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Expression and function of laminin and integrins on adhesion/migration of primary culture cells derived from rat oral epithelium

M. Sugisawa[1,2,3], T. Masaoka[1,2,3], Y. Enokiya[3], T. Muramatsu[1,3], S. Hashimoto[1,3], S. Yamada[1,2], M. Shimono[1,3]

[1]Oral Health Science Center HRC7, [2]Department of Periodontology and [3]Department of Pathology, Tokyo Dental College, Chiba, Japan

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Background and Objective: It remains controversial whether or not the junctional epithelium cells that are directly attached to teeth migrate on the enamel surface, as those cells are able to adhere firmly to the enamel. The aim of this study was to investigate the expression patterns of laminin γ_2, integrin β_4 and integrin α_3, and to examine their potential function in cell migration.

Material and Methods: Oral epithelium cells obtained from Sprague–Dawley rats were established in primary culture. We employed a wound-healing assay to characterize the direction of cell extension at the start of cell migration, and observed different localizations of laminin and integrins using immunofluorescence. For functional analyses of integrins, we employed a phosphatidylinositol-3-kinase (PI3K) activator to promote integrin β_4 function and used P1B5 to inhibit integrin α_3 function, and we analyzed the percentage of re-epithelialization as the migration function.

Results: Marked accumulation of laminin γ_2 was detected in the peripheral cytoplasm of cells adjacent to the wound area, as shown by the results of the migration assay. Integrin β_4 was detected in the distal cell processes of actively migrating cells, while integrin α_3 was found in cell membranes of cells adjacent to the wound area. In the functional analyses, the percentage of re-epithelialization was significantly lower in the PI3K-activator group and in the P1B5-treated group (2.5% and 7.2%, respectively) than in the control group (39.0%) ($p < 0.01$).

Conclusion: The results suggest that laminin γ_2 is secreted as a foothold for cell migration, that integrin β_4 participates in cell adhesion and that integrin α_3 is involved in cell migration in the primary culture cells.

Masaki Shimono, DDS, PhD, Department of Pathology, Tokyo Dental College, 1-2-2, Masago, Mihama-ku, Chiba 261-8502, Japan
Tel: +81 43 270 3781
Fax: +81 43 270 3784
e-mail: shimono@tdc.ac.jp

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The junctional epithelium adheres to the enamel surface and separates periodontal connective tissues from the external environment. Cells directly attached to the tooth (DAT cells) in the junctional epithelium that are aligned in the most superficial layer facing the enamel are thought to prevent the invasion of periodontal pathogens into the tissues as a result of this rigid attachment to the tooth. That attachment is characterized by an internal basal lamina (IBL) and hemidesmosomes that intervene between the enamel and DAT cells. Recently, the important functions of laminin and integrins have been recognized among the components of the IBL and hemidesmosomes (1–3).

The IBL is characterized by the fact that laminin 5 is its major adhesion protein and that neither type IV collagen nor laminin 1, which usually constitute basal lamina, are present (1–3). Laminin 5 is unique as it is the only protein in the laminin family that possesses a γ_2 chain (4).

By contrast, integrin β_4, which possesses an integrin β subunit with a large cytoplasmic domain, is the only member of that family to contain a heterodimer with an integrin α_6 subunit. In addition, integrin $\alpha_6\beta_4$ is involved in cell adhesion by forming hemidesmosomes as a result of its association with laminin 5 (5).

Integrin $\alpha_3\beta_1$, which has an integrin β_1 subunit, is widely expressed among the integrins and is a specific receptor for laminin 5 and laminin 10/11 ($\alpha_5\beta_1\gamma_1/\alpha_5\beta_2\gamma_1$). It is known that cell adhesion to laminin 5 can be strongly inhibited by an antibody to the integrin α_3 subunit (6). Moreover, it has been reported that abundant integrin $\alpha_3\beta_1$ is expressed in cancer cells with high invasive capacities that depend on cell migration (7).

The defense and homeostasis of periodontal tissues are maintained by the fast turnover of junctional epithelium cells. The primary junctional epithelium is originated from the reduced enamel epithelium, and thereafter mature junctional epithelium cells are supplied by the adjacent oral mucosa (8). Several studies have suggested that DAT cells are attached firmly to the enamel surface, and accordingly they may be nonmigratory cells that do not take part in the turnover of the junctional epithelium (1,2,9). By contrast, another study demonstrated that DAT cells possess microvilli-like structures at the enamel surface and stress fibers arranged in parallel to the tooth axis, which suggests that those structures may be involved in the adhesion and/or migration of DAT cells (10). Furthermore, an investigation using the 5-bromo-2-deoxyuridine-labeling method indicated that DAT cells have a high turnover rate (11). A study examining the expression of adhesion proteins in the regenerating epithelium after gingivectomy implied that both integrin β_4 and integrin α_3 are expressed in the marginal cells and in the IBL. That study further suggested that the binding of laminin 5 and integrin $\alpha_6\beta_4$ participates in the attachment of DAT cells to the enamel surface, while the combination of laminin 5 and integrin $\alpha_3\beta_1$ is involved in cell migration to the coronal side (12).

