J Periodont Res 2009; 44: 496–502

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01146.x

Effect of the dental adhesive, 4-META/MMA-TBB resin, on adhesion and keratinization of regenerating oral epithelium

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Tsuchiya Y, Muramatsu T, Masaoka T, Hashimoto S, Shimono M. Effect of the dental adhesive, 4-META/MMA-TBB resin, on adhesion and keratinization of regenerating oral epithelium. J Periodont Res 2009; 44: 496–502. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard

Background and Objective: The 4-META/MMA-TBB [4-(2-methacryloxyethyl)trimellitic anhydride/methyl methacrylate-tributylborane] resin is widely used as a dental adhesive. It has also been applied in the dressing of gingival wound surfaces following periodontal surgery. However, its effect on the regeneration and/or cell attachment of the oral epithelium remains to be clarified. To evaluate the effect of the resin applied as a wound dressing, we investigated expression of laminin 5, integrin β_4 and cytokeratin 14 in regenerating oral epithelium treated with this resin following gingivectomy from the viewpoint of cell attachment and differentiation.

Material and Methods: The resin was applied to the entire wound surface in rats after gingival surgery, and regenerating epithelium was examined immediately and at 1, 3, 5, 7 and 14 days later. The resin was removed 2 weeks after application in some animals and tissue further examined at 1, 3, 5 and 7 days later.

Results: Regenerating epithelium under the resin was not keratinized, but became keratinized immediately after removal of the resin. Laminin 5 and integrin β_4 were immunolocalized in the basal lamina, the internal basal lamina, in marginal cells of the regenerating epithelium and at the resin–regenerating epithelium interface. Cytokeratin 14 localized in the regenerating epithelium underneath the resin, as well as in healthy and regenerated junctional epithelial cells.

Conclusion: These results suggest that this resin covers the wound surface and that the regenerating epithelium biologically adheres to the resin during the initial process of its regeneration.

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Key words: 4-META/MMA-TBB resin; laminin; integrin; gingivectomy

Accepted for publication June 5, 2008

The 4-META/MMA-TBB [4-(2-methacryloxyethyl)trimellitic anhydride/methyl methacrylate-tributylborane] resin is widely employed as a dental adhesive. Numerous studies have reported its highly adhesive properties with enamel (1), dentin (1), cementum (2,3) and bone (4,5), and moderate biocompatibility with dentin–pulp complex (6,7) and periodontal tissue (8). Based on the results of those studies, it has also been applied in the dressing of gingival wound surfaces following flap surgery and intentional autotransplantation of teeth (9). However, little information on its biocompatibility with epithelium is available. How it influences gingival tissue when the resin monomers deeply infiltrate surrounding tissue, and its biocompatibility with the oral mucosa

during wound healing, in particular, remain to be fully clarified.

Junctional epithelium can completely regenerate following gingivectomy. Experiments using rats have indicated that the oral epithelium proliferates at 2 days post-gingivectomy; the regenerating epithelium then stratifies and keratinizes, with subsequent proliferation of connective tissue. Finally, regeneration of the junctional epithelium leads to completion of gingival regeneration by adhesion to the cemento-enamel junction (CEJ) and enamel surface (10,11).

Junctional epithelium has a unique structure and function, linking the tooth surface and connective tissue, thus sealing and protecting the tooth–gingiva interface. Previous studies have demonstrated that junctional epithelium cells adhere to the tooth by hemidesmosomes and the internal basal lamina (IBL; 12–14), and by laminins, type IV collagen and proteoglycans in the extracellular matrix (ECM) of the basal lamina. Laminins have been identified in the basal lamina of junctional epithelium by immunohistochemistry and *in situ* hybridization. However, only laminin 5 is found in the IBL, which lacks other elements found in the ECM, including type IV collagen (15). Laminin 5, in particular, contributes to cell adhesion associated with integrin $\alpha_6\beta_4$ at hemidesmosomes (16,17). A recent study on the expression of laminin 5 and integrin $\alpha_6\beta_4$ in junctional epithelium has demonstrated the localization of these proteins and mRNAs, which are produced by the tooth-facing cells where they make contact with the enamel surface (18).

