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Involvement of laminin and integrins in adhesion and migration of junctional epithelium cells

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KEYWORDS

laminin • integrin • junctional epithelium • migration • laser microdissection • BrdU

ABSTRACT

Background and Objective: The junctional epithelium attaches to the enamel surface with hemidesmosomes (of which laminin-5 and integrin- $\alpha 6\beta 4$ are the main components) in the internal basal lamina. Laminin-5 is also involved in cell motility with integrin- $\alpha 3\beta 1$, although their functions have not yet been clarified. The purpose of this study was to determine the functions of those adhesive components between the tooth and the junctional epithelium during cell migration. Because an idea has been proposed that directly attached to tooth cells (DAT cells) may not contribute to cell migration, 5-bromo-2-deoxyuridine staining was performed to confirm cell migration.

Material and Methods: We investigated laminin-5 (contained only in laminin-5), integrin- $\beta 4$ (involved in cell-extracellular matrix contact) and integrin- $\alpha 3\beta 1$ (inducing cell migration) in the junctional epithelium, oral gingival epithelium and gingival sulcus epithelium of 6-wk-old ICR mice using laser microdissection, quantitative real-time reverse transcription-polymerase chain reaction, immunofluorescence and 5-bromo-2-deoxyuridine staining.

Results: Laminin and integrins were clearly immunolocalized in the basal lamina of all epithelium. Quantitative analysis of laminin and integrin mRNAs by laser microdissection showed that they were more highly expressed in DAT cells than in basal cells in the oral gingival epithelium. In particular, a 12-fold higher expression of laminin-5 was observed in the junctional epithelium compared with the oral gingival epithelium. 5-Bromo-2-deoxyuridine staining showed rapid coronal migration of DAT cells.

Conclusion: These results suggest that the abundant expression of laminin-5 and integrin- $\alpha 6\beta 4$ is involved in the attachment of DAT cells to teeth by hemidesmosomes. Abundant expression of laminin-5 and integrin- $\alpha 3\beta 1$ might assist in DAT cell migration, confirmed by 5-bromo-2-deoxyuridine staining during the turnover of junctional epithelium.

Introduction

The junctional epithelium, a unique type of epithelium that forms the dento–epithelial junction, adheres to teeth by hemidesmosomes in the internal basal lamina (1,2). Laminin-5 has been identified in the internal basal lamina of the junctional epithelium by immunohistochemistry and by in situ hybridization (3,4). It is well known that the basal lamina is composed of extracellular matrix containing laminin, type-4 collagen and proteoglycan (5,6). However, only laminin-5 is found in the internal basal lamina, which also lacks type-4 collagen, and both of those elements are found in the extracellular matrix (3).

Laminin is a cross-shaped heterotrimer consisting of three subunits. Fifteen kinds of laminin have been identified thus far, all of which consist of combinations of three α , three β and three γ chains (7). Laminin-5, which consists of subunits of $\alpha 3$, $\beta 3$ and $\gamma 2$, in particular contributes to cell adhesion associated with integrin $\alpha 6\beta 4$ at hemidesmosomes (5,8). A recent investigation on the localization of laminin-5 and integrins in cultured gingival epithelium demonstrated the existence of these proteins that were produced by the epithelial cells where they contacted the surface of the culture dishes (9). However, the detailed localization and expressed quantity of these proteins in situ have not yet been confirmed.

As the internal basal lamina adheres to the enamel surface, it is difficult to analyze, in a quantitative manner, exclusive adhesion proteins of the junctional epithelium. For this reason, quantitative analysis of adhesion proteins, including laminin-5, integrins $\alpha 6\beta 4$ and $\alpha 3\beta 1$, has not been reported. Consequently, it remains to be determined why there is no laminin other than laminin-5 in the internal basal lamina. To determine and analyze the relative distribution of these adhesion proteins in the junctional epithelium, sulcus epithelium and oral gingival epithelium, laser microdissection would be useful to allow cells or tissues to be exclusively and accurately targeted under a microscope (10,11).

