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Differential localization of laminin γ_2 and integrin β_4 in primary cultures of the rat gingival epithelium

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Objectives: The aim of this study was to investigate the differential immunolocalization of laminin γ_2 and integrin β_4 in primary cultures of the rat gingival epithelium.

Methods: The gingival epithelium was obtained from Sprague-Dawley rats and was cultured in serum-free keratinocyte growth medium (DK-SFM). Western blotting analysis, immunofluorescence, confocal laser scanning microscopy (CLSM), and immuno-gold labeling for laminin γ_2 and integrin β_4 were employed. CLSM images for laminin and integrin were analyzed in horizontal (x–y axis) and in vertical (x–z axis) sections.

Results: Both laminin γ_2 and integrin β_4 were detected by Western blot analysis in the gingival epithelium. Immunolocalization of laminin γ_2 was distinct in the cytoplasm to form one or two irregular rings in gingival epithelial cells. By contrast, integrin β_4 was localized diffusely in the cytoplasm. F-actin (indicating actin filaments) was clearly discernible at the periphery of the cytoplasm to form a cellular fringe. In x–z axis images obtained by CLSM, laminin γ_2 was recognized as large foci in the most inner portion just above the basal plasma membrane. Integrin β_4 existed in the area where F-actin was labeled surrounding the membrane. Immuno-electron microscopy showed that 10nm colloidal gold particles indicating laminin γ_2 were mainly localized at the extracellular portion and in the peripheral cytoplasm, whereas integrin β_4 was distributed in the cytoplasm close to the basal plasma membrane but not in extracellular regions.

Conclusions: In primary cultures of the rat gingival epithelium, both laminin γ_2 and integrin β_4 may be produced by the epithelium, and irregular rings of laminin γ_2 are formed in areas where gingival cells adhere to the extracellular matrix.

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It is well known that the junctional epithelium participates in the firm attachment of epithelial cells to the tooth surface to separate the periodontal tissue from the external environment (1, 2). For that reason, the unique location of the junctional epithelium at the hard–soft tissue interface is a key to the initiation and progression of periodontal disease (3).

In the internal basal lamina, cell attachment of the junctional epithelium to the enamel surface is characterized by hemidesmosomes and the basal lamina exhibits an accumulation of extracellular matrix on the enamel surface (4–7). It has been proposed that cells aligned in the most superficial layer facing the enamel in the junctional epithelium should be termed as 'cells directly attached to the tooth' (DAT cells) (8, 9). Those cells possess firm adhesive structures that bind to

the tooth with integrin $\alpha_6\beta_4$ and laminin-5 in the internal basal lamina (4–6, 10).

Laminins are a family of extracellular matrix proteins that are cross-shaped heterotrimers of α, β and γ chains and that are mainly localized in the basal lamina of various tissues. Hormia *et al.* have proposed that integrin $\alpha_6\beta_4$ and its ligand, laminin-5, are expressed in the area of the internal basal lamina. Collagen IV and laminin-1, which are basic basal lamina components, have not been detected in the area of the internal basal lamina, which is different from all other basal lamina (4, 5, 6). It has been noted that laminin-5 is peculiar because it is the only protein in the family that has a γ_2 chain (11).

On the other hand, integrins are heterodimeric transmembrane proteins, which serve as receptors for extracellular matrix components and cell surface proteins (12, 13). Integrin β_4, which is unique among integrin β subunits because of its much larger cytoplasmic domain, may be involved in linking keratin filaments to these structures (14, 15). It has been proposed that the suprabasal distribution of α_6 is due to its association with β_1 in cell-to-cell contacts, whereas polarized basal expression is associated with β_4 in cell-to-extracellular matrix contacts (16). Based on those studies, we investigated laminin γ_2 and integrin β_4 in this study.

Nevertheless, little is known about the precise distribution of adhesive molecules and extracellular matrixes in gingival epithelial cells and in the internal basal lamina. It is also an open question whether laminin γ_2 and integrin β_4 of gingival cells exist on the attached surface of coverglass *in vitro*. Therefore, we examined the expression and immunolocalization of laminin γ_2 and integrin β_4 in the attachment face of primary cultures of rat gingival epithelium, spreading on the flattened glass surface lacking additional extra cellular matrixes and cellular elements, using western blotting, immunofluorescence, confocal laser scanning microscopy (CLSM) and immuno-gold labeling methods.