In contrast, cytokeratins (CK) are markers for the development and differentiation of epithelial tissue. CK 14, in particular, is understood to be a specific marker for junctional epithelium and the basal cells of the oral epithelium (19).

It is open question how 4-META/MMA-TBB resin influences regenerating epithelium, which types of adhesive protein are concerned in the epithelium, and how the resin affects the differentiation of the regenerating epithelium following its application combined with gingivectomy.

The purpose of this study was to investigate the expression of adhesive proteins (laminin 5 and integrin β_4) and CK 14 following experimental gingivectomy and direct application of 4-META/MMA-TBB resin to determine the effect of this resin on regeneration of oral epithelium and cell attachment to tooth.

Material and methods

Experimental design

Sixty-nine male Sprague–Dawley rats (6 weeks of age) were used in this study. The animals were divided into four groups: 18 rats each in C (control), G (gingivectomy), GR (gingivectomy plus resin application), and 12 rats in GRR (gingivectomy plus resin application and removal), as described below; another three rats were used to investigate healthy untreated animals. All animals were deeply anesthetized by intraperitoneal injection of sodium thiopental (Ravonal; Tanabe Seiyaku, Osaka, Japan). The maxillary first and second molars on both sides of the jaw were then etched with a phosphate agent (Red Activator; Sun Medical, Moriyama, Japan) and rinsed with distilled water, after which the following treatments were performed: in group C, 4-META/MMA-TBB resin (Super-Bond C&B; Sun Medical) was applied to the teeth through the neighboring palate; in group G, the palatal gingiva, including the coronal portion of the periodontal ligament, in the first and second molar regions was removed in a 1 mm width using a fine scalpel, and bleeding was staunched to maintain hemostasis; in group GR, following gingivectomy as described for group G, resin was applied to the entire wound surface and the teeth; and in group GRR, the animals were treated in the same way as group GR , after which the resin was removed 2 weeks later using an explorer and fine scissors, taking care that no bleeding occurred. The rats were fed powdered food (Oriental Yeast, Tokyo, Japan) during the experimental period, and were killed with overdose of the same anesthesia immediately, or at 1, 3, 5, 7 or 14 days after treatment in groups C, G and GR, and at 1, 3, 5 or 7 days after removal of the resin in group GRR . All experiments complied with the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College.

Histological and immunohistochemical analysis

Maxillae were resected *en bloc* from each animal and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Specimens were infiltrated with acetone to solubilize the resin, and decalcified with 10% ethylenediaminetetraacetic acid (pH 7.2) for 2 weeks. After dehydration in a graded series of alcohols and paraffin embedding, the specimens were serially sectioned at 3 μm in the bucco-lingual direction and stained with hematoxylin and eosin (HE), or were used for immunohistochemistry.

For immunohistochemistry, endogenous peroxidase was initially blocked with 0.3% hydrogen peroxide in methanol, after which the sections were either pretreated with 0.01% trypsin (Invitrogen, Carlsbad, CA, USA) in 50 mM Tris-HCl (pH 7.6) for 10 min at 37°C for laminin 5, or incubated in a microwave oven in 10 mM citrate (pH 6.0) for 15 min at 65°C for antigen retrieval of CK 14. The sections were then treated with 3% bovine serum albumin to prevent non-specific binding, followed by incubation with a rabbit polyclonal antibody against laminin 5 (Abcam, Cambridge, UK; diluted 1:100) or a monoclonal antibody against CK 14 (Progen, Heidelberg, Germany; diluted 1:10). After immunoreaction with the primary antibody overnight at 4°C, the sections were then incubated with horseradish-peroxidase-conjugated IgG [Histofine MAX-PO (MULTI); Nichirei, Tokyo, Japan] for 30 min. Finally, they were visualized using 0.01% 3,3′-diaminobenzidine tetrahydrochloride and counterstaining with Mayer's hematoxylin. Phosphate-buffered saline in place of the primary antibody was used for the negative controls.