On the other hand, it has been suggested that laminin-5, together with integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$, is involved in cell motility during the invasion of cancer cells and wound healing (12,13). It has been controversial whether directly attached to the tooth-facing cells (DAT cells) of the junctional epithelium can migrate, although the turnover time is believed to be faster in the junctional epithelium than in the oral gingival epithelium. Several investigators have proposed that DAT cells may be nonmigratory cells and that they do not participate in the turnover of the junctional epithelium (3,14,15). In contrast, other studies have reported evidence supporting a

high turnover of DAT cells (16–19). Thus, questions remain about whether DAT cells migrate to participate in cell turnover of the junctional epithelium (regardless of their expression of adhesion proteins), why there is no laminin (other than laminin-5) expressed in the internal basal lamina and how adhesion proteins are related to turnover of the junctional epithelium. In this study, we investigated the expression and immunolocalization of laminin $\alpha 2$ that possessed only laminin-5, integrin $\beta 4$ that can only form a heterodimer with integrin $\alpha 6$, and integrin $\alpha 3$ that can only form a heterodimer with integrin $\beta 1$, in DAT cells of the junctional epithelium, in basal cells of the oral gingival epithelium and in basal cells of the sulcus epithelium, using laser microdissection. We also measured cell migration using 5-bromo-2-deoxyuridine staining, and we discussed the involvement of laminin and integrins in the adhesion and migration of junctional epithelium cells.

Material and methods

Sample preparation (immunofluorescence microscopy and laser microdissection)

Gingival mucosa was obtained from 36-wk-old-male ICR mice, each weighing ~ 30 g. The animals were deeply anesthetized with 100 mg/kg of 25% sodium thiopental. After dislocating M1 and M2, the gingival epithelium surrounding the maxillary molar teeth, including the junctional epithelium and molar teeth, was detached. A spoon excavator was inserted from the gingival sulcus under the substance microscope and the molar teeth were then carefully exfoliated from the gingival epithelium. Afterwards, it was confirmed that there was no remaining gingival epithelium on the tooth surface. The palatal gingiva was excised linguo-buccally between M1 and M2, embedded in optical cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and frozen quickly in isopentane that had been refrigerated in liquid nitrogen. Frozen sections were cut at 6- μ m thickness for analysis using immunofluorescence. Other frozen sections were cut at 8- μ m thickness and were mounted on glass slides on which films were mounted for laser microdissection. All experiments were carried out according to the Guidelines for the Treatment of Animals established by the Tokyo Dental College.

Immunofluorescence microscopy

Sections for immunofluorescence were fixed in acetone for 5 min and

then dried for 30 min at 25°C. The sections were then incubated with polyclonal rabbit anti-laminin α 2 (diluted 1 : 100 with 3% bovine serum albumin) (Abcam, Cambridge, UK), polyclonal rabbit anti-integrin β 4 (diluted 1 : 100 with 3% bovine serum albumin) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal rabbit anti-integrin α 3 (diluted 1 : 100 with 3% bovine serum albumin) (Chemicon International, Temecula, CA, USA) for 2 h at room temperature. Next, the sections were incubated with a secondary antibody, goat anti-rabbit immunoglobulin G conjugated to Alexa 488 (diluted 1 : 100 with 3% bovine serum albumin) (Molecular Probes, Eugene, OR, USA), for 1 h at 25°C. Following this, the sections were then examined and photographed using a conventional fluorescence microscope (Axiophot 2; Carl Zeiss, München-Hallbergmoos, Germany). As controls, bovine serum albumin added to phosphate-buffered saline was used as the first antibody and no nonspecific reactions were observed.

Laser microdissection

Frozen sections were fixed in 100% methanol for 3 min, washed with 0.01% diethyl pyrocarbonate-treated water and stained with 0.1% toluidine blue. Basal cells of the oral gingival epithelium, basal cells of the sulcus epithelium and DAT cells in the sections were then microdissected using an AS Laser Microdissection System (Leica Microsystems, Wetzlar, Germany) (Fig. 1). Twenty-four cuts for each sample were collected in tubes to which 30 μ L of RNA denaturing solution had been added, containing 4 m guanidine thiocyanate (Sigma-Aldrich, St Louis, MO, USA), 25 mm sodium citrate (Wako, Osaka, Japan) and 0.5% sarcosyl (Sigma-Aldrich).

RNA extraction

Total RNA was extracted from the three types of cells in the laser-microdissected areas. One-hundred and seventy microlitres of RNA denaturing solution was added to each tube, which was then vortexed. After the addition of 20 μ L of 2 m sodium acetate, 220 μ L of water-saturated phenol and 60 μ L of chloroform-isoamyl alcohol, the tubes were centrifuged. The aqueous layer was transferred into a new tube. Two-hundred microlitres of isopropanol and 1 μ L of glycogen (10 mg/mL) were added, and the tubes were

stored at - 80°C overnight and then centrifuged. The pellets were washed with 70% ethanol, air dried for 10 min and then redissolved in diethyl pyrocarbonate-treated water.

