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Immunolocalization of laminin and integrin in regenerating junctional epithelium of mice after gingivectomy

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Background and Objective: The expression patterns of adhesive proteins and extracellular matrix proteins in regenerating gingival epithelium after gingivectomy are unknown. The aim of this study was to examine the expression of laminin 1, laminin γ_2 (a specific component of laminin 5), integrin β_4 and integrin α_3 in the regenerating gingival epithelium in order to understand the mechanism of wound healing during reconstitution of the sulcular environment.

Material and Methods: The palatal gingivae of the maxillary molars of Institute of Cancer Research mice were excised, and the regenerating tissues were examined 1, 3, 5, 7 and 14 days later. Fresh, non-fixed and non-decalcified frozen sections were prepared and stained using immunofluorescence.

Results: At 1 day post-surgery, intense expression of laminin γ_2, integrin β_4 and integrin α_3 was distinct in the frontal margin of the regenerating oral epithelium. Laminin γ_2 was diffusely detected on the root surface and in connective tissues beneath the regenerating oral epithelium at 3 and 5 days. At 7 days, laminin γ_2 was intermittently recognizable in the internal basal lamina (IBL) close to tooth-facing cells, while laminin γ_2, integrin β_4 and integrin α_3 were observed in the IBL and in the external basal lamina (EBL) of the regenerating junctional epithelium at 14 days.

Conclusion: These results suggest that secretion of laminin 5 in the connective tissue may induce epithelial cell migration, and that binding of laminin 5 to integrin $\alpha_6\beta_4$ and integrin $\alpha_3\beta_1$ in the IBL may provoke cell adhesion and migration of cells facing the tooth on the enamel surface of the regenerating junctional epithelium.

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The junctional epithelium is located between the cemento-enamel junction (CEJ) and the floor of the gingival sulcus, the epithelium of which connects consecutively to the gingival oral epithelium, forming the dento-epithelial junction (1). The attachment of the junctional epithelium to the enamel surface is accomplished by hemidesmosomes and by the internal basal lamina (IBL), which contains an accumulation of extracellular matrix. Basal cells of the junctional epithelium can also attach to the connective tissue by hemidesmosomes and by the

extracellular matrix in the external basal lamina (EBL). Components of the IBL are thought to be analogous to the EBL but are not absolutely identical (2–7). Hormia and colleagues (4–6) have demonstrated that integrins $\alpha_6\beta_4$ and $\alpha_3\beta_1$ and laminin 5, which is a ligand of integrin, are expressed not only in the EBL but also in the IBL. Collagen type IV and laminin 1, which are basic basal lamina components (8), have not been detected in the IBL. This is a characteristic of the IBL and distinguishes it from all other basal laminae (4–6). Laminins constitute a family of extracellular matrix proteins that are composed of cross-shaped heterotrimers of α, β and γ chains and are mainly localized in the basal laminae of various tissues. Among these, laminin 5, which is comprised of α_3, β_3 and γ_2 chains, is an unusual protein in the family because it contains a γ_2 chain (9). In contrast, integrins are heterodimeric transmembrane proteins which serve as receptors for extracellular matrix components and for cell surface proteins (10). Integrins β_4 and α_3 are detected both in the IBL and in the EBL, where they exist as integrin $\alpha_6\beta_4$ and $\alpha_3\beta_1$ heterodimers, respectively (4,6,10).

Numerous studies have characterized the regenerating junctional epithelium following gingivectomy. It has been demonstrated that the junctional epithelium regenerates completely following gingivectomy (2,11–14); however, the expression patterns of laminin and integrin have not yet been fully elucidated during the process of attachment of the regenerating junctional epithelium to the tooth. It can be expected that detection of the localization of laminin and integrin might be extremely difficult at the interface between the enamel surface and the regenerating junctional epithelium owing to the difficulty in sectioning whole mounts without fixation or decalcification of the tooth and maxillary bone.