For integrin–laminin double immunofluorescence labeling, the sections

were pretreated with trypsin and bovine serum albumin as described above, and then immunoreacted overnight with a mixture of anti-laminin 5 (diluted 1:100) and a mouse monoclonal antibody to integrin β_4 (Abcam; diluted 1:100) at 4°C. The sections were then incubated with Alexa Fluor® 488-conjugated anti-rabbit IgG (Invitrogen, Eugene, OR, USA; diluted 1:100) and Alexa Fluor® 568-conjugated anti-mouse IgG (Invitrogen; diluted 1:100) for 30 min at room temperature. Following counterstaining with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; Invitrogen), all of the specimens were examined and photographed using a conventional light/fluorescence microscope (Axio-Phot 2, Carl Zeiss, Oberkochen, Germany).

Results

Group C

The keratinized layer peeled off at the surface and in the middle region of the epithelium treated with resin. Eosinophilic and amorphous materials were detected at 5 days postoperatively, but not at 7 days. No inflammatory cell infiltration was apparent in the epithelium, except in the superficial layer and connective tissue. No distinct difference was observed between the resin-treated group and healthy untreated tissue (data not shown).

Laminin 5 was expressed in the external basal lamina (EBL) and IBL of the junctional epithelium and at the epithelium–connective tissue interface of the oral epithelium in healthy tissue. Laminin 5 expression in group C was similar to that in healthy tissue (data not shown).

Intense immunoreaction for CK 14 was detected in the basal cell layer and between the enamel and junctional epithelium in the palatal gingival epithelium of healthy tissue. Cytokeratin 14 was also expressed weakly in the outer cells of the junctional epithelium and intermediate cells of the oral gingival epithelium. In group C specimens, positive reactivity for CK 14 analogous to that in healthy tissue was observed (data not shown).

Group G

Observations on HE-stained sections — A substantial amount of gingival epithelium and submucosal tissue was removed during the gingivectomy, leaving clearly cut palatal gingival surfaces, with cells beneath the cut margin showing distinct degeneration (Fig. 1A). An accumulation of fibrin covered the cut surface, where abundant hemocytes and bacteria were detected at 1 day post-gingivectomy (Fig. 1G). Newly formed epithelial cells underneath the fibrin and inflammatory cells were observed in the cut margin at 3 days post-gingivectomy (Fig. 2A, arrow). Irregularly shaped basal cells were detected attached to the CEJ (Fig. 2G, open arrow), although their outlines were quite similar to those of junctional epithelium at 5 days post-gingivectomy. At 7 days, the regenerating epithelium reached the enamel to form new junctional epithelium (Fig. 3A). Keratinization was recognized in the regenerating epithelium at sites corresponding to the oral and sulcular epithelia, and abundant inflammatory cells infiltrated the connective tissues underneath the epithelium during the same period. At 14 days post-gingivectomy, no distinct difference was detected between the regenerated epithelium and the gingival epithelium in untreated animals (Fig. 3G).

Laminin 5 immunolocalization — Positive reactivity for laminin 5 was distinct in the epithelium–connective tissue interface of the residual epithelium immediately after the gingivectomy (Fig. 1B, arrowheads). Immunoreactivity for laminin 5 was detected at the frontal margin of the regenerating epithelium at 1 and 3 days post-surgery, and between the epithelium and the tooth surface at day 5 (Figs 1H and 2B,H). Intense expression of laminin 5 was apparent in the basal laminae, the EBL and the IBL at 7 and 14 days (Fig. 3B,H).

