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Ultrastructural study of tissues surrounding replanted teeth and dental implants

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Running title: Ultrastructure of tissues surrounding replanted teeth and implant

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Key words: tooth replantation, dental implant, junctional epithelium, attachment apparatus, electron microscopy

Abstract
Objectives: The aim of this study was to describe the ultrastructure of the dento-gingival border at replanted teeth and implants.
Material and methods: Wistar rats (8 weeks old) were divided into groups for replantation and implantation experiments. In the former, the upper right first molars were extracted and then immediately replanted. In the latter, pure titanium implants were used. All tissues were fixed, demineralized and embedded in epoxy resin for ultrastructural observations.
Results: One week after replantation, the junctional epithelium was lost, and the oral sulcular epithelium covered the enamel surface. The amount of the epithelium increased in 2 weeks, and resembled the junctional epithelium, and the internal basal lamina and hemidesmosomes were formed in 4 weeks. One week after implantation peri-implant epithelium was formed, and in 2 and 4 weeks this epithelium with aggregated connective tissue cells were observed. In 8 weeks the peri-implant epithelium receded, and aligned special cells with surrounding elongated fibroblasts and bundles of collagen fibers appeared to seal the implant interface.
Conclusion: In replantation of the tooth the internal basal lamina remained at the
surface of the enamel of the replanted tooth which is likely to be related to regeneration of the junctional epithelium and the attachment apparatus at the epithelium-tooth interface. Following implantation, a layer of cells with characteristics of connective tissue cells, but no junctional epithelium and attachment apparatus, was formed to seal the site of the implant.

Introduction

The interface between the gingiva and tooth enamel is characterized by the presence of an attachment apparatus composed of well-developed hemidesmosomes at the basal surface of the junctional epithelium, and internal basal lamina (Schroeder, 1986). This apparatus plays an important role in the firm attachment of the epithelium to the tooth, and in sealing the periodontal tissues from the oral environment. High resolution ultrastructural studies in our laboratory provided further evidence for this effective sealing (Sawada et al., 1996, 2001, 2003).

The techniques of both tooth replantation/transplantation and implantation are accepted and successfully applied as endodontic therapy in the field of dental medicine. In both cases, the original attachment apparatus is mechanically broken down immediately after the operation.

In previous experimental models, the attachment apparatus is regenerated at the dento-gingival border after gingival surgery (Listgarten, 1967; Taylor and Campbell, 1972; Marková, 1983). In the case of replantation of the tooth, whether the epithelial attachment apparatus is regenerated at the interface between the gingival epithelium and the surface of tooth enamel is not known. On the other hand, it is known that following implantation, the newly formed mucosa (peri-implant epithelium and connective tissue) makes close contact with the surface of the implant. In spite of a number of previous animal experiments, the detailed nature of the interface between the mucosa (especially the epithelium) and the implant, particularly whether the attachment apparatus is regenerated, remained to be determined.

The aim of the present study was therefore to describe the ultrastructure of the dento-gingival border at replanted teeth and implants.

Material and methods

Animals

The animals used in this study were 35 Wistar rats (male, 8 weeks old). All experiments were done in accordance with Tokyo Dental College laboratory animal facilities experimental guidelines. The animals were divided into two (15 rats and 20 rats for experiments I and II, respectively).
Surgical Procedures

Experiment I: Tooth replantation was done with a method as previously described (Ihara et al., 2007). Briefly, under general anesthesia with ketamine hydrochloride (125 mg/kg), upper right molars were luxated with a dental excavator and carefully extracted with forceps in order to avoid damaging surrounding tissues. Then, they were immediately replaced in their original sockets. Gingiva around the maxillary left first molars was used as control. All animals were allowed free access to water and a powdered diet (Oriental Yeast Co., Tokyo, Japan). For ultrastructural examination as described below, the animals (in 3 groups, 5 rats each) were sacrificed 1, 2 and 4 weeks after the procedure.