Kawamoto's method is characterized by the use of carboxymethyl cellulose (CMC) embedding, a tungsten-carbide knife and special adhesive films (15,16). To support the hard tissues, we used embedding in CMC, which is harder than the generally employed optimal cutting temperature compound. To cut the non-decalcified hard tissues, a tungsten-carbide knife was exceedingly useful, and to maintain the tissue morphology, we used films with a special adhesive agent.

Although the tissue dimensions and ultrastructures of mouse junctional epithelium and oral epithelium are different to those in humans, together with the turnover of their respective constituent cells, data pertaining to the former are very useful in understanding the mechanism underlying the wound healing process following gingivectomy in human periodontal tissue. Therefore, in this study we examined the expression patterns of laminin 1, laminin γ_2, integrin β_4 and integrin α_3 in regenerating junctional epithelium after gingivectomy using a mouse model. We used immunohistochemistry with non-fixed, non-decalcified frozen sections by Kawamoto's method (15,16) in order to characterize the wound healing mechanism during the reconstitution of the sulcular environment, with no artifacts of, or reductions in, antigenicity owing to decalcification.

Material and methods

Animals and surgical procedures

Sixty 8-week-old male Institute of Cancer Research mice, weighing about 30 g each, were used in this study. Under anesthesia induced by an intraperitoneal injection of sodium thiopental (Ravonal®; Mitsubishi Tanabe Pharma, Osaka, Japan), the animals were positioned with their mouths held open by the mouth opener for observation. The palatal gingivae both of the right and the left maxillary first to second molars were excised using a surgical knife (No. 12; Feather, Osaka, Japan). A piece of gingival tissue approximately 1 mm in width, including the junctional epithelium dentogingival fiber, was resected from the medial side of the first molar to the distal side of the second molar according to a method described in a previous study (17). Animals without any surgical and anesthetic treatment were used as controls. This study was conducted in accordance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College.

Preparation of freshly frozen and non-decalcified sections

The animals were divided into six groups consisting of five animals each. On days 1, 3, 5, 7 or 14 after gingivectomy and in control mice, the animals were deeply anesthetized and sacrificed with an intraperitoneal injection of overdose sodium thiopental, and the maxillas, together with the associated gingival tissues, were removed and immediately frozen in 2-methylbutane cooled with liquid nitrogen. Each specimen was rapidly embedded in 4% CMC (Finetec, Tokyo, Japan) and further frozen in 2-methylbutane. The frozen CMC blocks of tissues were set in a cryomicrotome (CM1900; Leica Microsystems, Wetzlar, Germany) kept at −25°C and were cut with a tungsten-carbide steel blade (Leica Microsystems). When the appropriate plane of CMC-embedded frozen tissue was exposed, an adhesive tape made of polyvinylidene chloride film (Cryofilm type I; Finetec) was glued on the cut surface. In this way, 6-μm-thick frozen sections supported by a polyvinylidene chloride film were collected one by one. These specimens were then used for immunofluorescence staining as detailed below.

Preparation of paraffin and decalcified sections

The same numbers of animals were used and were divided into groups as those used for the freshly frozen and non-decalcified sections. Animals, grouped as detailed in the previous subsection, were deeply anesthetized with an intraperitoneal injection of sodium thiopental and were then fixed with a transcardiac perfusion of 10% neutral buffered formalin. After the fixation, the maxillary jawbones were removed and were decalcified with 10% EDTA for 1 week at room temperature. Decalcified tissues were dehydrated with ethanol, cleared in xylene and embedded in paraffin blocks. Three-micrometer-thick sections were cut along the buccolingual plane and stained with

hematoxylin and eosin. Specimens were examined using a light microscope (Axiophot 2; Carl Zeiss, Oberkochen, Germany) and photographed.