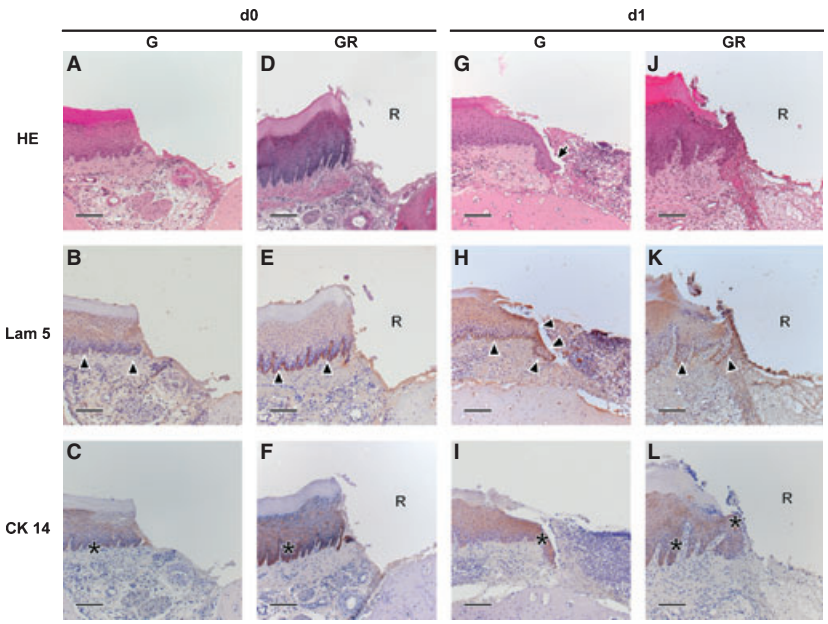

Fig. 1. Healing process and protein expression in G and GR groups: micrographs of samples immediately (A–F) and 1 day after surgery (G–L). The HE-stained specimens (top row) reveal that gingival epithelium and cut margin are distinct immediately and 1 day post-surgery in both treatments. Newly formed epithelial cells (arrow in G), exudates, inflammatory cells and fibrin can be observed 1 day post-gingivectomy. Laminin 5 immunolocalization (middle row) is distinct at the basal lamina of the remaining epithelium and at the frontal margin of the regenerating epithelium (arrowheads in B,E,H,K). Cytokeratin 14 (bottom row) is expressed in basal cells, parabasal cells and at the frontal margin of the regenerating epithelium (asterisks in C,F,I,L). R, resin space. Scale bars represent 100 μm.

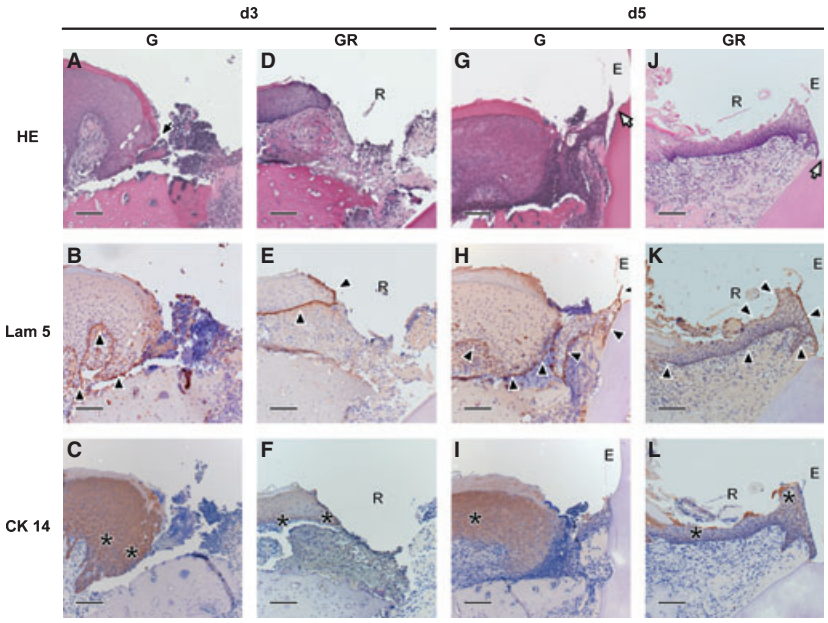

Fig. 2. Healing process and protein expression in groups G and GR: micrographs at 3 (A–F) and 5 days after surgery (G–L). The cut margin can be seen to be covered with new epithelium and inflammatory exudates 3 days post-surgery (A,D); thereafter basal cells of regenerating epithelium reach CEJ by 5 days post-surgery in both groups (open arrow in G,J). Laminin expression can be observed at the tooth–epithelium interface, as well as in basal lamina (arrowheads in H). In addition to these sites, it is also expressed at the resin–epithelium interface in group GR (E,K). Cytokeratin 14 is expressed at the frontal margin of the regenerating epithelium in group G, and further expressed in regenerating epithelium close to the resin in group GR (asterisks in C,F,I,L). E, enamel space; R, resin space. Scale bars represent 100 μm.