Quantitative real-time reverse transcription-polymerase chain reaction

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) using the TaqMan MGB probe (Applied Biosystems, Foster City, CA USA) was carried out. The mRNA expression levels of *lamc2* (AssayID Mm00500494), *itgb4* (AssayID Mm01266840) and *itga3* (AssayID Mm00442890), were determined by quantitative real-time RT-PCR and were normalized against 18s ribosomal RNA.

Total RNA was reverse transcribed using Qiagen Sensiscript Reverse Transcriptase (Qiagen, Valencia, CA, USA), and quantitative real-time RT-PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), according to the recommendations of the manufacturer. The RT-PCR conditions were as follows: reverse transcription at 50°C for 30 min, then PCR initial denaturation at 95°C for 15 min, 45 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 45 s. The results were analyzed using the $\Delta\Delta C_t$ method. The threshold cycle (C_t) value for each reaction, which reflects the amount of PCR needed to identify a target gene, and the relative levels of laminin $\alpha 2$, and of integrins $\beta 4$ and $\alpha 3 2$, for each sample were calculated according to the instructions of the manufacturer. 18s rRNA was used to normalize the amount of each mRNA, by subtracting its C_t value from that of each gene to obtain a ΔC_t value. The difference ($\Delta\Delta C_t$) between the ΔC_t value of each sample for the gene target and the ΔC_t value of the calibrator was determined. The dissections and RT-PCR reactions were repeated 10 times each per target area ($n = 10$). Values are expressed as means and standard deviations and were analyzed statistically using nonrepeated measures analysis of variance and the Student–Newman–Keuls test.

5-Bromo-2-deoxyuridine staining

5-Bromo-2-deoxyuridine staining was performed to determine DAT cell migration. Thirty, 6-wk-old male ICR mice, each weighing ~ 30 g, were injected intraperitoneally with 100 mg/kg of 5-bromo-2-deoxyuridine (Invitrogen, San Diego, CA, USA) in phosphate-buffered saline. The mice were killed at 2, 6, 12, 24 and 48 h after injection of 5-bromo-2-deoxyuridine by transcardial perfusion fixation with 10% neutral-buffered formalin under deep anesthesia. Maxillary jawbones were resected and were fixed in the same fixative solution for 24 h, decalcified in 10% EDTA-2Na (Wako Pure Chemical, Osaka, Japan) for 1 wk at 25°C, dehydrated and then embedded in paraffin. Paraffin sections were cut serially into sections ~ 5 µm thick. Deparaffinized sections were pre-incubated in 2 N HCl for 30 min at 25°C. The sections were treated in 0.3% H₂O₂ in methyl alcohol for 30 min at room temperature, incubated in 3% bovine serum albumin in phosphate-buffered saline for 30 min at room temperature, and then washed in phosphate-buffered saline for 5 min. The sections were then incubated with monoclonal rat anti-5-bromo-2-deoxyuridine (diluted 1 : 100 with 3% bovine serum albumin) (Abcam) for 2 h at 25°C. After washing with phosphate-buffered saline, the sections were incubated with a secondary antibody, Histofine SimpleStain MAX-PO (Rat) (Nichirei, Tokyo, Japan), for 2 h at 25°C. After washing in phosphate-buffered saline for 5 min, the sections were finally incubated with 0.05% 3,3'-diaminobenzidine for 1 min and were counterstained with Mayer's hematoxylin. Sections were examined and photographed using a conventional microscope (Axiophot 2; Carl Zeiss).

Results

Immunofluorescence

The expression patterns of laminin α 2, integrin β 4 and integrin α 3 were distinct in the gingival epithelia, including the junctional epithelium (Fig. 2).

Intense immunoreactivity for laminin α 2 was observed as straight, linear and green-colored fluorescence and was evident on the surface of DAT cells corresponding to the internal basal lamina. A fairly thick, interrupted immunopositive line was found in the internal basal lamina (Fig. 2A) but no positive reaction was detected in any part of the junctional epithelium. Weak immunolabeling of laminin α 2 was discernible in basal cells of the sulcus epithelium, which corresponds to the basal lamina, but no immunofluorescence was apparent in cells of the sulcus epithelium (Fig. 2B). Positive staining for laminin α 2 was restricted to a continuous and narrow zone parallel to the

epithelial ridge at the boundary between the basal cells of the oral gingival epithelium and the connective tissue corresponding to the basal lamina (Fig. 2C). The fluorescence was distributed diffusely along the boundary.