Immunofluorescence microscopy

Frozen sections for immunofluorescence were fixed in ethanol for 1 min at room temperature. After washing with phosphate-buffered saline (PBS), they were incubated with 3% bovine serum albumin (BSA) for 30 min at room temperature to prevent non-specific binding. After removal of the BSA, the sections were incubated with an anti-laminin γ_2 rabbit polyclonal antibody (Abcam, Cambridge, UK), an anti-integrin β_4 rat monoclonal antibody (Abcam), an anti-integrin α_3 goat polyclonal antibody (R&D Systems, Minneapolis, MN, USA) or an anti-laminin 1 rabbit polyclonal antibody (Progen, Heidelberg, Germany) overnight at 4°C. After washing in PBS, they were incubated with a secondary antibody, goat anti-rabbit immunoglobulin G (IgG), goat anti-rat IgG or rabbit anti-goat IgG conjugated with Alexa 488 and Alexa 568 phalloidin (Molecular Probes, Eugene, OR, USA) for F-actin for 1 h at room temperature. As controls, the specimens were treated with 3% BSA instead of antibodies to laminin γ_2, integrin β_4, integrin α_3 or laminin 1. They were examined and photographed using a confocal laser scanning microscope (MRC-1024UV; Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). In this study, we used Alexa 488 for laminin 1, laminin γ_2, integrin β_4 and integrin α_3, which were detected as a green fluorescence, and Alexa 568 F-actin, which produced red fluorescence. Frontal sections of regenerating junctional epithelium were acquired by scanning the x–y axis of each specimen (0.2 μm in thickness) from the apical to the basal sides. The Z-stack image was obtained by superimposing those green and red fluorescence images.

Results

Light microscopic observations

The untreated junctional epithelium was located between the CEJ (arrowheads in Fig. 1) and the floor of the gingival sulcus, the epithelium of which connects consecutively to the oral epithelium (Fig. 1A). At 1 day post-gingivectomy, the wound surface and exposed cervical root surface was clearly distinguished and was covered with necrotic tissue and a fibrin clot. A relatively large distance was apparent between the frontal margin of the regenerating oral epithelium and the root surface (Fig. 1B). Newly regenerating epithelium had migrated onto the wound and had attached around the CEJ at 3 days post-surgery (Fig. 1C). By 5 days, the regenerating epithelium had migrated onto the enamel surface from the CEJ to the coronal side, forming an immature junctional epithelium between the enamel and the keratinized oral epithelium (Fig. 1D). However, no obvious demarcation was discernible between the sulcus epithelium and the junctional epithelium. At 7 and 14 days post-surgery, the morphology of newly formed gingival tissues, including the junctional epithelium, appeared to be almost the same as that in the control animals (Fig. 1E,F).

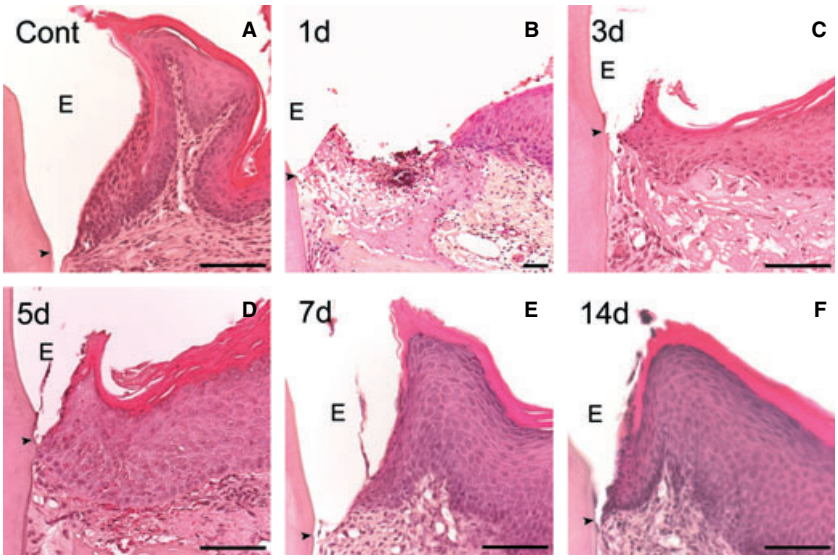

Fig. 1. Light micrographs of wound healing after gingivectomy. (A) Normal gingival epithelia (Cont), including junctional, sulcus and oral epithelia, can be detected in the control group. (B) At 1 day post-gingivectomy (1d), a fibrin clot and exudates are found in the wound area. (C) At 3 days (3d), the regenerating oral epithelium is attached to the CEJ. (D) At 5 days (5d), the regenerating epithelium in the vicinity of the enamel surface is also apparent. (E) At 7 days (7d), the morphology of the newly formed gingival tissue, including the junctional epithelium, appears to be similar to the control sample. (F) At 14 days (14d), the regenerating gingival epithelium appears almost identical to the control sample. E, enamel; arrowhead, CEJ; scale bar, 50 μm.