Cytokeratin 14 immunolocalization — At 1 day post-gingivectomy, immunoreactivity for CK 14 was detected intensely in the basal cells and weakly in the suprabasal cells of the regenerating epithelium. However, strong expression of CK 14 was also distinct at the frontal margin of the regenerating epithelium when the epithelium had not yet attached to the tooth at 3 days post-gingivectomy. At 7 days post-gingivectomy, CK 14 was immunoreactive in the entire cell layer of the oral epithelium, the oral sulcular epithelium and the junctional epithelium. The same expression pattern of CK 14 as that in healthy gingiva was observed at 14 days post-gingivectomy (asterisks in Figs 1C,I, 2C,I and 3C,I).

Group GR

Observations on HE-stained sections — At 1 days post-operatively, small leukocyte, fibrin and exudate accumulations were observed around the cut surface in the GR group (Fig. 1J). Incomplete regeneration of the epithelium and inflammatory reactions such as exudation became more marked in the cut margin at 3 days postoperatively (Fig. 2D). The regenerating epithelium consisted of only basal and suprabasal cells, and attached to the CEJ at 5 days (Fig. 2J, open arrow). Macrophage infiltration was still observed at 7 days postoperatively. The regenerating epithelium was still incomplete in outline, revealing a very thin intermediate layer and no keratinization (Fig. 3D,J).

Laminin 5 immunolocalization — Laminin 5 expression was discernible in the same manner as that in group G at 1 day postoperatively (Fig. 1E,K). However, a positive reaction for laminin 5 was also distinct not only in the basal lamina, but also in the resin-regenerating epithelium interface at 3 days post-gingivectomy (arrowheads in Figs 2E,K and 3E,K).

Cytokeratin 14 immunolocalization — Up to 3 days postoperatively, the same reactivity for CK 14 was seen as in group G. At 5 days postoperatively, positive reactions for CK 14 were observed in both the basal cells and the regenerating cells close to the resin (asterisks in Figs 2L and 3F,L).

Group GRR

Observations on HE-stained sections — Thin and keratinized regenerating epithelium was detected in the area of tissue where the resin had been applied 1 days after its removal. The basal cells had attached to the CEJ, but inflamed connective tissue was exposed where the thin regenerating epithelium had partly peeled off in some specimens (Fig. 4A, arrow). A keratinized layer was recognizable in all regenerating epithelia at 3 days after removal of the resin, and the regenerating epithelium showed morphology similar to that in healthy untreated tissue at 5 and 7 days after removal of the resin (Fig. 4D,G,J).

Laminin 5 immunolocalization — Immunoreactivity for laminin 5 was detected in the basal lamina, but not in the outermost layer of the regenerating epithelium throughout the experimental period. At 5 days after removal of the resin, a positive reaction for laminin 5 was also apparent in the IBL of the regenerating junctional epithelium (arrowheads in Fig. 4B,E,H,K).

Cytokeratin 14 immunolocalization — At 3 days after removal of the resin, intense expression of CK 14 was discernible in the basal cells of the regenerating epithelium and at the enamel surface of the regenerating junctional epithelium (asterisks in Fig. 4C,F,I,L).