Strong reactivity for integrin $\beta 4$ was seen in the cytoplasm of DAT cells and in three to four layers of cells located just beneath the cells facing the enamel (Fig. 2D). A linear positive reaction was also detected in part of the external basal lamina of the junctional epithelium. Weak labeling of integrin $\beta 4$ was distinct in the cytoplasm of basal cells and in the basal lamina of the sulcus epithelium (Fig. 2E). Positive reactions were evident at the interface between the oral gingival epithelium and in the connective tissue corresponding to the basal lamina. Faint immunoreactivity for integrin $\beta 4$ was also observed in the cytoplasm of basal cells of the oral gingival epithelium (Fig. 2F).

Interrupted and intense labeling for integrin $\alpha 3$ was clearly discernible on the surface of the DAT cells (Fig. 2G). A faintly positive reaction was also detected in the cytoplasm of the DAT cells and in three to four layers of cells located just beneath the DAT cells. Slightly positive reactions for integrin $\alpha 3$ were detected on the cell membranes of basal and suprabasal cells of the sulcus epithelium (Fig. 2H). Clear labeling was also found at the boundary between the basal cells of the oral gingival epithelium and the connective tissue. Weak reactivity for integrin $\alpha 3$ was also evident in the cytoplasm of the basal cells (Fig. 2I).

Quantitative real-time RT-PCR

The expression of laminin $\gamma 2$ (*lamc2*) and of integrins $\beta 4$ and $\alpha 3$ (*itgb4* and *itga3*) at the RNA level in the DAT cells of the junctional epithelium was higher than in the sulcus epithelium or in oral gingival epithelium (Fig. 3).

The highest level of gene expression of laminin $\gamma 2$ (*lamc2*) was found in DAT cells ($p < 0.01$) (Fig. 3B). This value was ~ 12 -fold higher than that present in the oral gingival epithelium. In basal cells of the sulcus epithelium, the level of *lamc2* was ~ 2.3 -fold higher than that present in the oral gingival epithelium.

The level of gene expression of integrin $\beta 4$ (*itgb4*) in the DAT cells was ~ 1.3 -fold higher than in basal cells of the oral gingival epithelium (Fig. 3C), although the difference was not statistically significant ($p < 0.05$). On the other hand, the expression level of integrin $\beta 4$ in the sulcus epithelium was lower

than in the other two types of epithelia.

The expression level of integrin $\alpha 3$ (*itga3*) in DAT cells of the junctional epithelium was ~ four-fold higher than in the oral gingival epithelium (Fig. 3D). However, the expression level of integrin $\alpha 3$ in the sulcus epithelium was lower than in the oral gingival epithelium (Fig. 3D).

5-Bromo-2-deoxyuridine staining

Numerous 5-bromo-2-deoxyuridine-positive cells were observed in basal cells of the junctional epithelium and in DAT cells near the cemento–enamel junction after 2 h (Fig. 4A). 5-Bromo-2-deoxyuridine-positive cells were detected over time at the coronal portion of the junctional epithelium (Fig. 4B,C). 5-Bromo-2-deoxyuridine-positive cells were found in the middle of the DAT cells and in the basal cells of the external basal lamina after 24 h (Fig. 4D). However, the 5-bromo-2-deoxyuridine stain was no longer distinct in DAT cells after 48 h (Fig. 4E). In the basal cells of the sulcus epithelium, cells labeled with 5-bromo-2-deoxyuridine were localized in the basal layer after 2 h (Fig. 4A) and they were detected within three layers of the basal side after 48 h (Fig. 4E). On the other hand, 5-bromo-2-deoxyuridine-positive cells were found in the basal layer of the oral gingival epithelium after 2 h (Fig. 4F). These cells were found in two or three layers of the basal lamina after 24 h (Fig. 4I) and were also observed in the spinous layer of the oral gingival epithelium after 48 h (Fig. 4J).

Discussion

It is thought that hemidesmosomes are composed of laminin-5 and integrin $\alpha 6\beta 4$ (20), and thus both laminin-5 and integrin $\alpha 3\beta 1$ may participate in cell migration (12,13). In this study, we investigated laminin $\alpha 2$ and integrins $\beta 4$ and $\alpha 3$, because laminin-5 only possesses a $\alpha 2$ subunit, integrin $\beta 4$ (which may be involved in cell–extracellular matrix contact) and integrin $\alpha 3$ (which induces cell migration) (7,12,21). The expression of laminin-5 and integrins has already been demonstrated in human oral tissues by immunofluorescence staining (4,21), in mouse oral tissues by immunofluorescence staining and by in situ hybridization (3) and in rat oral tissues by immunofluorescence (3). However, there have been no comparative studies on the levels of expression of these adhesive proteins in DAT cells of the junctional epithelium, or in basal cells of the sulcus epithelium or oral gingival

epithelium, which are restricted areas containing small amounts of protein. Therefore, we analyzed the RNA expression of laminin $\alpha 2$, and integrins $\beta 4$ and $\alpha 3$ in murine gingival epithelium, including the junctional epithelium and the sulcus epithelium, by immunofluorescence and by quantitative real-time RT-PCR from samples collected using laser microdissection.