Materials and methods

Gingival epithelial tissue

The oral mucosa was obtained from 30 male Sprague-Dawley rats, each weighing about 60 g (3 weeks old). The gingival epithelium, including junctional epithelium surrounding molar teeth of the upper and lower jaws, was dissected. The epithelial layer was removed from the underlying connective tissues by digestion with dispase (25 U/ml: Roche, Mannheim, Germany) for 3 h at 37°C (17).

Preparation of cytosolic and total membrane fractions

Rat gingival epithelial tissues were minced into small pieces using a razor blade. The tissues were homogenized in 320 mM sucrose, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 20 mM HEPES (pH 7.5) using a Teflon-pestle homogenizer (As One, Tokyo, Japan). The nuclear fraction was removed from the homogenate by centrifugation at 750 *g* for 10 min, and the postnuclear supernatant was centrifuged at 200,000 *g* for 120 min. The resultant supernatant and pellet were used as the cytosolic and total membrane fractions, respectively (18).

Western blot analysis

Proteins were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to PVDF membrane (Bio-Rad Laboratories Ltd, Hercules, CA, USA) and blotted with anti-laminin γ_2 mouse monoclonal antibody (1:100: Chemicon International, Inc., Temecula, CA, USA) or with anti-integrin β_4 rabbit polyclonal antibody (1:100: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 24 h at 4°C. Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) were used as secondary antibodies for 60 min at room temperature, and these proteins were detected by ECL Western Blotting Analysis System (Amersham Biosciences). X-ray film (Hyperfilm ECL, Amersham Biosciences) was used to analyze the blots.

Cell and cell culture

The gingival epithelium obtained by a manner mentioned above was dissolved with 0.05% trypsin-EDTA (Invitrogen Corp., Carlsbad, CA, USA) after mincing for 30 min at room temperature in order to obtain a single cell suspension. After passage through a cell strainer (100μm mesh: Becton Dickinson and Company, San Jose, CA, USA) the cells were seeded on 35-mm uncoated cell culture dishes (Iwaki, Chiba, Japan) in serum-free keratinocyte growth medium (DK-SFM: Invitrogen Corp.). After 3 days, the epithelia began to grow in islands and showed typical epithelial morphology. When they were close to confluent (usually 10 days after incubation), the cells were harvested and used for the experiments.

Immunofluorescent microscopy

Cells used for immunofluorescent studies were placed onto prepared cover glass surfaces, cultured at 37°C for 10 days, and fixed with 2% paraformaldehyde in 0.1 M sodium cacodylate, for 30 min at room temperature. After washing with phosphate-buffered saline, the cells were incubated with an anti-laminin γ_2 mouse monoclonal antibody (Chemicon International, Inc.) or with anti-integrin β_4 rabbit polyclonal antibody (Chemicon International, Inc.) for 2 h at room temperature. After washing with phosphate-buffered saline, the cells were incubated with a secondary antibody, goat anti-mouse IgG or goat anti-rabbit IgG conjugated with Alexa 488 (Molecular Probes, Inc., Eugene, OR, USA), Alexa 568 phalloidin (Molecular Probes, Inc.) for F-actin, and TO-PRO-3 iodide (Molecular Probes, Inc.) for nuclear counter staining, respectively. As a control, specimens were treated with normal goat serum instead of antibodies of laminin γ_2 and integrin β_4

in the control. The cells were examined and photographed using a conventional fluorescence microscope (Axiophot 2: Carl Zeiss Co. München-Hallbergmoos, Germany) and a CLSM (MRC-1024UV: Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Horizontal sections of gingival epithelial cells were acquired by scanning the *x–y* axis of each specimen (0.4 μm in thickness) from the apical 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.