The results of these studies strongly suggest that DAT cells migrate on the enamel surface while they are attached to the enamel. However, the relationship between cell adhesion or migration and the localization of adhesive factors has not yet been clarified. In order to elucidate the mechanism(s) involved in the adhesion and migration of oral epithelium cells, we investigated the expression patterns of laminin γ_2 (which is involved in cell adhesion and migration), integrin β_4 (which participates in cell adhesion) and integrin α_3 (which takes part in cell migration), using primary culture cells derived from the rat oral mucosa. We studied the direction of cell spreading and immunolocalization of these adhesive proteins using immunofluorescence and a wound-healing assay. We also examined how integrins are involved in cell migration by using a phosphatidylinositol-3-kinase (PI3K) activator to promote integrin β_4 function and using P1B5 to inhibit the function of integrin α_3.

Material and methods

Materials

Oral mucosa tissue was obtained from 50 male Sprague–Dawley rats (3 wk of age), each weighing about 60 g. The mucosal epithelium from the gingiva, including the junctional epithelium surrounding the molar teeth of the upper and lower jaws, the cheek and the palate, was dissected. The tissue specimens were soaked for 30 min at room temperature 24°C in serum-free Dulbecco's modified Eagle's medium containing 1% penicillin–streptomycin, 0.1% amphotericin B and 0.01% gentamicin (all from Invitrogen Corp., Carlsbad, CA, USA), to prevent contamination. Thereafter, the epithelial layer was removed from the underlying connective tissues by digestion with dispase (1.1 U/mL; Godo-shusei, Chiba, Japan) for 24 h at 4°C. This study was conducted in accordance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College.

Cells and cell culture

A primary culture of rat oral mucosal epithelium, collected as described above, was established based on the method of Tanno *et al*. (13). The specimens were dissolved with 0.05% trypsin–EDTA (Invitrogen), and oral mucosa cells were collected by centrifugation. The epithelial cells were seeded on cover glasses in a culture dish (Iwaki, Chiba, Japan), and cultures were initiated in defined keratinocyte serum-free medium (Invitrogen). After 3 d, the epithelia began to grow, and groups of 5–10 cells aggregated to form small islands. When they were close to confluence (usually after 7 d in culture), the cultured cells were used for the various experiments detailed below.

Wound-healing assay

After 7 d of culture, we confirmed that the oral mucosal cells grown on cover glasses were subconfluent, then aspirated the medium and scraped the cell sheets with the tip of a pair of sterile forceps, approximately 400 μm in diameter. After scraping, the wounded surface was washed twice with phosphate-buffered saline (PBS) and was then used for immunofluorescence studies and for functional experiments. This wound-healing assay was employed because the direction of cell extension was distinct when the cells around the scraped area began to migrate to the wound site (14).

Immunofluorescence microscopy

The scraped-wound cell sheets and the unscraped controls were washed twice with PBS and then cultured in defined keratinocyte serum-free medium at 37°C under an atmosphere of 5% CO_2. After 2 or 3 d, the cells were fixed with 4% paraformaldehyde for 20 min at

room temperature. After washing with PBS, the cells were incubated with 3% bovine serum albumin (BSA) for 30 min at room temperature to prevent nonspecific reactions. After removal of the BSA, the cells were incubated, overnight at 4°C, with a rabbit polyclonal antibody to laminin γ_2 (diluted 1:100 with 1% BSA; Abcam, Cambridge, UK), a mouse monoclonal antibody to integrin β_4 (diluted 1:100 with 1% BSA; Abcam), a mouse monoclonal antibody to integrin α_3 (diluted 1:100 with 1% BSA; Abcam), or a rabbit polyclonal antibody to integrin α_3 (diluted 1:100 with 1% BSA; Acris GmbH, Herford, Germany). After washing in PBS, the cells were incubated with an appropriate secondary antibody – either goat anti-rabbit immunoglobulin G (IgG) (diluted 1:100 with 1% BSA) or goat anti-mouse IgG (diluted 1:100 with 1% BSA) (both from Molecular Probes, Eugene, OR, USA) – conjugated with Alexa 488 or Alexa 568, then with phalloidin conjugated with Alexa 568 (diluted 1:100 with 1% BSA) (Molecular Probes) to stain F-actin, for 1 h at room temperature. Subsequently, the cells were incubated for 10 min at room temperature with 4-6-diamino-2-phenylindole (DAPI) (diluted 1:50,000 with PBS) (Molecular Probes) to counterstain the nuclei. As controls, the cells were treated with 3% BSA instead of with the primary antibodies. The cells were then examined and photographed using a conventional fluorescence microscope (Axiophot 2; Carl Zeiss, München-Hallbergmoos, Germany) and a confocal laser scanning microscope (MRC-1024UV; Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). Horizontal sections of the epithelial cells were acquired by scanning the *x–y* axis of each specimen (0.4 μm in thickness) from the apical side to the basal side. The total image was obtained by superimposing those images. The *x–z* axis images (vertical sections) of the cells were acquired by reconstructing the *x–y* images.

