Title

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

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Running title: Oral epithelium adheres to 4-META/MMA-TBB resin

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Abstract

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 wound dressing, we investigated expression of laminin 5, integrin β4 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 at immediately, and 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 β4 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 the regenerating epithelium biologically adheres to the resin during the initial process of its regeneration.
Introduction

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 α6β4 at hemidesmosomes (16, 17). A recent research on the expression of laminin 5 and integrins in junctional epithelium demonstrated the localization of these proteins and mRNAs, which were produced by the tooth-facing cells where they contacted 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 β4) 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.
Materials and methods

Experimental design

Sixty-nine male Sprague-Dawley rats (six weeks of age) were used in this study. The animals were divided into 4 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 3 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 the C group, 4-META/MMA-TBB resin (Super-Bond C&B, Sun Medical, Moriyama, Japan) was applied to the teeth through the neighboring palate; in the G group, 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 the GR group, following gingivectomy as described for the G group, resin was applied to entire wound surface and the teeth; in the GRR group, the animals were treated in the same way as the GR group, after which the resin was removed 2 weeks later using an explorer and fine scissors, making sure to incur no bleeding. The rats were fed powdered food (Oriental Yeast, Tokyo, Japan) during the experimental period, and were sacrificed at immediately, or 1, 3, 5, 7 or 14 days after treatment in the C, G and GR groups, and at 1, 3, 5 or 7 days after removal of the resin in the GRR group. 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 and paraffin embedding, the specimens were serially sectioned at 3 µm in the bucco-lingual direction and stained with hematoxylin-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 37ºC for laminin 5, or incubated in a microwave oven in 10 mM citrate (pH 6.0) for 15 min at 65ºC for antigen retrieval of CK 14. The sections were then treated with 3% bovine serum albumin (BSA) 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 4ºC, 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 BSA as described above, and then immunoreacted overnight with a mixture of anti-laminin 5 (diluted 1:100) and a mouse monoclonal antibody to integrin β4 (Abcam, diluted 1:100) at 4ºC. 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 (AxioPhot 2, Carl Zeiss, Oberkochen, Germany).
Results

C group

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 post-operatively, but not at 7 days. No inflammatory cell infiltration was apparent in the epithelium, except for 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. CK 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).

G group

HE observations – 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 cemento-enamel junction (CEJ; Fig. 2g, open arrow), although their outline was quite similar to those of junctional epithelium at 5 days post-gingivectomy. Keratinization was recognized in the regenerating epithelium corresponding to the oral and the sulcular epithelia, and abundant inflammatory cells infiltrated the connective tissues underneath the epithelium during the same period. At 7 days, the regenerating epithelium reached the enamel to form new junctional epithelium, but leukocyte infiltration was still present in the connective tissue (Fig. 3a). 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, 1h, 2b, 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 (Fig. 2h). Intense expression of laminin 5 was apparent in the basal laminae, the EBL and the IBL at 7 and 14 days (Fig. 3b, 3h).

**CK 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 (Fig. 1c, 1i, 2c, 2i, 3c, 3i, asterisks).

**GR group**

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

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

**CK 14 immunolocalization** – Up to 3 days post-operation, the same reactivity for CK 14 was seen as in the G group. At 5 days post-operation, positive reactions for CK 14 were observed in both the basal cells and the regenerating cells close to the resin (Fig. 2i, 3f, 3l, asterisks).
**GRR group**

*HE observations* – Thin and keratinized regenerating epithelium was detected in the area of tissue where the resin had been applied 1 day 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, 4g, 4j).

*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 (Fig. 4b, 4e, 4h, 4j, arrowheads).

*CK 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 (Fig. 4c, 4f, 4i, 4l, asterisks).

**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 fluorescence at the frontal margin of the regenerating epithelium in the G group and in the cells facing the resin in the GR group. Integrin β4 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 in all experimental periods (Fig. 5a–d, arrowheads).

After removal of the resin, expression of laminin 5 and integrin β4 at basal lamina was not changed, however, their expressions 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 post-operation in both the G group (Fig. 2g) and the GR group (Fig. 2j). Furthermore, keratinization of the regenerating epithelium was observed within 2 days after the regenerating basal cells had attached to the CEJ in the G group (Fig. 3a), although no keratinization was detected in the GR group. On the other hand, keratinization took place immediately after removal of the resin in the GRR group (Fig. 4a). These results indicate that, while resin application inhibits keratinization, it does not affect the healing and 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 γ2-laminin subunit, in cells directly attached to the tooth, rather than 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 (Fig. 1g, 2a). 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 the GR group (Fig. 2e, 2k, 3e, 3k), but disappeared after removal of the resin in the GRR group (Fig. 4b, 4e, 4h, 4j). As mentioned above, among laminins, only laminin 5 is expressed in IBL of junctional epithelium. This phenomenon assumes that, regenerating epithelium under resin took on biological character of junctional epithelium, and once resin removed, regenerating epithelium changed its character to other oral epithelium. Furthermore, this suggests that regenerating epithelium does not recognize the resin as foreign body and 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 α6β4 heterodimer is believed to function as a receptor for laminin 5, and integrin β4 is known to dimerize...
only with the α6 chain (23). In this study, laminin-integrin double immunofluorescence staining revealed expression of integrin β4 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 α6β4 implies strong adhesion via hemidesmosomes between regenerating epithelium and resin.