Integrin–laminin double immunofluorescence labeling

Integrin–laminin double immunofluorescence was performed after detection of expression of laminin 5 at the resin interface. Under double immunofluorescence microscopy, a positive reaction for laminin 5 was detected as red

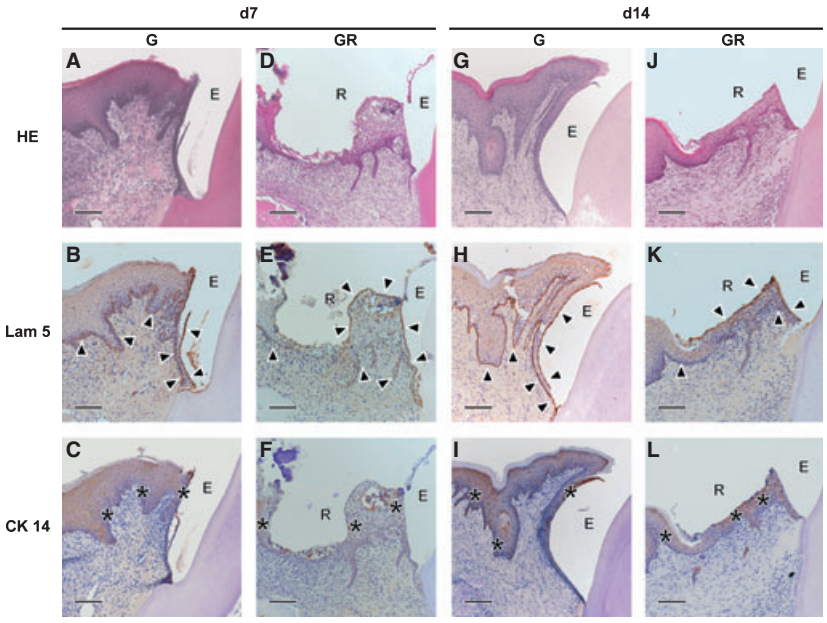

Fig. 3. Healing process and protein expression in groups G and GR: micrographs at 5 (A–F) and 14 days post-surgery (G–L). In group G, keratinization can be observed in regenerated epithelium corresponding to oral and sulcular epithelium 7 days post-gingivectomy, and the epithelium is fully regenerated morphologically by 14 days (A,D). In contrast, the morphology is incomplete in outline, revealing a very thin intermediate layer and no keratinization, even 14 days postoperatively in group GR (J). While laminin 5 is immunolocalized at the basal lamina, regenerated EBL and IBL (B,H), it is also expressed at the resin-regenerating epithelium interface in group GR (arrowheads in E,K). Cytokeratin 14 (bottom row) is immunopositive in regenerated junctional epithelium and in parabasal cells of the oral epithelium (asterisks in C,I,) in group G. It is also expressed in regenerating cells close to resin (asterisks in L). E, enamel space; R, resin space. Scale bars represent 100 μm.

fluorescence at the frontal margin of the regenerating epithelium in group G and in the cells facing the resin in group GR. Integrin β_4 was expressed as green fluorescence not only at the interface between regenerating epithelium and resin, but also in the cytoplasm of regenerating epithelial cells and inflammatory cells at all experimental time points (arrowheads in Fig. 5A–D).

After removal of the resin, expression of laminin 5 and integrin β_4 at the basal lamina showed no change. However, their expression at the interface between the regenerating epithelium and resin, where keratinization was taking place, disappeared (Fig. 5E–H).

Discussion

The results of the present study demonstrated that regenerating epithelial cells reached the CEJ and covered the connective tissue at 5 days postoperatively in both group G (Fig. 2G) and group GR (Fig. 2J). Furthermore, keratinization of the regenerating epithelium was observed within 2 days after the regenerating basal cells had attached to the CEJ in group G (Fig. 3A), although no keratinization was detected in group GR (Fig. 3D). In contrast, keratinization took place immediately after removal of the resin in group GRR (Fig. 4A). These results indicate that, while resin application inhibits keratinization, it does not affect healing or the rate of regeneration in gingivectomized epithelium.

The junctional epithelium is attached to the tooth via the basal lamina and hemidesmosomes that seal and protect the dento-gingival junctions from the oral cavity (12–14,20), thus reinforcing the attachment itself. Among the constituent elements of the basal lamina and hemidesmosomes, laminin 5, a matrix protein, specifically induces the promotion and maintenance of epithelial adhesion at the tooth–epithelium interface (15,21). A study using RT-PCR has also demonstrated intensive expression of *lamc2*, which codes for the laminin 5-specific γ_2-laminin subunit, in cells directly attached to the tooth, rather than in the oral epithelium (18).