Confocal laser scanning microscopic observations

Immunolocalization of laminin 1 — In the untreated junctional epithelium, laminin 1 was immunoreactive linearly in the EBL. However, immunoreactivity for laminin 1 was not expressed in the IBL (Fig. 2A). No positive reaction for laminin 1 was seen in the apical area of the newly formed epithelium at 1 and 3 days post-surgery (Fig. 2B,C). Linear immunoreactivity of laminin 1 was distinct in the basal portion corresponding to the basal lamina of the regenerating oral epithelium at 5 days post-surgery (Fig. 2D). At 7 and 14 days post-surgery, linear reactivity of laminin 1 at the same intensity as in the untreated junctional epithelium was detected in the EBL of the regenerating oral epithelium (Fig. 2E,F).

Immunolocalization of laminin γ_2 — Linear expression of laminin γ_2 was distinct in both the IBL and the EBL of

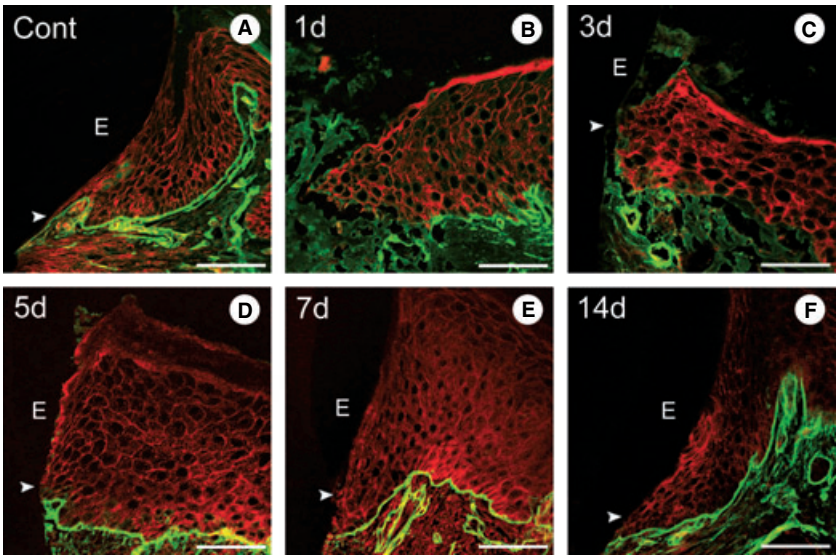

Fig. 2. Immunofluorescence localization of laminin 1. (A) Untreated gingival epithelium. Linear immunoreactivity for laminin 1 is clearly observed as green fluorescence in the EBL, but not in the IBL. F-actin is detected as red fluorescence. (B) At 1 day post-gingivectomy, laminin 1 is not expressed at the apical area of marginal cells in the newly formed epithelium. (C) At 3 days, laminin 1 is apparent in the connective tissue but not in the basal lamina of the regenerating epithelium. (D) At 5 days, linear expression of laminin 1 is found in the basal lamina and in connective tissues of the regenerating epithelium. (E) At 7 days, linear immunolocalization of laminin 1 is discernible in the basal laminae of the epithelium and blood vessels. (F) At 14 days, immunolocalization of laminin 1 is comparable to the control sample. E, enamel; arrowhead, CEJ; scale bar, 50 μm.

the untreated junctional epithelium (Fig. 3A). Intense immunoreactivity for laminin γ_2 was detected in the peripheral portion of the head cells of the regenerating oral epithelium at 1 day post-surgery (Fig. 3B). At 3 days post-surgery, positive reactions for laminin γ_2 were detected not only around the CEJ, where the head cells of the regenerating oral epithelium had approached and attached, but also on the exposed root surface and in connective tissues beneath the regenerating epithelium. In addition, immunoreactivity was also recognized in the surface layer of the regenerating epithelium (Fig. 3C).