Immuno-electron microscopy

For ultra-thin immuno-gold labeling, the cells were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, and were collected and embedded in a 1% agarose gel. These cells were embedded in 2.3 M sucrose and were rapidly frozen by liquid nitrogen. Ultra-thin cryo-sections were cut using an ultra-cryo-microtome at −100°C (FC-4S, Reichert Ultracut-S: Reica, Co., Heidelberg, Germany). The cryo-sections were mounted on carbon-coated formvar nickel grids and were incubated with the mouse monoclonal primary antibody for laminin γ_2 (Chemicon International, Inc.) for 2 h at 37°C. After rinsing as noted above, the sections were incubated with secondary antibodies anti-mouse IgG conjugated with colloidal gold (10 nm: British Biocell International Ltd, Cardiff, UK) for 45 min at 37°C. The sections were treated with the rabbit polyclonal primary antibody for integrin β_4 (Chemicon International, Inc.) for 2 h at 37°C, then reacted with a secondary antibody anti-rabbit IgG conjugated with colloidal gold (10 nm: British Biocell International Ltd) for 45 min at 37°C. After staining with uranyl acetate and lead citrate, the sections were examined and photographed using a transmission electron microscope (H7100: Hitachi, Co., Tokyo, Japan).

Results

Western blot analysis

Western blot analysis demonstrated distinct bands of laminin γ_2 (at 105 and 150 kDa) and integrin β_4 (at 150 kDa) in the cytosolic supernatant and in the total membrane fractions (Fig. 1). Both laminin γ_2 and integrin β_4 were more abundant in the cytosolic supernatant than in the total membrane fraction. In the total membrane fraction, integrin β_4 was present at higher levels than laminin γ_2 (Fig. 1).

Epithelial cell culture

Primary cultures of rat gingival epithelium began to attach to the culture dishes in small and round shapes after 24 h. After 3 days, the cells grew in small islets and showed typical epithelial sheets. After 7–10 days, the cells became confluent and a small number of large cells were surrounded by several small cells (Fig. 2a). Those cells peeled off from the dishes in about 2 weeks. Figure 2(b) shows F-actin stained fluorescent red by phalloidin. Filaments reactive for F-actin were detected clearly in the epithelial cells as bundles of red fluorescence aligned with the cell contour. Numerous small dots indicating accumulations of actin filaments were also distinct in the cytoplasm of the epithelial cells (Fig. 2b).

Immunofluorescent microscopy

Expression of laminin γ_2 Laminin γ_2 was detected as diffusely distributed green fluorescence and nuclei stained by TO-PRO-3 were observed as round areas of red fluorescence in the centers of the epithelial cells.

Laminin γ_2 immunoreactivity was observed obviously as one or two irregular rings or peripheral fringe of immunoreactivity in the cytoplasm. These rings were observed approximately 25 μm in diameter. Fibers reactive for F-actin were also observed in the cytoplasm, except for the area of laminin γ_2 localization. In specimens examined by double immunofluorescent labeling for F-actin and laminin γ_2, positive reactions for both were detected in the peri-nuclear cytoplasm, but not in the nucleus. F-actin filaments were strongly reactive at the periphery of the cytoplasm to form cellular fringes (Figs 2c and d, arrowheads).

Expression of integrin β_4 Immunoreactivity of integrin β_4 was also recognized as green fluorescence. Integrin β_4 was localized diffusely in the cytoplasm, but the circular immunoreactive areas observed for laminin γ_2 were not distinct. Various-sized foci were mingled (Fig. 2e, open arrowheads). Relatively large foci of integrin β_4, about 5 μm in diameter, were frequently located in the peripheral cytoplasm (Fig. 2f, open arrowheads). F-actin stained by phalloidin was discernible at the periphery of the cells to form the cell-frame.

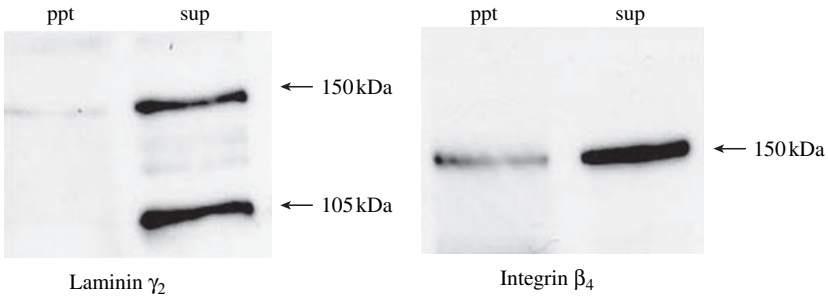

Fig. 1. Immunoblot of laminin γ_2 and integrin β_4. In Western blot analysis, the ppt lane shows the total membrane fraction and the sup lane shows the cytosolic supernatant. Bands of laminin γ_2 (at 105 and 150 kDa) and integrin β_4 (at 150 kDa) can be detected in both samples. In the total membrane fraction, integrin β_4 is more abundant than laminin γ_2.