Experiment II: After extraction of the tooth with the method described above, a screw-type implant was immediately placed in the socket. The implant used in this study (Fig. 1) was custom-made pure titanium implant with no surface treatment and of the size of 1.6 mm in diameter and 4 mm in length (Platon Japan Co., Tokyo, Japan). Sufficient space was left between the opposing lower first molar and the implant to avoid occlusal stimuli during mastication. After the operation, the animals were given water and a powdered diet ad libitum. They (in 4 groups, 5 rats each) were sacrificed in groups at 1, 2, 4 and 8 weeks after the operation.

Preparations of tissues for light and electron microscopy

Under anesthesia with ketamine hydrochloride, the animals were perfused with a cold fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 20 min. Isolated upper jaws were further fixed by immersing them into the same fresh fixative for 5 hr at 4°C. They were washed with sodium phosphate buffer, and demineralized in 10% EDTA for 4 weeks at 4°C. In experiment II, implants were mechanically separated from the surrounding tissue according to the method of Ikeda et al. (2000). Both replanted teeth with gingiva, and peri-implant tissues were postfixed with 1% osmium tetroxide in 0.1 M sodium phosphate buffer for 1 hr, dehydrated in a graded series of ethanol, and embedded in Epoxy resin. Ten blocks/group (5 each for experiments and control) were prepared. Semi-thin sections were stained with toluidine blue for light microscopy. Thin sections were stained with uranyl acetate and lead citrate before observation (average 5 sections/block) in an H-7100 electron microscope (Hitachi, Tokyo, Japan).

Results

Experiment I

Light microscopy
One week after replantation, the greater part of the junctional epithelium was lost at the
dento-gingival interface, and the enamel at the coronal side was seen covered by the oral sulcular epithelium (OSE). Beginning at the tip of the OSE and advancing towards the apical side a thin layer of epithelium was formed along the surface of the enamel (Fig. 2a, bracket). The lost junctional epithelium was replaced by the connective tissue of the lamina propria in direct contact with the enamel (Fig. 2a). Numerous inflammatory cells invaded this area.

In 2 weeks, the epithelium newly formed during the first week was further extended to approximately halfway between gingival crest and cemento-enamel junction. Inflammatory cells had almost entirely disappeared in the connective tissue of the lamina propria (Fig. 2b).

In 4 weeks this new epithelium was further extended to a point approximately two thirds between the gingival crest and cemento-enamel junction. The morphology of this new epithelium resembled that of the junctional epithelium in the control gingiva (micrograph not shown). No inflammation remained in the lamina propria, and bundles of collagen fibers were oriented parallel to the dental axis (Fig. 2c).

Electron microscopy
One week after replantation, a new epithelium was formed as an extension of the OSE along the surface of the enamel of the replanted tooth (Fig. 3). The cells of this epithelium closely resembled the cells of the junctional epithelium. A basal lamina-like structure emerged along the surface of this new epithelium at the interface between these epithelial cells and the surface of the replanted tooth (Fig. 3, inset).

In two weeks, numerous hemidesmosomes appeared on the enamel side of the new epithelium which was further advanced towards the apical direction and closely associated with a newly produced internal basal lamina composed of a lamina lucida and lamina densa. In addition, a basal lamina-like structure of slightly higher electron density emerged between the lamina densa of the internal basal lamina and the surface of the enamel (Fig. 4a, arrows with asterisks). Connective tissue fibroblasts were adhered along this basal lamina-like structure (Fig. 4b, arrows with asterisks). Judging from the way of its emergence, this basal lamina-like structure at the surface of the replanted tooth was probably preexisting basal lamina produced on the junctional epithelium prior to extraction.

Four weeks after replantation, the epithelial cells covering the enamel showed morphology almost identical to that of the cells of the junctional epithelium in control animals. That is, the epithelial cells had many cytoplasmic projections and were connected by desmosomes. These cells had flattened oval nuclei and contained many vacuoles in their cytoplasm. Numerous neutrophils had invaded their intercellular spaces (Fig. 5).

Experiment II
Light microscopy

One week after implantation the mucosal tissue surrounding the implant was similar to the mucosa surrounding the one week old replant. That is, a peri-implant epithelium was observed to be contiguous with the oral mucosa epithelium at the surface of the implant (Fig. 6a). The structure of the peri-implant epithelium was similar to that of the oral sulcular epithelium of the tooth replant. A few inflammatory cells invaded peri-implant connective tissue, and were in direct contact with the implant (Fig. 6a).