Expression of laminin-5

Our results for immunofluorescence showed a strongly positive reaction for laminin $\alpha 2$ in the internal basal lamina. RT-PCR revealed that the expression of *lame2* in DAT cells was ~ 12 -fold higher than in the oral gingival epithelium. This implies that this large amount of laminin-5 may contribute to the strong adhesion between the tooth surface and DAT cells, although there is no laminin other than laminin-5 in the internal basal lamina. In fact, laminin possesses the ability to self-assemble *in vitro* by forming a felt-like sheet through interactions between the ends of its arms (22).

The expression of laminin-5 was found to be much higher (~ 12 -fold) in the internal basal lamina than in the oral gingival epithelium. This suggests that laminin-5 constitutes a unique adherence system between hard tissues and the epithelium in the basal lamina of the tooth surface, as well as an adhesive structure at the first stage of development, as suggested by Bruce et al. (23).

Expression of integrin $\beta 4$

We characterized integrin $\beta 4$ to determine the expression of integrin $\alpha 6\beta 4$, because integrin $\beta 4$ can only form a heterodimer with integrin $\alpha 6$ (24). Our results showed that the expression of integrin $\beta 4$ mRNA in DAT cells is similar to that in basal cells of the oral gingival epithelium (1.3-fold higher in DAT cells). The immunolocalization of integrin $\beta 4$ was distinct in the cytoplasm of the DAT cells and was detected diffusely in three to four layers of junctional epithelium cells located just beneath the cells facing the enamel. Therefore, integrin $\beta 4$ may not be specifically localized in DAT cells. Tanno et al. demonstrated that both laminin $\alpha 2$ and integrin $\beta 4$ are produced by cultured gingival epithelium, and that irregular rings of laminin are formed in areas where the cells adhere to the culture dish (9).

A study of integrin $\beta 4$ in basal cells indicates that integrin $\beta 4$, which is a receptor of laminin and consists of hemidesmosomes associated with laminin-5,

plays a role in cell adhesion (24). The results of this previous study were supported by the results of the present study demonstrating the expression of integrin $\beta 4$ in junctional epithelium cells. Even in DAT cells on the enamel surface, integrin $\beta 4$ participates in cell–tooth adherence. Our finding, that the level of expression of integrin $\beta 4$ is similar to that in connective tissue, implies that integrin $\beta 4$ has no particular role in DAT cells, except for adhesion as in other tissues.

Expression of integrin $\alpha 3$

In our study, the expression of integrin $\alpha 3$ in DAT cells was five-fold higher than in the oral gingival epithelium. Moreover, strong immunolabeling was detected throughout the entire junctional epithelium and in the internal basal lamina facing the enamel. It has been reported that integrin $\alpha 3\beta 1$ is involved in cell migration together with laminin-5 (12). To confirm the relationship between the high expression of integrin $\alpha 3$ and cell migration, we investigated migration changes in 5-bromo-2-deoxyuridine-labeled DAT cells over time. Our results showed that the 5-bromo-2-deoxyuridine-labeled cells moved coronally on the enamel surface and disappeared after 48 h, whereas the labeled basal cells remained in the spinous layer of the oral gingival epithelium even after 48 h. This indicates that DAT cells can migrate on the enamel surface and that the turnover time of DAT cells is faster than that of the oral gingival epithelium. Taking the abundant expression of laminin $\gamma 2$ and integrin $\alpha 3$ in the junctional epithelium into account, these adhesive proteins may be involved in the cell migration and faster turnover of the junctional epithelium (12).

It has been proposed that the 190-kDa $\alpha 3$ chain of laminin-5 is not processed immediately after secretion and is involved in migration in association with integrin $\alpha 3\beta 1$. The contact of integrin $\alpha 3\beta 1$ with laminin-5 promotes the expression of plasmin. Laminin-5 is subsequently cleaved by the plasmin, and the α chain changes into a 160-kDa fragment to build hemidesmosomes associated with integrin $\alpha 6\beta 4$ (25). Goldfinger et al. suggested that the up-regulation of laminin-5, and the down-regulation of plasmin, induces cell migration at the vanguard of wound healing, whereas hemidesmosomes are organized behind that edge by laminin-5-processed proteolysis (26).