Functional assays

We investigated, using a promoter or an inhibitor of integrins, the effects on migration after scraping the cultured epithelial cells. We employed the blocking-antibody P1B5 (10 μg/mL; Chemicon International, Temecula, CA, USA) to inhibit the function of integrin α_3 (15). We used a PI3K activator (10 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) to enhance the expression of integrin β_4 (16). The cells were then washed in PBS and incubated for 48 h at 37°C with defined keratinocyte serum-free medium containing the respective factors at a concentration of 10 μg/mL. Before treatment with inhibitor or activator, the wound sites of the cell sheets were photographed immediately after scraping using a phase-contrast microscope (IMT-2F; Olympus, Tokyo, Japan), and the extent of each wound width was quantified using a digital camera system (Pixera Corp., Los Gatos, CA, USA). After treatment, the cells were cultured for 48 h at 37°C and then fixed with 4% paraformaldehyde for 20 min at room temperature. The wound widths after the scraping and treatment were measured again at the same site that had been quantified before the treatment, and the percentage of re-epithelialization before treatment vs. after treatment was calculated. As a control, the same volume of PBS, as of inhibitor or promoter, was added to the medium, and comparisons of percentage re-epithelialization were measured. For statistical analyses of the results, the Kruskal–Wallis *H*-test and the Mann–Whitney *U*-test with Bonferroni correction were used to assess the differences between each group, and a *p*-value of < 0.01 was considered statistically significant.

Results

Epithelial cell culture

Small and round-shaped cells cultured from the rat oral epithelium were found to attach diffusely to the culture dishes after 24 h. After 3 d, groups of 5–10 cells had aggregated and resembled small epithelial islands (Fig. 1A). After 7–10 d, the cultured cells grew to subconfluence and several large cells (approximately 50 μm in diameter) were each surrounded by numerous small cells (approximately 20 μm in diameter) (Fig. 1B). These cells detached from the culture dishes after about 2 wk.

Immunofluorescence microscopy

Expression of laminin γ_2 — Laminin γ_2 was detected as green immunofluorescence, and DAPI-labeled nuclei were discernible as blue by confocal laser scanning microscopy. In the majority of cells in unscraped control culture sheets, a marked accumulation of laminin γ_2 was distinct in the cell periphery and in intercellular spaces, but not in the peri-nuclear region (Fig. 2A). However, laminin γ_2 was detected extensively from the peri-nuclear region to the cell membrane in several cells. Intense immunoreactivity for laminin γ_2 was discernible in cells located proximally to the scraped area, namely it was found in the peripheral cytoplasm of cells adjacent to the scraped (wound) site (Fig. 2B,D arrows). In *x–z* axis images, remarkably strong expression of laminin γ_2

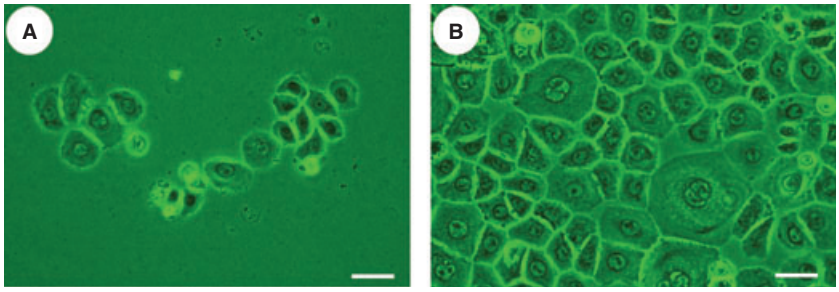

Fig. 1. Primary culture cells of the rat oral epithelium. In primary culture cells of the rat oral epithelium, 5–10 cells aggregate to form small epithelial islands that seem to attach to the culture dishes after 3 d (A). The cells are subconfluent, and several large cells (approximately 50 μm in diameter) are surrounded by numerous small cells (approximately 20 μm in diameter) after 7–10 d (B). Bar: 20 μm.