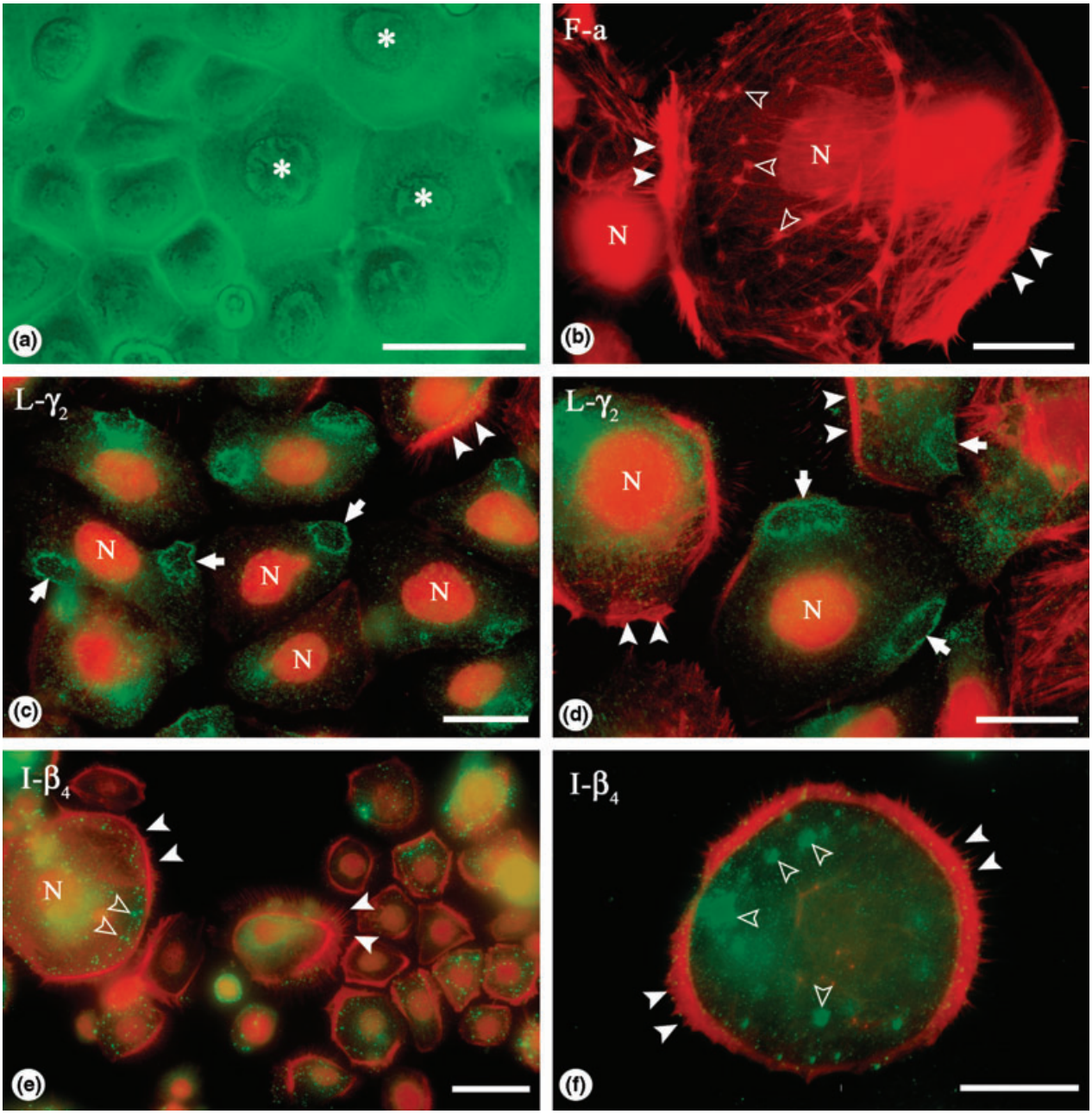

Fig. 2. (a–f) Immunofluorescence study. Primary cultures of rat gingival epithelium in small islets and displaying typical epithelial sheets. The cells become confluent, and fewer large cells (asterisks) were each surrounded by several small cells (a). F-actin filaments can be detected clearly as bundles of red fluorescence to form the cell contour (arrowheads). A large number of small dots are distinct in the cytoplasm of gingival cells (open arrowheads) (b). Laminin γ_2 is detected as green fluorescence and nuclei are observed as round areas of red fluorescence (N). Laminin γ_2 can be observed as one or two irregular rings or peripheral fringes of immunoreactivity, approximately 25 μm in diameter (arrow). Fibers reactive for F-actin are observed in the cytoplasm except for areas of laminin γ_2 localization (arrowheads) (c, d). Immunoreactivity of integrin β_4 is detected as green fluorescence. It can be observed diffusely in the cytoplasm, and various sized foci are mingled (e). Foci about 5 μm (open arrowheads) exist in the cytoplasm of epithelial cells (f). Original magnification: (a) × 460, (b) × 650, (c) × 275, (d) × 650, (e) × 250, (f) × 750. Bar: (a), (c), and (e), 50 μm; (b), (d), and (f), 25 μm. F-a: F-actin; I-β_4: integrin β_4; L-γ_2: laminin γ_2; N: nucleus.

No immuno-reaction could be found in control (the data was not shown).

Confocal laser scanning microscopy observation

Expression of laminin γ_2 In *x–y* axis images of CLSM, laminin γ_2 was strongly expressed in the cytoplasm to form one to two ring-like structures as green fluorescence (Fig. 3a, arrow). The F-actin fibers were observed to form protuberances and fragments around the cells, except for areas where the laminin γ_2 circular structures were localized (Fig. 3a, arrowheads). In *x–z* axis images of CLSM, laminin γ_2 