Our results thus suggest that a large amount of unprocessed laminin-5

which can contact with integrin $\alpha 3\beta 1$ expressed in the junctional epithelium is a prerequisite for cell migration and those adhesions cause cell migration by a focal contact in the tip of the DAT cell. We surmise further that enhancement of plasmin secretion promotes the proteolysis of laminin-5 to assist in the formation of hemidesmosomes in association with integrin $\alpha 6\beta 4$ (26).

To understand the details of the regulatory mechanism in adhesion and migration of junctional epithelium is important for the reliable acquisition of epithelial adhesion following dental implants and periodontal surgery. It is probably easier to obtain the connective tissue attachment when we can regulate and suppress the apical migration of gingival epithelium.

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FIGS

Fig. 1. Cells collected from mouse gingival tissue by laser microdissection. Directly attached to tooth (DAT) cells of the junctional epithelium (A– C), basal cells of the sulcus epithelium (D– F), and basal cells of the oral gingival epithelium (G– I). Three areas were dissected and collected.

Fig. 2. (A–C) Immunofluorescence staining of laminin $\gamma 2$. In the internal basal lamina, strong expression was observed as linear and green-colored fluorescence along the surface of the enamel (A). In the sulcus epithelium, weak immunolabeling was discernible along the basal lamina (B). In the oral gingival epithelium, positive reactions were observed at the boundary between the basal cells and the connective tissue (C). (D–F) Immunofluorescence of integrin $\beta 4$. In the junctional epithelium, immunoreactivity was seen in the cytoplasm of directly attached to tooth (DAT) cells and in three to four layers of cells located facing the enamel. Linear positive reactions were also detected in the internal basal lamina (D). In the sulcus epithelium, weak expression was observed along the basal lamina and in the cytoplasm of basal cells (E). In the oral gingival epithelium, faint immunoreactivity was found along the basal

lamina and cytoplasm of basal cells (F). (G–I)_ Immunofluorescence of integrin $\alpha 3$. In the junctional epithelium, immunoreactivity was detected in the cytoplasm of DAT cells and in three to four layers of cells located just beneath the DAT cells. In the internal basal lamina, strong staining in a line along the surface of the enamel was seen (G). In the sulcus epithelium, a positive reaction was slightly apparent on cell membranes of basal and suprabasal cells (H). In the oral gingival epithelium, distinct labeling was found at the boundary between the basal cells of the oral gingival epithelium and the connective tissue. A weak reaction was also evident in the cytoplasm of basal cells (I). Arrowheads indicate positive reactions. CT, connective tissue; ES, enamel space; JE, junctional epithelium; OE, oral gingival epithelium; SE, sulcular epithelium. Bar = 20 μm .

Fig. 3. (A) Areas of collection. Comparison of mRNA expression levels of laminin $\gamma 2$ (B), integrin $\beta 4$ (C) and integrin $\alpha 3$ (D) by real time reverse transcription-polymerase chain reaction in three parts of the gingival epithelium. Laminin $\gamma 2$ and integrin $\alpha 3$ expression was higher in the junctional epithelium than in the sulcus epithelium or oral gingival epithelium ($p < 0.01$). JE, junctional epithelium; OE, oral gingival epithelium; SE, sulcular epithelium.

Fig. 4. Several 5-bromo-2-deoxyuridine-positive cells were localized at the basal layer after 2 h (A,F). In directly attached to tooth (DAT) cells, many 5-bromo-2-deoxyuridine-positive cells were observed at the coronal side after 48 h and 5-bromo-2-deoxyuridine-labeled cells were hardly discernable after 48 h (A, 2 h; B, 6 h; C, 12 h; D, 24 h; E, 48 h). 5Bromo-2-deoxyuridine-positive cells were observed in the basal layer of the oral gingival epithelium after 2 h (F). These cells were found in two or three layers beneath the basal lamina after 24 h (I). They were also observed in the spinous layer of the oral gingival epithelium after 48 h (J). (F, 2 h; G, 6 h; H, 12 h; I, 24 h; J, 48 h.) Bar = 20 μm .