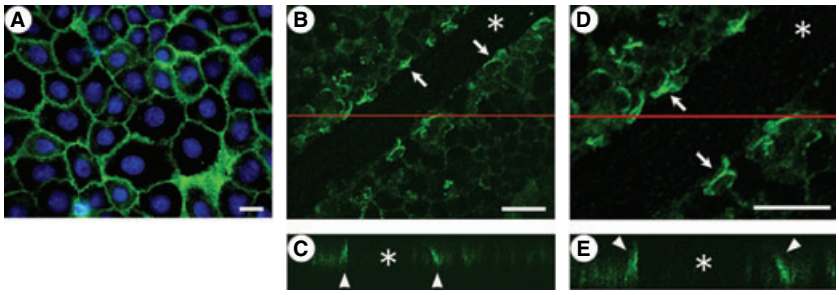

Fig. 2. Expression of laminin γ_2. Laminin γ_2 is observed as green immunofluorescence, and 4-6-diamino-2-phenylindole (DAPI)-labeled nuclei are discernible as blue in confocal laser scanning microscopy images. In most cells of the unscraped control sheets, marked accumulation of laminin γ_2 is found at the cell periphery and in intercellular spaces (A). Intense immunoreactivity for laminin γ_2 is observed in cells located proximally around the wound area, specifically in the peripheral cytoplasm of cells adjacent to the scraped area (arrows in panels B and D). In *x–z* axis images (indicated by the red line), laminin γ_2 is concentrated in basal regions of cells adjacent to the wound area (arrowheads in panels C and E). Asterisks indicate wound areas. Bar: (A) 20 μm (B, D) 50 μm.

was evident in the basal regions of cells adjacent to the scraped area (Fig. 2C,E arrowheads).

Expression of integrin β_4— Integrin β_4 could be detected as green immunofluorescence using conventional fluorescence microscopy. In untreated culture cells, integrin β_4 immunoreactivity was apparent in the entire cytoplasm and was particularly strong in the peri-nuclear region. Numerous and short cell processes were formed at the cell periphery (Fig. 3A). However, in cells around the wound area, integrin β_4 was distinct as intense immunofluorescence in the long, thick processes of cells located distally to the scraped area (Fig. 3B arrows). Intense immunoreactivity for integrin β_4 was found to occur diffusely in the central cytoplasm of several cells. F-actin fibers stained as linear red fluorescence from the cell membranes to the central cytoplasm. Large numbers of cell processes were seen at the cell periphery, and some of these extended towards the scraped area (Fig. 3C arrows). In triple immunofluorescence images, the DAPI-stained nuclei were discernible as blue fluorescence in the center of each cell. Actin fibers (red) could be distinctly observed in cell processes extending towards the wound area, whereas integrin β_4 (green) was detected in contralaterally localized cell processes (Fig. 3D).

Expression of integrin α_3— Positive reactivity for integrin α_3 was observed as punctate green fluorescence and was detected most strongly in the peri-nuclear regions of unscraped control cells. However, the fluorescence reactivity of integrin α_3 was weaker than that of integrin β_4, and accordingly it did not extend to the peripheral region (Fig. 4A). By contrast, in cells around the wound area, punctate immunoreactivity for integrin α_3 extended into the proximal cytoplasm of cells in the scraped area (Fig. 4B arrows). Immunoreactivity for integrin α_3 was also detected in the peri-nuclear cytoplasm. F-actin fibers labeled as linear red fluorescence and were observed in the peripheral cytoplasm and in cellular processes. Fine cellular protrusions were discernible in cells extending towards the scraped area (Fig. 4C arrows). Triple immunofluorescence images demonstrated distinct localization of integrin α_3 (green), actin fibers (red) and nuclei (blue). Both integrin α_3 and actin fibers were discernible in cells extending towards the scraped area (Fig. 4D).