was recognized distinctly as round areas of accumulation and linear deposits of immunoreactivities in the most inner portions of the cells just above the culture dish surface (arrow). However, little reactivity could be found in other portions of the cytoplasm facing the dish. F-actin reactivities were observed along the boundary between the epithelial cells and the culture dish (arrowheads), except for areas where laminin γ_2 was expressed (Fig. 3b).

Expression of integrin β_4 In *x–y* axis images of CLSM, integrin β_4 was localized in the entire cytoplasm as green fluorescence, but rarely in the nucleus (Fig. 3c). In *x–z* axis images, integrin β_4 was diffusely located in the cytoplasm and colocalized with F-actin. F-actin was recognized along the cell membrane as linear structures at the boundary between the cell and the culture dish (Fig. 3d, arrowheads).

Immuno-electron microscopical observation

By immuno-electron microscopy, 10nm colloidal gold particles indicating laminin γ_2 were detected mainly at the basal extracellular portion and in the peripheral cytoplasm of the cells (Fig. 4a, arrowheads). Gold particles reacting with integrin β_4 were localized in the cytoplasm close to the basal plasma membrane but not in extracellular regions (Fig. 4b, arrowheads).

Discussion

It is well known that firm adhesive structures between surface cells of the junctional epithelium and the enamel are composed of laminin-5 and integrin $\alpha_6\beta_4$. Among these adhesive proteins, we examined laminin γ_2 and integrin β_4 because laminin-5 only possesses the γ_2 subunit and integrin β_4 may be involved in cell-to-extracellular matrix contacts (11, 16). Thus, the expression of laminin γ_2 signifies the existence of laminin-5 and integrin β_4 seems to be more important than integrin α_6 in relation to cell-to-extracellular matrix contacts.