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Fig.1

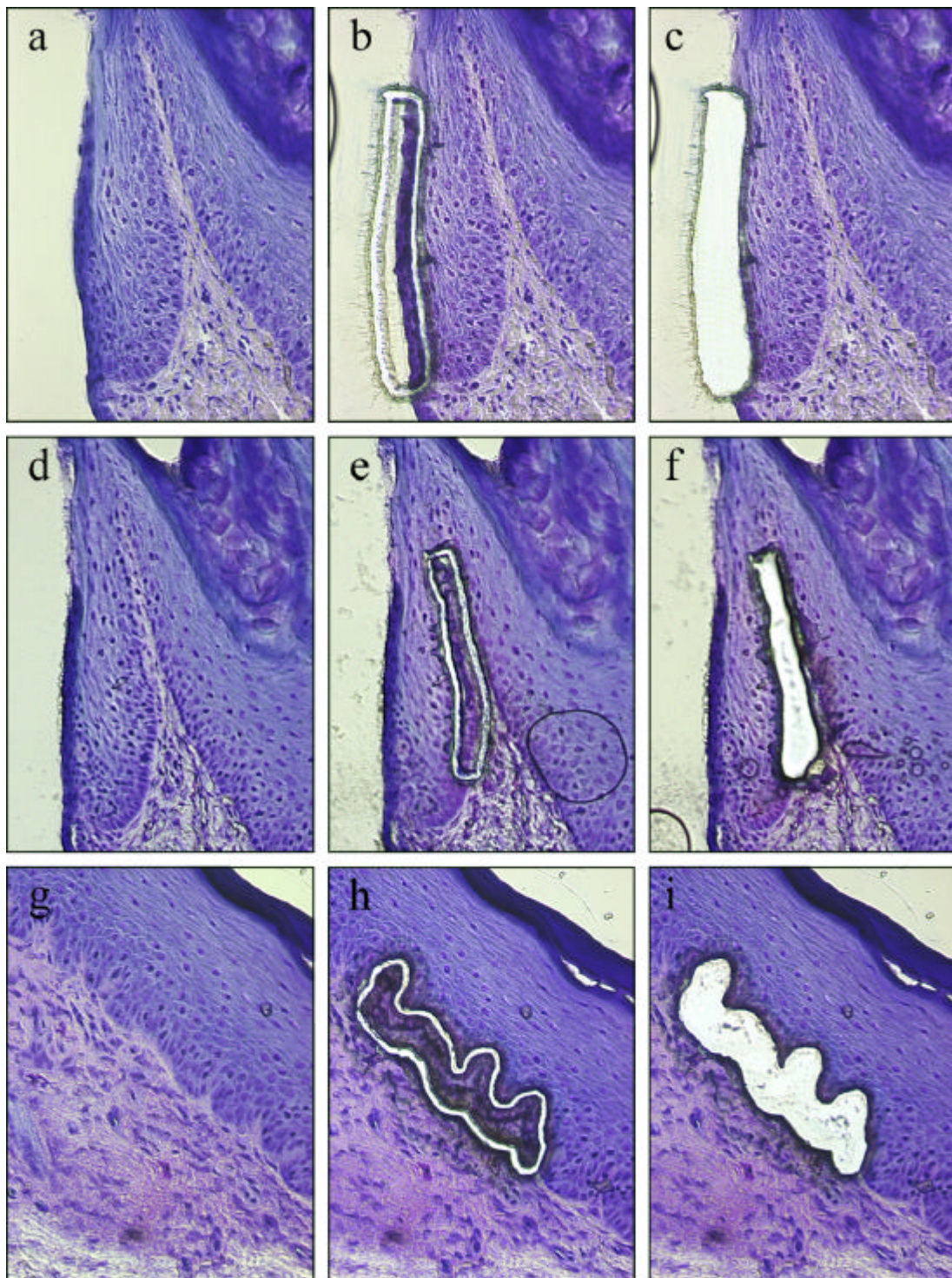