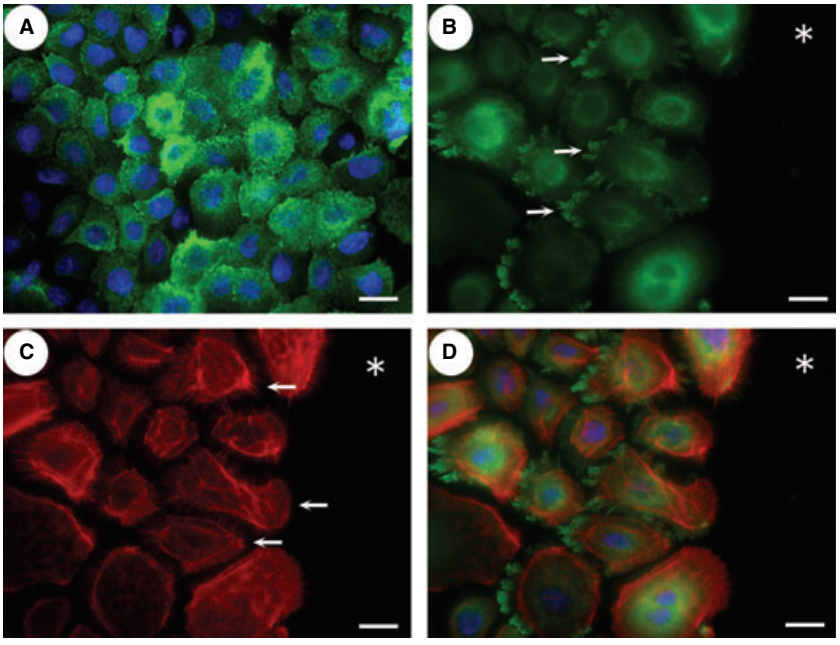

Fig. 3. Expression of integrin β_4. In untreated control cells, integrin β_4 is observed as green fluorescence: it is distributed throughout the cytoplasm and is concentrated particularly in the peri-nuclear region (A). In the scraped area, integrin β_4 is apparent as intense immunofluorescence in the long processes of cells located distally around the wound area (arrows in panel B). F-actin fibers, stained as linear red fluorescence, are present from the cell membranes to the central cytoplasm and in cell processes formed at the cell periphery, and some of those extended towards the scraped area (arrows in panel C). In triple immunofluorescence images, the 4-6-diamino-2-phenylindole (DAPI)-stained nuclei are seen as blue immunofluorescence in the center of each cell. Actin fibers (red) can be observed in cell processes extending towards the wound area. Integrin β_4 (green) is detected in the contralaterally localized cell processes (D). Asterisks display wound areas. Bar: 20 μm.

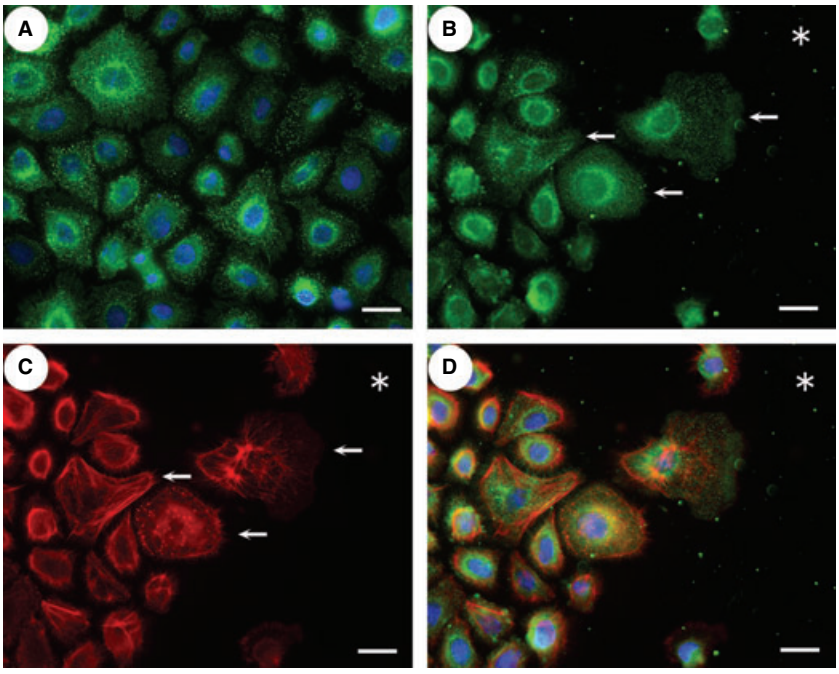

Fig. 4. Expression of integrin α_3. The expression of integrin α_3, observed as punctate green fluorescence, is strongest in the peri-nuclear region of unscraped control cells (A). In cells around the wound area, punctate immunoreactivity for integrin α_3 extends in the proximal cytoplasm to the scraped area (arrows in panel B). F-actin fibers (linear red fluorescence) can be observed in the peripheral cytoplasm, and cellular processes are discernible that extend towards the scraped area (arrows in panel C). Triple immunofluorescence images demonstrate the distinct localization of integrin α_3 (green), actin fibers (red) and nuclei (blue). Both integrin α_3 and actin fibers extend towards the scraped area (D). Asterisks indicate wound areas. Bar: 20 μm.

Relationship between the expression of integrin α_3 and integrin β_4— In specimens double-labeled for integrins α_3 and β_4, integrin α_3 was stained green and integrin β_4 was stained red (Fig. 5A-I). Strong reactivity of integrin α_3 was detected in the cytoplasm and in cell membranes of cells adjacent to the wound area (Fig. 5A arrowheads). By contrast, integrin β_4 was observed in the distal processes of cells in the scraped area (Fig. 5B arrows). The differences in expression of both proteins can be seen in the merged image (Fig. 5C).

Images of the *x–z* axis (Fig. 5G,H,I) were acquired using confocal laser scanning microscopy by reconstructing the region noted by the red line of the *x–y* axis images (Fig. 5D,E,F). Strong reactivity for integrin α_3 was distinct in the basal region of cells localized proximally to the wound area (Fig. 5I green arrow), while integrin β_4 found to be present at high levels (Fig. 5I red arrow).

Functional analysis of integrins

The mean percentage of re-epithelialization was significantly lower (2.5%) in cells treated with the PI3K activator, which promotes integrin β_4, than in the control group (39.0%) ($p < 0.01$). Similarly, in cells treated with P1B5, which inhibits integrin α_3, the mean percentage of re-epithelialization was also significantly lower (7.2%) than in the untreated control ($p < 0.01$). There was no significant difference in the percentage of re-epithelialization between the PI3K activator or P1B5 treated cells. In conclusion, re-epithelialization was strongly inhibited by the promotion of integrin β_4 or by the inhibition of integrin α_3 in primary culture-cells derived from the oral epithelium (Fig. 6).