Tanno et al demonstrated that laminin 5 and integrin β4 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). CK 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). On the other hand, 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. 2i, 3c, 3i). Furthermore, we observed that CK 14 was also detected in the resin-facing cells of the regenerating epithelium (Fig. 2f, 2l, 3f, 3l), whereas CK 14 expression disappeared and keratinization took place following removal of the resin (Fig. 4i–l). An experiment by Caffesse et al revealed that the biological characteristics of oral epithelium, i.e. as 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.
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References


Legends for Figures

Figure 1.
Healing process and protein expression in G and GR groups: micrographs of at immediately (a–f), and at 1 (g–l) day after surgery. HE-stained specimens (upper row) revealed that gingival epithelium and cut margin were distinct at immediately and at 1 day post-surgery in both treatment. Newly-formed epithelial cells (arrow), exudates, inflammatory cells and fibrin observed at 1 day post-gingivectomy (g). Laminin 5 immunolocalization (middle row) was distinct at basal lamina of remaining epithelium and at frontal margin of regenerating epithelium (arrowheads). Immunoreactivity showed expression of CK 14 (bottom row) in basal cells, parabasal cells and at frontal margin of regenerating epithelium (asterisks). E, enamel space; R, resin space. Bar = 100 µm.

Figure 2.
Healing process and protein expression in G and GR groups: micrographs of at 3 (a–f) and 5 (g–l) days after surgery. Cut margin was covered with new epithelium and inflammatory exudates at 3 days post-surgery (a, d), thereafter basal cells of regenerating epithelium reached CEJ at 5 days post-surgery in both groups (g, j, open arrow). Laminin expression was observed at tooth-epithelium interface, as well as in basal lamina (h, arrowheads). In addition of these sites, it was also expressed in resin-epithelium interface in GR group (e, k). CK 14 was expressed at frontal margin of regenerating epithelium in G treatment and further expressed in regenerating epithelium close to the resin in GR group (asterisks). E, enamel space; R, resin space. Bar = 100 µm.

Figure 3.
Healing process and protein expression in G and GR groups: micrographs of at 5 (a–f) and 14 (g–l) days post-surgery. In G treatment, keratinization was observed in regenerated epithelium corresponding to the oral and sulcular epithelium at 7 days post-gingivectomy and the epithelium was fully regenerated in morphologically at 14 days (a, d). On the other hand, it was incomplete in outline, revealing very thin intermediate layer and no keratinization even at 14 days after GR treatment (j). While laminin 5 was immunolocalized at basal lamina, regenerated EBL and IBL (b, h), it is also expressed at resin-regenerating epithelium interface in GR group (e, k, arrowheads). CK 14 (bottom row) was immunopositive in regenerated junctional epithelium and in parabasal cells of oral epithelium (c, i, asterisks) in G group. It is also expressed in regenerating cells close to resin (l, asterisks). E, enamel space; R, resin space. Bar = 100 µm.
Figure 4.
Regeneration process in GRR group. Thin and keratinized regenerating epithelium was detected at 1 (a–c), 3 (d–f), 5 (g–i) and 7 (j–l) days post-treatment. Basal cells were attached to CEJ at 1 day post-resin removal. Inflamed connective tissues were partly exposed where thin regenerated epithelium had peeled off (arrows; a). A keratinized layer was recognizable in all regenerating epithelia (tow row). Laminin 5 expression (arrowheads) disappeared in outermost area of regenerated epithelium, as was seen in healthy epithelium, whereas it was localized in EBL, IBL and basal lamina (middle row). CK 14 was immunolocalized in basal cells, parabasal cells and junctional epithelium cells (bottom row, asterisks). E, enamel space. Bar = 100 µm.

Figure 5.
Laminin 5-integrin β4 double immunofluorescence labeling in GR (top row) and GRR group (bottom row). Laminin 5 was strongly expressed as red fluorescence at interface between regenerative epithelium and resin, as well as in basal lamina in GR group. Integrin β4 was expressed as green fluorescence not only at interface between regenerative epithelium and resin (arrowheads), but also in cytoplasm of regenerating epithelial cells and inflammatory cells (a–d). Once resin had been removed, regenerating epithelium was then keratinized and expression of laminin and integrin β4 disappeared (e–h). KL, keratinized layer; R, resin space. Bar = 25 µm.