-specific antibody (28). Because blood vessels were not sutured at the transplantation, the life of the graft was dependent on tissue fluid reflux (29). Several days are required for revascularization. Therefore, capillary vessels inside the graft may be formed by angiogenesis of mouse cells. From these immunohistochemical profiles, it was suggested that epithelium and subepithelial connective tissue, except for capillary vessels, retained human characteristics rather than mouse characteristics in the grafts of gingival tissue. Because growth and differentiation of epithelial tissues are dependent on epithelial–mesenchymal interactions mediated by inflammatory cytokines and growth factors (5,30), it is important for subepithelial connective tissue to preserve its epithelial characteristics (19). Connective tissue cells are more likely to become murine cells (31), but these graft cells maintained their human characteristics, thus suggesting that the present method can be used in the analysis of epithelial–mesenchyme interactions.

In conclusion, the present *in vivo* experimental model appears to be useful for analyzing human gingival mucosa. We believe that this model has various uses for examining the association between bacterial infection and host reaction in marginal periodontitis.

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References

1. Breivik T, Opstad PK, Gjermo P, Thrane PS. Effects of hypothalamic-pituitary-adrenal axis reactivity on periodontal tissue destruction in rats. *Eur J Oral Sci* 2000;**108:**115–122.
2. Nakajima K, Hamada N, Takahashi Y *et al*. Restraint stress enhances alveolar bone loss in an experimental rat model. *J Periodont Res* 2006;**41:**527–534.
3. Sfakianakis A, Barr CE, Kreutzer DL. *Actinobacillus actinomycetemcomitans*-induced expression of IL-1a and IL-1b in human gingival epithelial cells: role in IL-8 expression. *Eur J Oral Sci* 2001;**109:**393–401.
4. Schenkein HA. Host responses in maintaining periodontal health and determining periodontal disease. *Periodontol 2000* 2006;**40:**77–93.
5. Salvi GE, Lang NP. Host response modulation in the management of periodontal diseases. *J Clin Periodontol* 2005;**32:**108–129.
6. Boehncke WH. The SCID-hu xenogeneic transplantation model: complex but telling. *Arch Dermatol Res* 1999;**291:**367–373.
7. Bonyhadi ML, Kaneshima H. The SCID-hu mouse: an *in vivo* model for HIV-1 infection in humans. *Mol Med Today* 1997;**3:**246–253.
8. Hioki K, Kuramochi T, Endoh S, Terada E, Ueyama Y, Ito M. Lack of B cell leakiness in BALB/cA-nu, scid double mutant mice. *Exp Anim* 2001;**50:**67–72.
9. Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature* 1983;**301:**527–530.
10. Schuler W, Weiler IJ, Schuler A *et al*. Rearrangement of antigen receptor genes is defective in mice with severe combined immune deficiency. *Cell* 1986; **46:**963–972.
11. Nonoyama S, Smith FO, Bernstein ID, Ochs HD. Strain-dependent leakiness of mice with severe combined immune deficiency. *J Immunol* 1993;**150:**3817–3824.
12. Takizawa Y, Saida T, Tokuda Y *et al*. New immunodeficient (nude-scid, beige-scid) mice as excellent recipients of human skin grafts containing intraepidermal neoplasms. *Arch Dermatol Res* 1997; **289:**213–218.
13. Urano K, Katakai Y, Tokuda Y, Ueyama Y, Nomura T, Yamamoto S. Failure of genotoxic carcinogens to produce tumors in human skin xenografts transplanted to SCID mice. *Carcinogenesis* 1995;**16:**2223–2226.
14. Takizawa Y, Saida T, Tokuda Y, Dohi S, Ikegawa S, Ueyama Y. Engraftment of precursor lesions of human cutaneous neoplasms onto C.B-17 SCID mice: a useful *in vivo* experimental model of carcinogenesis in human skin. *Arch Dermatol Res* 1995;**287:**237–241.
15. Sugai J, Iizuka M, Kawakubo Y *et al*. Histological and immunocytochemical studies of human psoriatic lesions transplanted onto SCID mice. *J Dermatol Sci* 1998;**17:**85–92.

16. Salvi GE, Spets-Happonen S, Singer RE, Offenbacher S. Reconstitution of a hyperinflammatory prostaglandin E2 response to *Porphyromonas gingivalis* challenge in severe combined immunodeficient mice. *J Periodontol* 2005;**76:**16–21.
17. Holmstrup P, Stoltze K, Bretlau P, Dabelsteen E. Human buccal mucosa transplants in nude mice. *Scand J Dent Res* 1981;**89:**89–96.
18. Caproni M, Torchia D, Pestelli E, Volpi W, Fabbri P, Schincaglia E. Clinical and immunohistochemical study of the effect of a cosmetic product in patients with asteatotic eczema. *Int J Tissue React* 2005;**27:**23–29.
19. Inokuchi S, Shimamura K, Tohya H *et al*. Effects of fibroblasts of different origin on long-term maintenance of xenotransplanted human epidermal keratinocytes in immunodeficient mice. *Cell Tissue Res* 1995;**281:**223–229.
20. Holmstrup P, Dabelsteen D, Reibel J, Harder F. Normal keratinized mucosa transplants in nude mice. *Scand J Dent Res* 1981;**39:**187–193.
21. Zhang Z, Jin L, Champion G, Seydel KB, Stanley SL Jr. Shigella infection in a SCID mouse-human intestinal xenograft model: role for neutrophilis in containing bacterial dissemination in human intestine. *Infect Immun* 2001;**69:**3240–3247.
22. Barrett AW, Morgan M, Nwaeze G, Kramer G, Berkovitz BK. The differentiation profile of the epithelium of the human lip. *Arch Oral Biol* 2005;**50:**431–438.
23. Sumitomo S, Kumasa S, Iwai Y, Mori M. Involucrin expression in epithelial tumors of oral and pharyngeal mucosa and skin. *Oral Surg Oral Med Oral Pathol* 1986;**62:**155–163.
24. Lotan R. Squamous cell differentiation markers in normal, premalignant, and malignantepithelium: effects of retinoids. *J Cell Biochem Suppl* 1993;**17F:**167–174.
25. Tsukinoki K, Yasuda M, Shimamura K *et al*. Odontogenic myxofibroma arising in an elderly male: cell kinetics of epithelium and stroma. *Histopathology* 1999;**35:**280–281.
26. Nagle RB. Intermediate filaments: a review of the basic biology. *Am J Surg Pathol* 1988;**12:**4–16.
27. Altmannsberger M, Osborn M. Mesenchymal tumor markers: intermediate filaments. *Curr Top Pathol* 1987;**77:**155–178.
28. Miettinen M. Immunohistochemistry in tumour diagnosis. *Ann Med* 1993;**25:**221–233.
29. Okada T. Revascularisation of free full thickness skin grafts in rabbits: a scanning electron microscope study of microvascular casts. *Br J Plast Surg* 1986;**39:**183–189.
30. Tsukinoki K, Yasuda M, Asano S *et al*. Association of hepatocyte growth factor expression with salivary gland tumor differentiation. *Pathol Int* 2003;**53:**815–822.
31. Holmstrup P, Hansen IL, Harder F, Dabelsteen E. Identification of human connective tissue in transplant of human oral mucosa in nude mice. *Acta Pathol Microbiol Immunol Scand [A]* 1984;**92:**31–37.