Discussion

Recent investigations have clarified the molecular mechanism by which the junctional epithelium adheres to the enamel surface (17,18) through the IBL and hemidesmosomes, the latter consisting of laminin 5 and integrin $\alpha_6\beta_4$ (5,19). A 12-fold higher expression of laminin 5 contributes to the firm adhesion between each tooth and the junctional epithelium, although type IV collagen, nidogen and other proteins constituting the external basal lamina are not present in the IBL (2,11).

Turnover, involving cell migration, occurs in the junctional epithelium to maintain the homeostasis of periodontal tissues (8,20). However, several studies have proposed that DAT cells in the junctional epithelium may be nonmigratory and that they do not participate in the turnover because the junctional epithelium is tightly bound to the enamel surface by laminin 5 and integrin $\alpha_6\beta_4$ (1,2,9). By contrast, other studies have suggested that DAT cells participate in the turnover involving cell migration. Those studies note that laminin 5 and integrin $\alpha_6\beta_4$ are involved in adhesion between epithelial cells and enamel, while laminin 5 and integrin $\alpha_3\beta_1$ are engaged in cell migration (10–12,21–23).

In order to clarify the functional details of adhesion and migration of epithelial cells, we characterized the expression patterns of laminin 5, as well as of integrins $\alpha_6\beta_4$ and $\alpha_3\beta_1$, in those cells. In addition, we investigated the effects of a PI3K activator, which up-regulates integrin β_4, and of P1B5, which down-regulates integrin α_3, on cell migration.

Expression of integrins β_4 and α_3

It has been demonstrated previously that laminin 5 and integrin β_4 are produced by primary culture cells derived from the rat oral mucosa (13); our study supports the results of that previous report and also shows that integrin α_3 is produced by primary culture cells of the oral mucosa.

In cells of the untreated group, immunoreactivities for both integrins

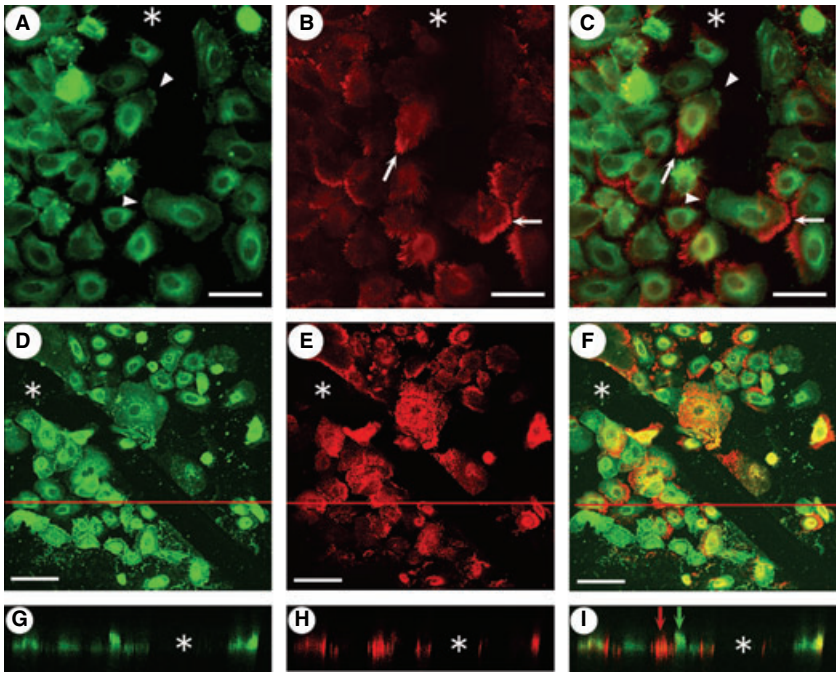

Fig. 5. Relationship between expression of integrins α_3 and β_4. In double-labeled specimens, integrin α_3 is seen as green fluorescence and integrin β_4 is observed as red fluorescence (A–C). The immunoreactivity for integrin α_3 is located in the cytoplasm and in cell membranes of cells adjacent to the wound area (arrowheads in panel A). Integrin β_4 is observed in cell processes distal to the scraped area (arrows in panel B). Arrowheads indicate integrin α_3, and arrows indicate integrin β_4. Differences in expression can be seen in panel C, which superimposes panels A and B. Images of the *x–z* axis (G, H, I) were acquired by reconstructing the region at the red lines of *x–y* axis images (D, E, F). Intense reactivity for integrin α_3 is observed in the basal region of cells localized proximally to the wound area (green arrow in panel I). Integrin β_4 is concentrated in the distal region (red arrow in panel I). Asterisks indicate wound areas. Bar: 50 μm.