Studies on the expression of laminin and integrin by cells cultured from the oral mucosa have been examined using

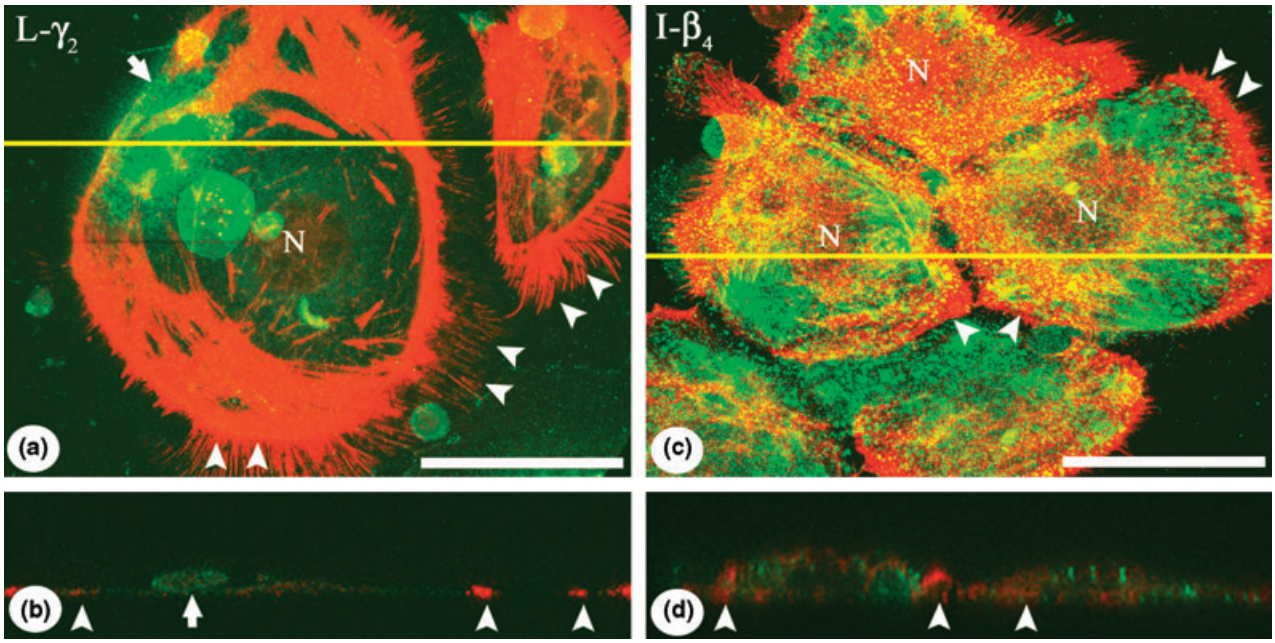

Fig. 3. (a–d) Confocal laser scanning microscopy (CLSM) observation. In *x–y* axis images of CLSM, laminin γ_2 is strongly expressed in the cytoplasm to form one to two ring-like structures as green fluorescence (arrow). F-actin filaments are observed to form fragments around the cells (arroweads) (a). In *x–z* axis images of CLSM (cut at the yellow line), laminin γ_2 is recognized distinctly as round accumulations (arrow) and linear depositions of weak immunoreactivities in the most inner portions just above the surface of the culture dish. F-actin reactivities were observed along the boundary between the epithelial cells and the culture dish (arrowheads), except for areas where laminin γ_2 was expressed (b). In *x–y* axis images, integrin β_4 is localized in the entire cytoplasm as green fluorescence (c). In *x–z* images cut at the yellow line, integrin β_4 is diffusely located in the cytoplasm and coexists with F-actin. F-actin is observed on the cell membrane at the boundary between the cell and the dish (arrowheads) (d). Original magnification: (a–d) × 550. Bar: 50 μm. I-β_4: integrin β_4; L-γ_2: laminin γ_2; N: nucleus.