Fig.2

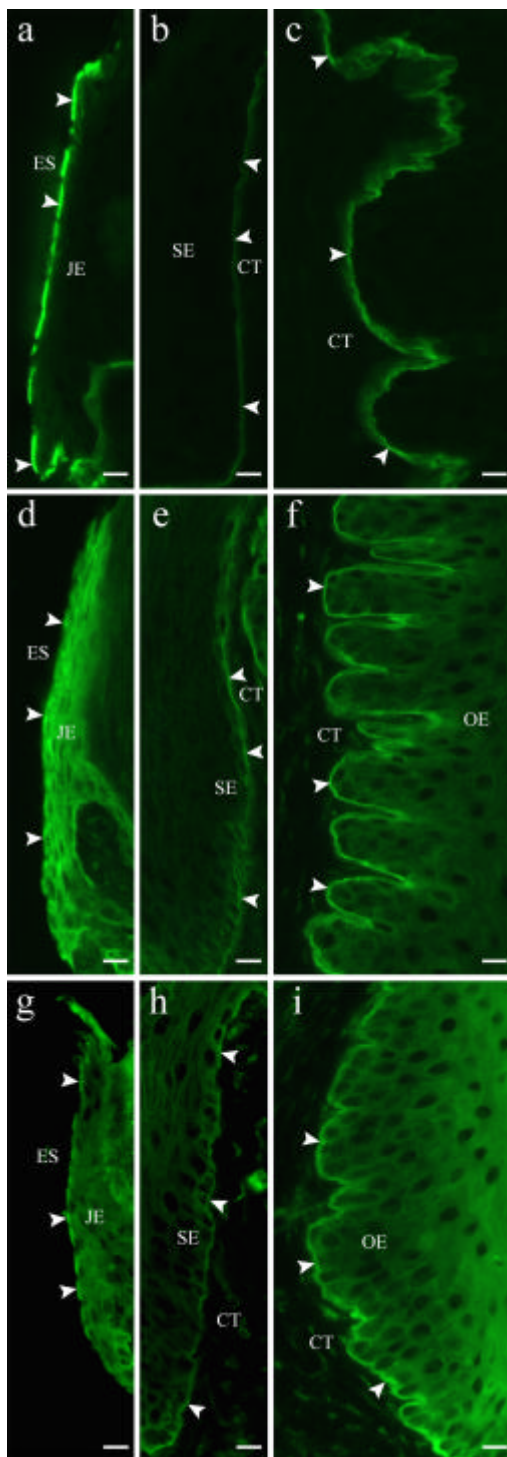


Fig.3

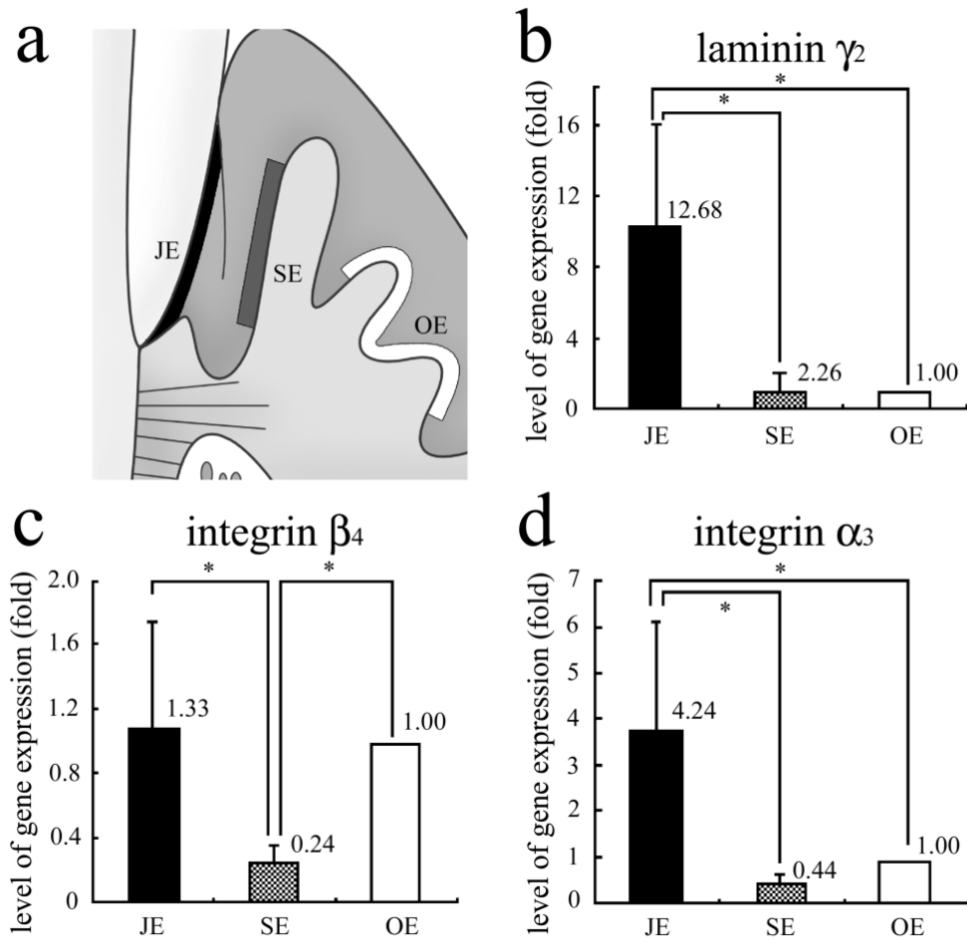


Fig.4