At 2 and 4 weeks later, the position of the leading edge of the peri-implant epithelium was approximately the same as that at one week (Fig. 6b, arrowhead). No extension of the epithelium towards the apical side such as observed in replantation occurred. Dense packing of oval or flattened cells was observed at the interface between the implant and the connective tissue (Fig. 6c). On the outer side of this dense mass of cells, elongated fibroblastic cells were observed running almost parallel to long axis of the implant.

Eight weeks after implantation, the leading edge of the peri-implant epithelium receded in the direction of the gingival crest (Fig. 6d, arrowhead). This epithelium showed the characteristics of the oral sulcular epithelium (Fig. 6e). A broad space on the side of the implant was covered by peri-implant connective tissue. Careful examination of this interface showed the presence of a zone with dense aggregation of superimposed flat or cubic cells displaying an epithelium-like alignment (Fig. 6f). On the outer side of the cell layer, elongated fibroblasts among bundles of collagen fibers, were seen arranged parallel to the long axis of the implant (Fig. 6f).

Electron microscopy

One week after implantation, an alignment of the flattened cells with numerous interdigititated microvilli facing the implant was observed. The interface of cells and implant was relatively straight, but hemidesmosomes and internal basal lamina were not present (Fig. 7a). The surface of the implant was covered by a mixture of connective tissue cells and inflammatory cells which invaded into the space between the connective tissue and the implant. Sparse, irregularly aligned collagen fibers were distributed around the connective tissue cells (Fig. 7b).

Two weeks later, the morphology of the peri-implant epithelium began showing a resemblance to that of the oral sulcular epithelium, and inflammatory cells had assembled along its underside (Fig. 8a). No cells having the morphology of the cell of the junctional epithelium were observed. In the peri-implant connective tissue, fibroblast-like cells of irregular form had accumulated enough to cover the surface of the implant. In places fragments of cells and collagen fibrils were interspersed in the narrow intercellular spaces in the cell layer (Fig. 8b).

Four weeks later the arrangement described above was not altered (data not
Eight weeks after implantation, the peri-implant epithelium showed characteristics of the oral sulcular epithelium. Both hemidesmosomes and basal lamina were not present at the interface between the epithelium and the implant (Fig. 9a, arrows). Cells showing epithelium-like alignment with extremely narrow intercellular spaces (Fig. 9b) were seen in the peri-implant connective tissue which was closely attached to the implant. The cells had numerous ribosomes and amounts of rough endoplasmic reticulum. The cells grew flatter and came to resemble fibroblasts more closely the further away they were from the implant (Fig. 9b). Surrounding the cell layer, abundant collagen fiber bundles as well as elongated fibroblasts ran almost parallel with the long axis of the implant.

**Discussion**

**Dento-gingival epithelium interface in replanted teeth**

The first part of this study is ultrastructural observation of the area of the interface between the gingival epithelium and the enamel of replanted teeth. The results clearly indicated that the junctional epithelium was almost fully restored to the condition seen in controls 4 weeks after replantation. In addition, numerous hemidesmosomes and internal basal lamina were observed in the interface with the enamel. Other laboratories (Taylor and Campbell, 1972; Maríková, 1983) reported rapid restoration of the junctional epithelium and the attachment apparatus in experiments involving mechanical removal of the junctional epithelium by means of a small steel blade or silk ligature inserted in the border between the gingival junctional epithelium and the enamel. The origin of restored epithelium observed in this study is not clear, but a possibility is that it is formed by the new proliferation of preexisting cells of the junctional epithelium. Atsuta et al. (2005a) made observation on the healing process of the epithelium following extraction of the rat molars. According to the report of these authors, the junctional epithelium was almost completely lost one day after extraction. After that, the number of cells arising from the oral sulcular epithelium increased and covered the upper surface of the extraction wound, and further expanding epithelium fused to cover the wound completely. In this study, although the junctional epithelium was largely lost one week after implantation, epithelium from the lower border of the oral sulcular epithelium advanced in the apical direction along the replant. This suggests that the restored junctional epithelium arose from the cells of the oral sulcular epithelium that survived the mechanical damage of extraction. Preexisting basal lamina which had remained attached to the enamel surface of the replanted tooth is thought to be significantly related to the proliferation and differentiation of epithelial cells and the reconstruction of the attachment apparatus.
Interface between dental implant and epithelium