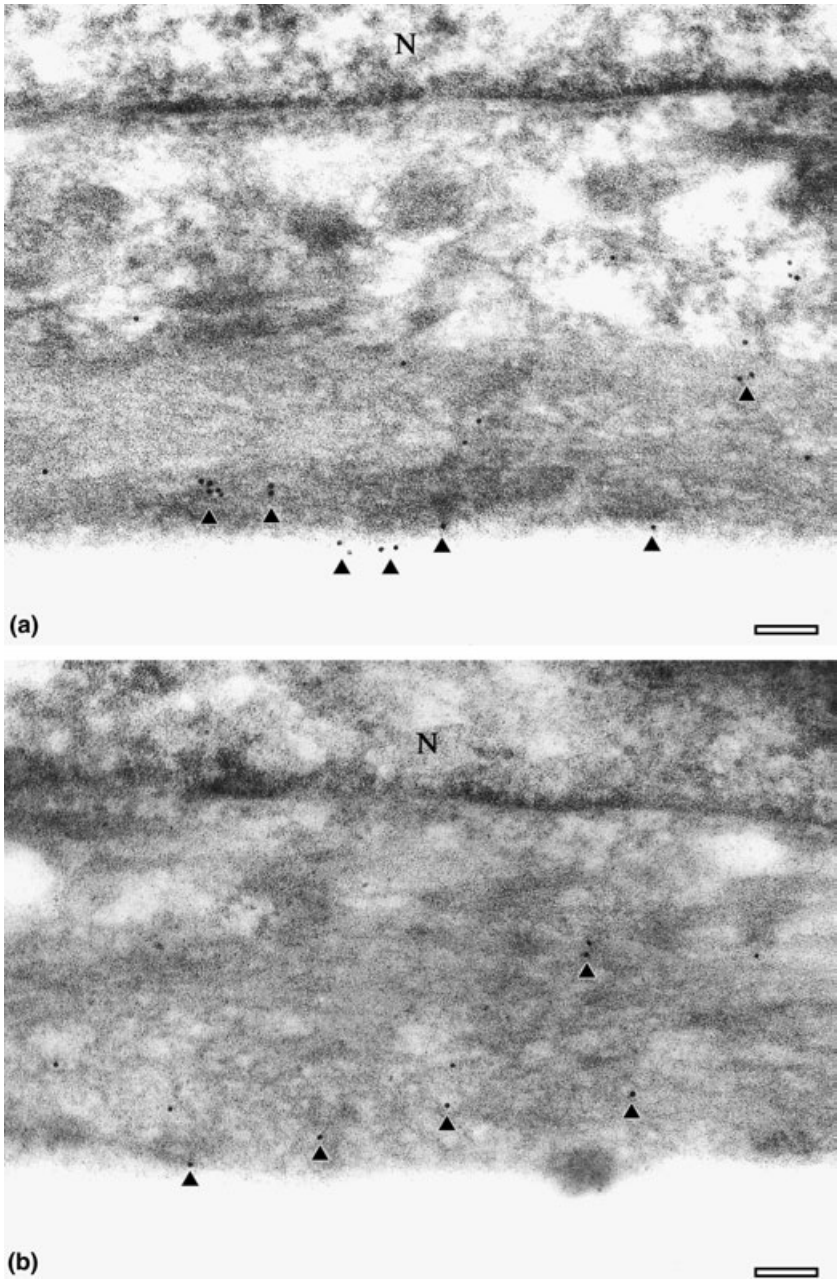

Fig. 4. (a and b) Immuno-gold staining for laminin γ_2 and integrin β_4. Colloidal gold particles of 10 nm indicating laminin γ_2 were mainly detected at the basal extracellular portion and in the peripheral cytoplasm of the cells (arrowheads) (a). Colloidal gold particles of 10 nm reacting with integrin β_4 were localized in the cytoplasm close to the basal plasmamembrane but not in extracellular regions (arrowheads) (b). Original magnification: (a) and (b) × 60,000. Bar: 0.1 μm. N: nucleus.

rat oral mucosa (19), human oral mucosa (20), and human gingival keratinocytes (21). Among those studies, Pakkala *et al.* investigated the distribution of laminin and integrin using Western blotting and immunofluorescence (21). They demonstrated bands of laminin γ_2 at 105 kDa and at 150 kDa. By Western blot analysis, we also detected the expression of laminin γ_2 (at 105 and 150 kDa) and integrin β_4 (at 150 kDa) to support those previous studies (21, 22). In our studies, laminin γ_2 and integrin β_4 are more abundant in the cytosolic fraction than in the total membrane fraction, which suggests that both laminin and integrin are localized more intensively in the cytoplasm than on the membrane. In this condition, large fragments of the membrane including plasma membrane were accumulated into the pellet, and mostly small vesicles including laminin γ_2 may still remain in supernatant as soluble fraction. Furthermore, laminin-5 expression was observed in the whole cytoplasm of the enamel-faced junctional epithelial cells, which were probably 'cells directly attached to the tooth' (DAT cells) (7, 23). In the membrane fraction, integrin β_4 is more abundant than laminin γ_2, which may reflect the fact that laminin is a secretory/regulatory protein, whereas integrin is a membrane-penetrating protein (13, 24).