β_4 and α_3 were distinct at the periphery and in the whole cytoplasm. Strong reactivity for integrin β_4 was detectable in the distal processes around the scraped areas, while integrin α_3 was distinctly observed to extend into the proximal cytoplasm. In addition, the *x–z* images acquired by confocal laser scanning microscopy demonstrated the distal expression of integrin β_4 and the proximal localization of integrin α_3 in cells located on both sides of the scraped area. Those results imply that integrin α_3 is expressed at the side towards the extension of the cell, whereas integrin β_4 is localized at the opposite side. It has been demonstrated that integrin β_4 is composed of the heterodimer integrin $\alpha_6\beta_4$ with the integrin α_6 subunit, while integrin α_3 is only composed of integrin $\alpha_3\beta_1$ with the β_1 subunit. Both integrins $\alpha_6\beta_4$ and $\alpha_3\beta_1$ exist as receptors of laminin 5 (24). Moreover, the interaction between laminin 5 and integrin $\alpha_6\beta_4$ provokes cell adhesion, whereas the interplay between laminin 5 and integrin $\alpha_3\beta_1$ gives rise to cell migration (21–23). Our results on the different localizations of integrins β_4 and α_3 in cells on both sides of the scraped area imply the roles of both integrins in cell adhesion and migration. Namely, it suggests that integrin β_4 is expressed at the distal (backward) regions of the cells to form basic points for adhesion, while integrin α_3 is localized at the proximal (frontal) regions to promote cell migration.

Expression of laminin 5

Laminin 5 is characteristic as the only protein possessing the γ_2 chain in the laminin family (4). Therefore, we examined the expression of the laminin γ_2 subunit to observe the localization of laminin 5. Our results demonstrate that laminin γ_2 accumulates at the cell periphery and at the intercellular space, but not in the peri-nuclear region, in the large groups of cells in the untreated sheets. Taking the characteristic of laminin 5 as a secretory regulation protein into account, this accumulation of laminin γ_2 in the center of each cell mass may indicate the completion status of the production and secretion of laminin 5 at the stabilized period of cell adhesion.

By contrast, in the scraped group, immunoreactivity for laminin γ_2 was found at the proximal (frontal) cytoplasm of the cells extending towards the wound area. This result is consistent with the results of the study by Pakkala *et al.* (25) that reported intense expression of laminin γ_2 as a belt-and-bow-like immunofluorescence at the peripheral cytoplasm. Likewise, the *x–z* axis images revealed intense immunoreactivity for laminin γ_2 in the basal region of cells adjacent to the wound area. This suggests strongly that cells adjacent to the wound area secrete laminin γ_2 as a foothold for cell migration after the scraping.

Functional analysis of integrins β_4 and α_3

We investigated the functions of integrins β_4 and α_3 in cell migration using a PI3K activator and P1B5. PI3K is an enzyme that phosphorylates the inositol ring of phosphatidylinositols at their 3′ position and promotes the accumulation of adhesion structures to the basal plasma membrane (26). In addition, PI3K regulates cell adhesion through integrin $\alpha_6\beta_4$ (16), and the activation of PI3K increases the level of expression of integrin $\alpha_6\beta_4$ on the surface of epithelial cells (27). By contrast, P1B5 is a blocking antibody that inhibits the action of integrin α_3 (6). We analyzed the effects of enhancing integrin β_4, and of inhibiting integrin α_3, on the percentage of re-epithelialization (scraped area), using the PI3K activator to promote the expression of integrin β_4 and P1B5 to suppress the action of integrin α_3. The results