A positive reaction was discernible around the CEJ where the regenerating epithelium had attached at 5 days post-surgery. Relatively weak immunoreactivity was observed in the connective tissue beneath the regenerating epithelium; however, immunoreactivity for laminin γ_2 was not distinct in the surface layer of the regenerating epithelium (Fig. 3D).

At 7 days post-surgery, a weaker reaction than the control was detected in the IBL, and linear expression restricted to the basal lamina was evident in the EBL. Intense immunoreactivity for laminin γ_2 was recognizable around the CEJ. However the reaction became indistinct in the connective tissue underneath the regenerating epithelium (Fig. 3E). At 14 days post-surgery, immunoreactivity of laminin γ_2 was recognized as a linear expression in the apical half of the IBL, and at the EBL, linear immunolabeling of laminin γ_2 was equivalent to the control group (Fig. 3F).

Immunolocalization of integrin β_4 — In the untreated junctional epithelium, intense immunoreactivity for integrin β_4 was clearly observed in the IBL adjacent to the tooth-facing cells and also in the EBL close to basal and suprabasal cells (Fig. 4A). Positive reactivity for integrin β_4 was seen in the frontal margin and surface cells of the regenerating oral epithelium at 1 day post-surgery (Fig. 4B). At 3 days post-surgery, intense immunoreactivity for integrin β_4 was detected in the basal lamina of the regenerating oral epithelium of basal and suprabasal cells at apical areas (Fig. 4C). At 5–7 days post-surgery, the margin of regenerating oral epithelium cells had attached to the tooth surface, and positive reactivity was observed only in the IBL around the CEJ of newly attached epithelial cells. In contrast, intense reactivity was seen in the EBL of basal cells of the regenerating oral epithelium (Fig. 4D,E). At 14 days post-surgery, equivocal immunoreactivity of integrin β_4 was found in the IBL of regenerating cells attached to the tooth. Meanwhile, linear and intense reactivity was distinct in the EBL and basal lamina of the regenerating basal cells (Fig. 4F).

Immunolocalization of integrin α_3 — In the untreated junctional epithelium, immunoreactivity for integrin α_3 was recognized in the IBL of the tooth-facing cells. However, expression of integrin α_3 was more distinct in the EBL of basal cells (Fig. 5A). At 1 day post-surgery, expression of integrin α_3 was seen in the regenerating oral epithelium. In particular, intense and belt-like immunoreactivity was apparent in the cytoplasm of basal and suprabasal cells (Fig. 5B). At 3 and 5 days post-surgery, the margin of regenerating oral epithelium cells had attached to the tooth in close proximity to the CEJ. The intense and belt-like expression of integrin α_3 was discernible in the cytoplasm of basal and suprabasal cells (Fig. 5C,D).

At 7 days post-surgery, no reactivity for integrin α_3 was observed in the periphery of regenerating cells attached to the tooth. A belt-like immunoreactivity was distinct in the EBL of basal and suprabasal cells. Notably, intense immunolabeling for integrin α_3 was detected in cell membranes and cytoplasm of basal and suprabasal cells close to the CEJ (Fig. 5E). At 14 days post-surgery, positive reactivity was apparent not only in the IBL but also in the cell membranes and cytoplasm of the tooth-facing cells. The immunoreactivity for integrin α_3 was detected in both the cell membranes and the EBL of basal or suprabasal cells. The expression of integrin α_3 was more intense in the EBL than in the IBL (Fig. 5F).