The distinct localization of laminin γ_2 and integrin β_4 could be seen not only in Western blots but also in CLSM images. Using CLSM, Shiraiwa *et al.* investigated the expression of laminin γ_2 in cells of rat oral mucosa cultured on titanium (19). However, they only analyzed the horizontal but not the three-dimentional distribution. In *x–z* axis images of CLSM, we detected laminin γ_2 localized at the boundary between the cells and the culture dish. Little reactivity could be found in other portions of the cytoplasm facing the dish. On the other hand, integrin β_4 is localized diffusely in the cytoplasm and numerous small accumulations of immunoreactivity could be detected near the boundary. These differences in localization of both proteins might result from the different natures of laminin and integrin.

Few studies on the expression of laminin and integrin in the rat gingival epithelium have been reported employing immunogold labeling with ultra-thin frozen sections. Our immuno-electron microscopy results confirm the differential distribution of laminin and integrin. Laminin γ_2 is mainly localized at the extracellular portion and in the peripheral cytoplasm, whereas integrin β_4 is distributed in the cytoplasm close to the basal plasma membrane but not in extracellular regions. This may reflect the fact that laminin is located in the

extracellular matrix, whereas integrin is distributed in the plasma membrane.

In immunofluorescence studies, Pakkala *et al*. have also observed positive reactions for laminin-5 as peripheral belt-like arrays underneath cell islands (21). In our results using primary cultures of rat gingival epithelium, laminin γ_2 immunofluorescence is detected as circular rings about 25 μm in diameter, rings that were not mentioned by Pakkala *et al*. (21). The rings indicating laminin γ_2 aggregation are peculiar structures and do not contain F-actin. This implies that there is no actin filament in the area where laminin is involved in the adhesion of cells to the extracellular matrix. Taking the nature of laminin as a secretory/regulatory protein into account, large amouts of laminin may be produced and secreted to form ring structures when cells adhere.

On the other hand, integrin β_4 immunoreactivity is observed diffusely in the cytoplasm. This pattern of integrin distribution correlates with the fact that integrin is a non-regulated membrane-penetrating protein and indicates that integrin is produced and distributed constantly in the cytoplasm toward the plasma membrane.

Altman *et al*. firstly succeeded to collect pure junctional epithelium by curetting after flap operation in rats, and established the primary culture method by coculture with 3T3 fibroblasts (25). They examined the keratin expression in junctional epithelium, sulcular epithelium, and gingival oral epithelium. They pointed out that a 57 kDa band of keratin indicating characteristics of gingival epithelium disappeared when the cells were cultured, namely the keratin expression of gingival epithelium was markedly altered into those of junctional epithelium. Furthermore, a 48 kDa band of keratin disappeared when the cultured cells were confluent, and differences of both cell types were subsequently lost.

From these results, we considered that the primarily cultured cells derived from sulcular, oral gingival, and junctional epithelia in our experiment had similar characteristics to the cultured cells of junctional epithelium. In addition, it is well known that the junctional epithelium is physiologically replaced by the oral epithelium during its turnover (1).

An experiment by Caffesse *et al*. has indicated that the biologic characteristics of the junctional epithelium are induced by attachment to the tooth (sulcular environment) (26). This may imply that the features of cultured cells from oral epithelium are probably changed into those of cells from junctional epithelium when the cells attach to the surface of a culture dish, which is analogous to the enamel from the lack of extracellular matrix and cellular elements point of view.

Our studies employing cultured cells on non-coated dishes were designed to model the situation of the junctional epithelium facing the enamel surface by internal basal lamina where collagens do not exist. Because we could confirm the existence of laminin γ_2 and integrin β_4 in the cultured cells, we were able to evaluate whether our experimental model is appropriate to study the interface between the junctional epithelium and the enamel surface.

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