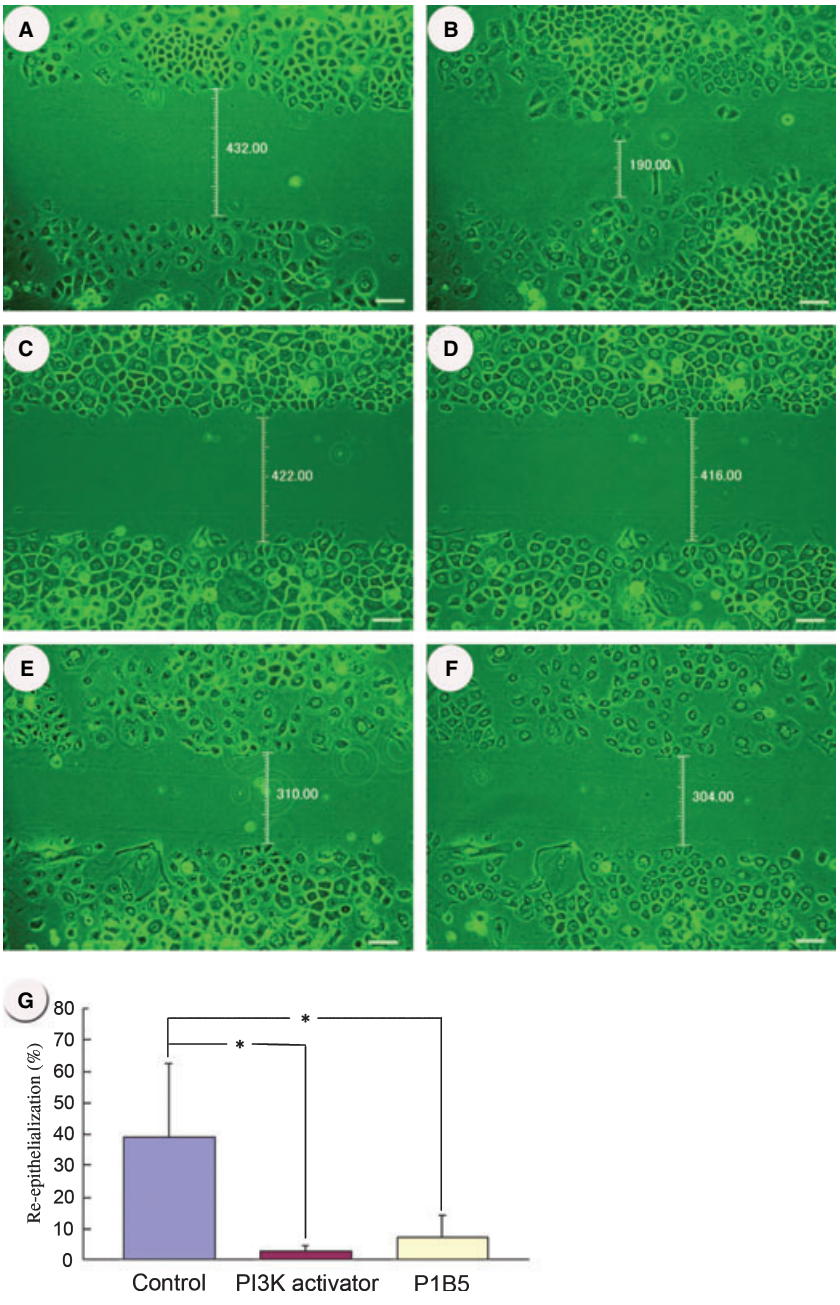

Fig. 6. Functional analysis of integrins β_4 and α_3. The involvement of integrins in re-epithelialization was analyzed using a phosphatidylinositol-3-kinase (PI3K) activator to enhance integrin β_4 and using P1B5 to inhibit integrin α_3. Observations using an inverted stereomicroscope show the control (top), the PI3K activator-treated (middle) and the P1B5-treated (bottom) wounded cells. Left panels (A, C, E) show cells immediately after scraping, and right panels (B, D, F) show cells 48 h later. The mean percentage of re-epithelialization in PI3K activator-treated cells and in the P1B5-treated cells is shown in panel G. (n = 22) Bar: 100 μm.

showed that the percentage of re-epithelialization was significantly suppressed both in the PI3K activator-treated and the P1B5-treated groups compared with the control group. Increasing the expression of integrin $\alpha_6\beta_4$ enhances the formation of hemidesmosomes with laminin 5 and promotes cell adhesion to the basal surface by stimulating PI3K. As a result of the enhanced cell adhesion, the dissociative reaction between cell and surface of cover glasses would be inhibited and cell migration may be suppressed. An alternative possibility could be envisaged in which the integrin α_3 antibody inhibits the interaction of integrin α_3 with laminin 5, suppressing the initial cell spreading, and thus inhibiting the percentage of re-epithelialization.

From our functional analyses, we confirm the action of integrin α_3 on cell migration. It has been suggested that abundant expression of laminin 5 and integrin α_3 is involved in the attachment and migration of DAT cells to teeth by hemidesmosomes and might contribute to the migration of DAT cells in order to prevent the invasion of bacteria (11). Currently, the mode of adhesion and migration of the regenerative epithelium following gingivectomy has been demonstrated by Masaoka *et al.* (12). They observed that laminin γ_2 is initially expressed to serve as a foothold, after which integrins β_4 and α_3 are detected in the frontal margins of the regenerating epithelial cells, and finally the expression of laminin γ_2, integrins β_4 and α_3 are observed in DAT cells of the regenerating epithelial cells. The expression patterns of laminin γ_2, integrin β_4 and integrin α_3 following gingivectomy are analogous to those of the cultured cells on both sides of the scraped area seen in our study. Those changes can be explained by the hypothesis of Goldfinger *et al.*, who proposed a functional model of laminin 5, integrin $\alpha_6\beta_4$ and integrin $\alpha_3\beta_1$ in wound healing (19,28). Following scraping of the cell sheets, intensive expression of laminin γ_2 was distinct at the frontal (proximal) cytoplasm extending towards the wound area, because those epithelial cells secreted extracellularly unprocessed laminin 5 (α_3 subunit: 190 kDa) to make a foothold. Subsequently, integrin α_3 was strongly expressed in the peripheral cytoplasm extending towards the wound area and might combine with the unprocessed laminin 5 just secreted. It is implied that integrin β_4 forms hemidesmosomes by binding to processed laminin 5 (α_3 subunit:

160 kDa), and is expressed strongly at the distal (backward) regions of cells to form adhesion points to assist cell migration.

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