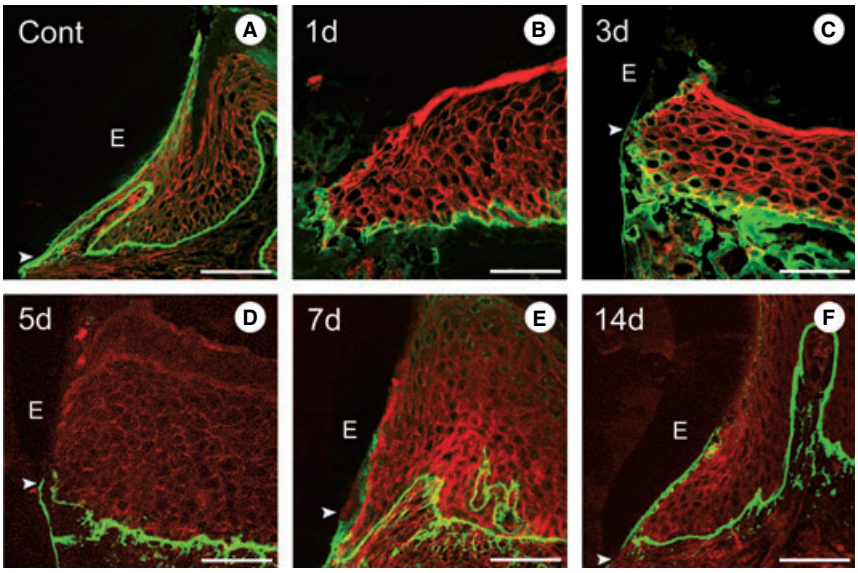

Fig. 3. Immunofluorescence localization of laminin γ_2. (A) In gingival epithelium of the control group, linear immunoreactivity for laminin γ_2 is detected as green fluorescence in both the EBL and the IBL. (B) At 1 days post-gingivectomy, intense immunolocalization of laminin γ_2 is detected in the peripheral portion of the head cells in the regenerating epithelium. (C) At 3 days, laminin γ_2 was diffusely detected in the connective tissue and at the root surface. The immunoreactivity is apparent not only in marginal but also in basal cells of the regenerating epithelium. (D) At 5 days, expression of laminin γ_2 is detected around the basal lamina of the epithelium and the root surface. (E) At 7 days, linear immunoreactivity of laminin γ_2 is found along the EBL and weaker reaction is detected in the IBL. (F) At 14 days, immunoreactivity of laminin γ_2 is observed as a linear expression and is distinct in the apical half of the IBL, and at the EBL. E, enamel; arrowhead, CEJ; scale bar, 50 μm.