In this study, we demonstrated expression of laminin 5 in the frontal margin of the regenerating epithelium at 1 and 3 days post-gingivectomy, when the regenerating epithelium had not yet attached to the tooth (Figs 1H and 2B). This may explain why the leading cells of the regenerating epithelium were activated to migrate onto the wound bed, and why laminin 5 was expressed in the provisional basal lamina at the early stage of wound healing, as described in a previous study (22).

Laminin 5 was also expressed at the interface between the regenerating epithelium and the resin in group GR (Figs 2E,K and 3E,K), but disappeared after removal of the resin in group GRR (Fig. 4B,E,H,K). As mentioned above, among laminins, only laminin 5 is expressed in the IBL of junctional epithelium. This phenomenon suggests that regenerating epithelium under resin takes on the biological character of junctional epithelium, and once resin is removed, the regenerating epithelium changes its character from that of junctional epithelium to other oral epithelium. Furthermore, this suggests that regenerating epithelium does not recognize the resin as foreign body, and that the resin participates not only in covering the entire gingivectomized area, but also constitutes the microenvironment in the dento-epithelial interface.

Integrins, a component of hemidesmosomes, are heterodimeric transmembrane glycoproteins that are formed by the non-covalent association of α and β subunits. Among integrins, the $\alpha_6\beta_4$ heterodimer is believed to function as a receptor for laminin 5, and integrin β_4 is known to dimerize only with the α_6 chain (23). In this study, laminin–integrin double immunofluorescence staining revealed

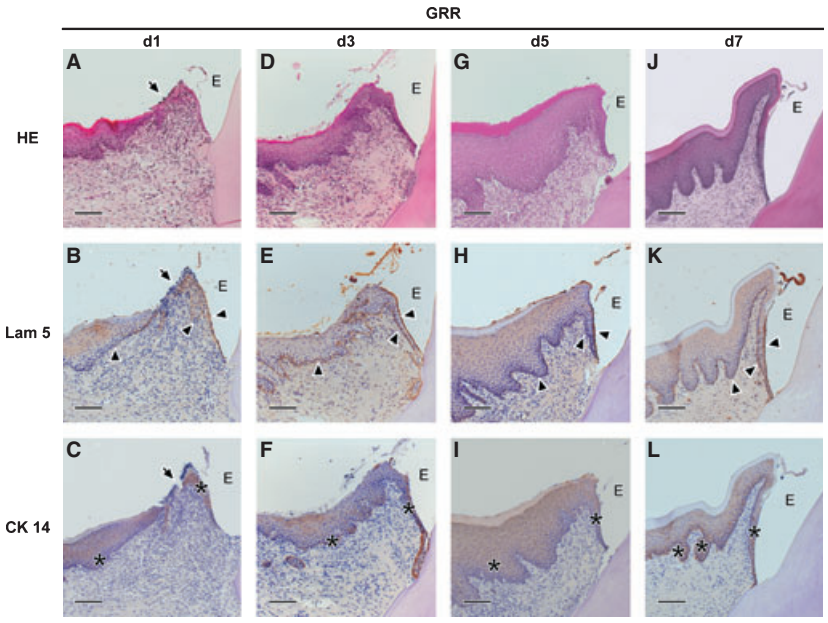

Fig. 4. Regeneration process in group GRR. Thin and keratinized regenerating epithelium is detected at 1 (A–C), 3 (D–F), 5 (G–I) and 7 days postoperatively (J–L) . Basal cells are attached to the CEJ 1 day post-resin removal. Inflamed connective tissues are partly exposed where thin regenerated epithelium has peeled off (arrow in A). A keratinized layer is recognizable in all regenerating epithelia (top row). Laminin 5 expression (arrowheads) disappears in the outermost area of the regenerated epithelium, as is the case in healthy epithelium, whereas it is localized in EBL, IBL and basal lamina (middle row). Cytokeratin 14 is immunolocalized in the basal cells, parabasal cells and junctional epithelial cells (bottom row, asterisks). E, enamel space. Scale bars represent 100 μm.