The second part of this study is related to short term and long term therapeutic (healing) processes following implantation of the pure titanium dental implants. As to the surface topography of the implant, titanium implants with no surface treatment were used in this study. Shirakura et al. (2003) did not find any difference in bone formation in the rat maxilla with the condition of the surface of titanium implants. The attachment of soft tissue onto titanium implants was found not to be influenced by the roughness of the surface of the implant (Abrahamsson et al., 2002). The observation of implant-epithelium interface was done with the method of implant removal. According to Ikeda et al. (2000) the quality of peri-implant epithelium which was prepared with implant removal methods was same as that preserved by the use of cryofracture technique believed to be the method of superior preservation of the tissue.

The peri-implant epithelium was preserved in the form of the oral sulcular epithelium, and neither junctional epithelium nor attachment apparatus were restored. A number of other researchers (Berglundh et al., 1991, 2007; Schüpbach et al., 1994; Abrahamsson et al., 1996, 1999, 2002; Fujii et al., 1998; Moon et al., 1999) reported restoration of the junctional epithelium around implants at the light-microscope level. A few reports dealt with the in vivo reconstruction of the attachment apparatus at the electron microscopic level (Gould et al., 1984; McKinney et al., 1985; Hashimoto et al., 1989). According to a recent ultrastructural study of Ikeda et al. (2000) on titanium-alloy implantation in rats, hemidesmosomes and internal basal lamina are restored only in the apical one third of the peri-implant epithelium and not in the coronal two thirds. These authors also reported the presence of laminin 1 (Ikeda et al., 2000) and laminin 5 (Atsuta et al., 2005b), components of the basal lamina, in the areas of the interface with immunohistological examinations. In their reports the importance of laminin 5 in the formation of hemidesmosomes and the basal lamina was emphasized.

The results in this study differ from that of these authors. The reason for this disagreement is not yet clearly understood. By the use of plasma-sprayed ITI implants in dogs Fujiseki et al. (2003) reported that no attachment apparatus was formed in the peri-implant epithelium and that the nature of the epithelium was closer to that of the oral mucosal epithelium than to the junctional epithelium on the basis of differences in immunohistochemical results.

Interface between dental implant and connective tissue

The connective tissue around the implants was reported to be a highly fibrillated scar-like tissue with less blood vessels and cellular components (Buser et al., 1992). Previously there were no reports clearly describing the emergence of distinctively aligned epithelial cells in the peri-implant tissue, and the results of this study demonstrated the presence of such epithelium-like layer for the first time. Ultrastructurally the assembly of these cells was not accompanied by any features
characteristic of the epithelium such as desmosomes, hemidesmosomes, tonofilaments, or the basal lamina. Also, no specific structures which might be related to attachment were observed at the interface between these cells and the implant.

Bundles of well-developed collagen fibers and elongated fibroblasts were observed surrounding this cell layer and were arranged parallel to the long axis of the implant. Similar arrangement was observed in other laboratories (Moon et al., 1999; Abrahamsson et al., 2002; Berglundh et al., 2007) and it was believed to provide an effective sealing of peri-implant tissues from the oral environment. The biological significance of the epithelium-like layer of cells is not clear, but it may, as Abrahamsson et al. (2002) suggested, cooperate with fibroblasts to help stabilize peri-implant tissues.

References


**Figure legends**

**Fig. 1.** Photograph of pure-titanium implant used in this study. Bar = 1 mm.

**Fig. 2a-c.** Rat molar gingiva at 1 week (a), 2 weeks (b), and 4 weeks (c) after tooth replantation. (a) The coronal side of the tooth is covered by the oral sulcular epithelium (OSE). Typical junctional epithelium has almost completely disappeared in the dento-gingival interface. The enamel surface is in direct contact with the connective tissue (CT) of the lamina propria. Bracket indicates elongated epithelium. (b)
Epithelium is elongating in the apical direction. (Inset) Higher magnification view of boxed area. Arrowhead indicates a border between the leading edge of elongating epithelium and connective tissue. (c) The leading edge (arrowhead) of the epithelium in continuation from the oral sulcular epithelium (OSE) located near the cement-enamel junction. The epithelium resembles typical junctional epithelium in control tissues. ES, enamel space; OE, oral epithelium; D, dentin; CEJ, cement-enamel junction; asterisk, gingival crest. Toluidine-blue staining. Bars = 100µm (a, b, c), 25µm (inset).