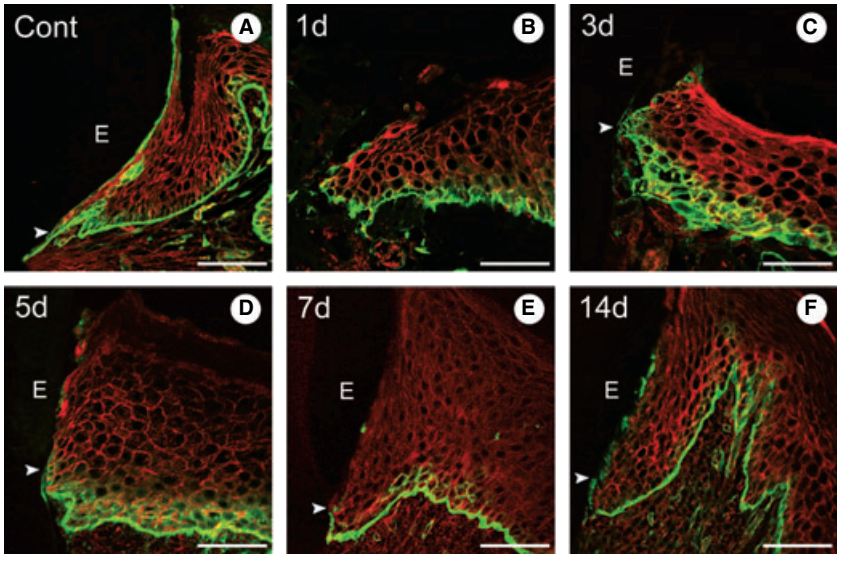

Fig. 4. Immunofluorescence localization of integrin β_4. (A) In the untreated junctional epithelium, immunoreactivity for integrin β_4 is seen as green fluorescence in both the IBL and the EBL. (B) At 1 day post-gingivectomy, integrin β_4 is detected in the frontal margin and surface cells of the regenerating epithelium. (C) At 3 days, expression of integrin β_4 is distinct in the basal and suprabasal cells and in the marginal cell layer of the regenerating epithelium. (D) At 5 days, immunolabeling of integrin β_4 is discernible in both the basal lamina and in the basal and/or suprabasal cells. However, no positive reaction was found at the interface between the enamel and the regenerating epithelium. (E) At 7 days, linear expression of integrin β_4 was evident in the EBL, but not at the enamel–epithelium interface except in the IBL around the CEJ. (F) At 14 days, a linear expression of integrin β_4 was observed in the EBL and discontinuous with that in the IBL. E, enamel; arrowhead, CEJ; scale bar, 50 μm.

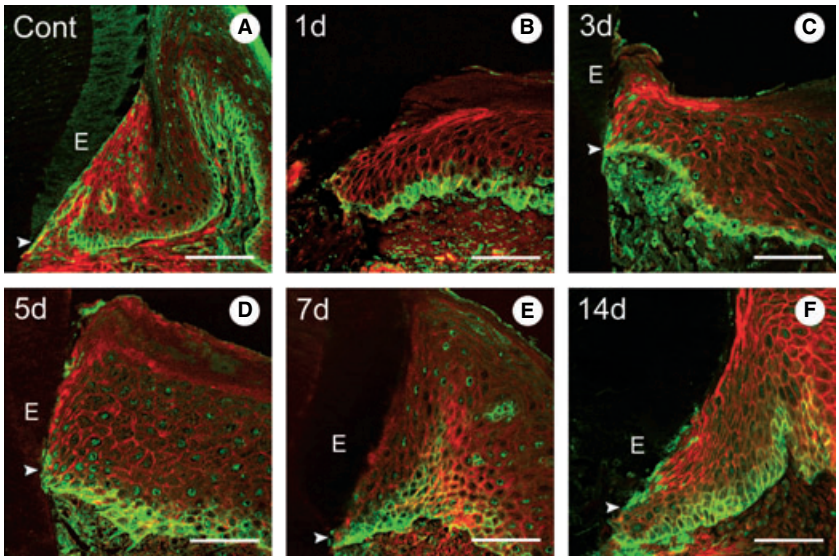

Fig. 5. Immunofluorescence localization of integrin α_3. (A) In gingival epithelium in the control group, immunolabeling of integrin α_3 as green fluorescence is localized in the EBL and in the basal lamina of basal and suprabasal cells, but is expressed only indistinctly in the IBL. (B) At 1 day post-gingivectomy, intense immunolabeling of integrin α_3 is recognized as a belt-like fluorescence in basal cells of the regenerating epithelium. (C) At 3 days, intense labeling for integrin α_3 is detected in the cytoplasm of basal cells and underneath the connective tissues. (D) At 5 days, a belt-like fluorescence of integrin α_3 is apparent in basal and suprabasal cells. (E) At 7 days, a belt-like fluorescence of integrin α_3 is recognized in the EBL of basal and suprabasal cells, but not at the enamel–epithelium interface. (F) At 14 days, belt-like immunolabeling for integrin α_3 is observed in the EBL of basal and suprabasal cells and also in the IBL. E, enamel; arrowhead, CEJ; scale bar, 50 μm.

Discussion

It is well known that the regenerative junctional epithelium after gingivectomy is derived from the oral epithelium and that maturation of the junctional epithelium is induced by epithelial cell attachment to the tooth surface that forms the sulcular environment (18). Previous studies on the wound healing process of oral mucosa have demonstrated that epithelial migration to the injured surface is activated. However, laminin 1 expression was not detected at the frontal margin of the regenerating oral epithelium within 2–4 days post-operation, but was observed in the basal portion of the epithelium at days 5–7 post-operation (14,19). In the present study, we demonstrated changes in expression of laminin 1, laminin γ_2, integrin β_4 and integrin α_3 in the regenerative oral epithelium and in the junctional epithelium after gingival surgery. Laminin 1 expression was not detected in the regenerative epithelium until 3 days, but its immunoreactivity was recognized in the newly formed basal lamina and EBL at 5–14 days post-gingivectomy. Since the morphology of newly formed gingival tissues appeared to be almost the same as that in the control mice, we used basal lamina and EBL for 1–5 and 7–14 days in the experimental group, respectively.