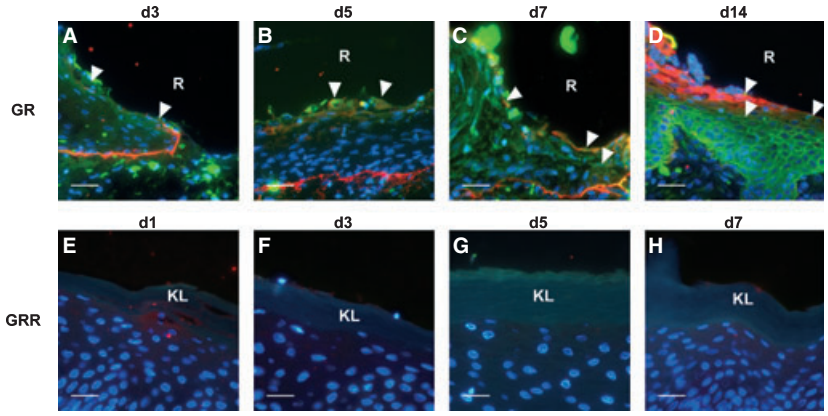

Fig. 5. Laminin 5–integrin β_4 double immunofluorescence labeling in GR (top row) and GRR groups (bottom row). Laminin 5 is strongly expressed as red fluorescence at the interface between regenerative epithelium and resin, as well as in the basal lamina in group GR. Integrin β_4 is expressed as green fluorescence not only at the interface between regenerative epithelium and resin (arrowheads), but also in the cytoplasm of regenerating epithelial cells and inflammatory cells (A–D). Once resin has been removed, regenerating epithelium becomes keratinized and expression of laminin and integrin β_4 disappears (E–H). KL, keratinized layer; R, resin space. Scale bars represent 25 μm.

expression of integrin β_4 chain in the resin-facing cells, as well as in the connective tissue-facing cells of the regenerating epithelium (Fig. 5A–D), although this disappeared after removal of the resin (Fig. 5E–H). This immunolocalization of integrin $\alpha_6\beta_4$ implies strong adhesion via hemidesmosomes between regenerating epithelium and resin.

Tanno *et al.* demonstrated that laminin 5 and integrin β_4 were expressed in the basal side of cells cultured from rat oral epithelium (24). Other studies have indicated the presence of hemidesmosomes and expression of laminin 5 at the interface between epithelial cells and titanium alloy (a bio-inert dental material; 25,26). These earlier reports strongly indicate that regenerating epithelium adheres to resin by means of the basal lamina and hemidesmosomes.

Hemidesmosomes are transmembrane cell-matrix junctional complexes that are able to intracellularly connect the CK filaments of epithelial cells with the ECM (27). Cytokeratin 5 and CK 14 are mainly expressed in the undifferentiated basal cells of stratified squamous epithelium (28,29). It has also been shown that hemidesmosomes are specifically composed of CK 5 and CK 14 (28). In contrast, in junctional epithelium, a non-keratinized epithelium, both CK 14 and CK 19 are expressed (19,30–32). Hormia *et al.* have reported that CK 14 is more intensely expressed in the tooth-facing cells of the junctional epithelium than is CK 19 (20). This is supported by our immunohistochemical results regarding CK 14 (Fig. 3C,I). Furthermore, CK 14 was also detected in the resin-facing cells of the regenerating epithelium (Figs 2F,L and 3F,L), whereas CK 14 expression disappeared and keratinization took place following removal of the resin (Fig. 4F,I,L). An experiment by Caffesse *et al.* revealed that the biological characteristics of oral epithelium, i.e. keratinized or non-keratinized, are induced by its attachment to the enamel (33). Once the resin was removed, the regenerating epithelium lost its ability to adhere to the resin and modified its expression of CK 14, provoking subsequent differentiation and keratinization.

Acknowledgements

We would like to thank Associate Professor Jeremy Williams, Tokyo Dental College, for his assistance with the English of this manuscript.

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