**Fig. 3.** Electron micrograph of the bracketed area in Figure 2a. (Inset) Higher magnification view of boxed area. Note occurrence of a basal lamina-like structure (BL) at the enamel surface. ES, enamel space; PM, polymorphonuclear leukocytes. Bars = 5µm, 1µm (inset).

**Fig. 4.** Electron micrograph of the inset in Fig. 2b, showing the interface at 2 weeks after replantation. (a) Well-differentiated hemidesmosomes (HD) are evident. Newly deposited basal lamina is composed of lamina lucida (LL) and lamina densa (LD). Arrows with asterisks indicate electron-dense basal lamina-like structure. (b) A connective tissue fibroblast (FB) located next to dense basal lamina-like structure (arrows with asterisks). ES, enamel space; DE, desmosome; TF, tonofilaments. Bars = 0.5µm.

**Fig. 5.** Cells of regenerated junctional epithelium (JE) 4 weeks after replantation. Many vacuoles and tonofilaments are observed in the cytoplasm. ES, enamel space; PM, polymorphonuclear leukocyte. Bar = 5µm.

**Fig. 6.** Light micrographs of peri-implant mucosa at 1 week (a), 2 weeks (b, c), 8 weeks (d-f) postimplantation. (a) The coronal side of the implant is covered by peri-implant epithelium (PIE). A large area of implant surface is in direct contact with connective tissue (CT). (b) No cells of junctional epithelium-like structure are observed at the implant interface. Arrowhead indicates the leading edge of the peri-implant epithelium. (c) Higher magnification view of the boxed area of (b) showing the interface between implant and connective tissue. Note accumulation of fibroblast-like cells. (d) Arrowhead indicates the leading edge of the peri-implant epithelium. (e) The apical part of the peri-implant epithelium (PIE) resembles oral sulcular epithelium in its appearance. (f) Higher magnification view of the interface of implant and connective tissue. Cells (arrows) accumulated close to the implant resemble epithelium cells. Many fibroblasts (FB) among bundles of collagen fibers are oriented parallel to the surface of the implant. AB, alveolar bone; CT, connective tissue; IS, implant space; OE, oral epithelium. Toluidine-blue staining. Bars = 100µm (a, b, d), 25µm (c, e, f).
Fig. 7. (a) Electron micrograph of the interface of peri-implant epithelium (PIE) 1 week after implantation. Internal basal lamina is not observed at the interface between the peri-implant epithelium and implant surface. (b) Peri-implant connective-tissue interface. Many inflammatory cells occur close to the implant. Collagen fibers (Coll) are randomly distributed among fibroblasts (FB). IS, implant space. Bars = 5µm.

Fig. 8. (a) Electron micrograph of junction between leading edge of the peri-implant epithelium (PIE) and peri-implant connective tissue (PCT) 2 weeks after implantation. (b) Electron micrograph of the area indicated by a square in Fig. 6b. Fibroblast-like cells closely accumulate at the peri-implant interface. CD, cell debris; Coll, collagen fibrils; IS, implant space. Bars = 5µm.

Fig. 9. Eight weeks after implantation. (a) Peri-implant epithelial cells (PIE) resemble the oral sulcular epithelial cells of control tissue. No attachment apparatus is observed at the implant surface (arrows). (b) Interface of peri-implant connective tissues. The cytoplasm of a cell close to the surface of the implant contains an irregularly shaped nucleus (N) and large amount of ribosomes with narrow intercellular spaces. Fibroblasts (FB) among bundles of collagen fibers (Coll) are oriented parallel to long axis of the implant. IS, implant space. Bars = 5µm.
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