In contrast, intense expression of laminin γ_2 was recognized not only in basal cells of the regenerating oral epithelium but also at the tooth surface and in connective tissues at 3–5 days post-surgery. At 7 and 14 days post-surgery, when the newly formed junctional epithelium had attached to the enamel surface, laminin γ_2 expression was apparent at the IBL close to the CEJ, while its expression in connective tissue was reduced. Our results on the immunoreactivity of laminin 1 and laminin γ_2 indicate that the production of laminin 1 is slower than that of laminin γ_2. It is interesting to note that both laminin 1 and laminin γ_2 were expressed in the EBL, whereas no laminin 1 expression was noted in the IBL.

Our results indicate that the expression of laminin 1, which is an essential component of the basal lamina, is not distinct in the early stage of the wound healing process, but is found in the late stage. These findings suggest that movement of the regenerative gingival epithelium to the wound takes place first, after which the reconstruction of hemidesmosomes and attachment of the epithelium and connective tissue may occur (8,20,21). These observations suggest that the expression of laminin 1 is related to the degree of cell differentiation and that laminin 1 is not expressed in the undifferentiated epithelium, including the margin of the regenerating epithelium and the junctional epithelium.

The immunoreactivity of integrin β_4 and integrin α_3 (the specific receptors of laminin 5) was distinct at cell membranes in the margins of both the regenerating basal and suprabasal cells and of the newly formed tooth-facing cells. However, those immunoreactions were indistinct in the IBL. These results suggest that the regenerating epithelium migrates to the wound surface and the tooth surface with an incomplete basal lamina in the early stage of wound healing (14,20,22).

Immunoreactivities for laminin γ_2, integrin β_4 and integrin α_3 were observed in cell membranes at the

frontal margin of the regenerating oral epithelium at 1–3 days post-surgery. Subsequently, cell polarity was determined after establishment of the basal lamina and hemidesmosomes; finally, the expression of laminin γ_2, integrin β_4 and integrin α_3 became apparent at both the EBL and the IBL in the basal cells and in tooth-facing cells. From these observations, it is tempting to suggest that there is a close relationship between the expression of adhesive proteins and cellular polarity.

Concerning the relationship between cell adhesion and cell migration, Goldfinger and colleagues (23) proposed a model for the functions of laminin 5, integrin $\alpha_3\beta_1$ and integrin $\alpha_6\beta_4$ in epithelial wound healing. In that model, cell adhesion is induced by laminin 5 binding to integrin $\alpha_6\beta_4$, and cell migration is provoked by laminin 5 binding to integrin $\alpha_3\beta_1$. Furthermore, Kinumatsu and colleagues demonstrated that the expression of laminin γ_2 (*lamc2*) was higher (about 12 times) in the IBL than in the EBL (24).

We observed a positive reaction for laminin γ_2 in the apical half of the IBL at 7 days post-surgery. However, the expression of integrin β_4 and integrin α_3 was more intermittent than that of laminin γ_2. Sabag and colleagues reported that the IBL and hemidesmosomes are formed at the tooth surface at 14 days post-surgery (14). We previously showed that microvilli-like structures exist at the attachment surface of cells directly attached to the tooth (DAT cells) using the osmium maceration method (25).

Taking these results together, we propose that integrins β_4 and α_3 are expressed after the formation of hemidesmosomes on the tooth surface, after which the tooth-facing cells adhere and migrate on the enamel surface. The binding of laminin 5 to integrin $\alpha_6\beta_4$ takes place, which prompts hemidesmosome formation, and the cells begin to migrate from the CEJ to the coronal side following the binding of laminin 5 to integrin $\alpha_3\beta_1$.

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