Development of a Novel Model for the Investigation of Implant–Soft Tissue Interface

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Background: In dental implant treatment, the long-term prognosis is dependent on the biologic seal formed by the soft tissue around the implant. The in vitro investigation of the implant–soft tissue interface is usually carried out using a monolayer cell-culture model that lacks a polarized-cell phenotype. This study developed a tissue-engineered three-dimensional oral mucosal model (3D OMM) to investigate the implant–soft tissue interface.

Methods: A 3D OMM was constructed using primary human oral keratinocytes and fibroblasts cultured on a skin-derived scaffold at an air–liquid interface. A titanium implant was inserted into the engineered oral mucosa and further cultured to establish epithelial attachment. The 3D OMM was characterized using basic histology and immunostaining for cytokeratin (CK) 10 and CK13. Histomorphometric analyses of the implant–soft tissue interface were carried out using a light-microscopy (LM) examination of ground sections and semi-thin sections as well as scanning electron microscopy (SEM).

Results: Immunohistochemistry analyses suggests that the engineered oral mucosa closely resembles the normal oral mucosa. The LM and SEM examinations reveal that the 3D OMM forms an epithelial attachment on the titanium surface.

Conclusion: The 3D OMM provided mimicking peri-implant features as seen in an in vivo model and has the potential to be used as a relevant alternative model to assess implant–soft tissue interactions. J Periodontol 2010;81:1187-1195.

KEY WORDS
Cell; cultured techniques/methods; dental implants; epithelial attachment; immunohistochemistry; mouth mucosa/anatomy & histology; tissue engineering.

Dental implant systems have been in clinical use for almost 5 decades. The long-term success of the dental implant treatment was first reported in the late 1970s by Dr. Per-Ingvar Brånemark.1 It is well accepted that osseointegration is vital for the success of implant treatment. Although the integrity of the implant–hard tissue interface is essential, the interaction between the gingival/mucosal soft tissue and the implant must also be considered. This biologic soft-tissue seal with the implant is critical to ensure the long-term prognosis of dental implants.2 The peri-implant mucosa responds to plaque in a fashion similar to the healthy gingiva that surrounds teeth.3,4 When the integrity of the biologic seal around an implant is disturbed by the inflammatory process, the underlying bone is exposed to the oral bacterial environment and leads to peri-implantitis.

Histomorphometric analyses5,6 from animal models show that the soft tissue attachment around natural teeth and implant surfaces had similar features but differed in terms of the quality of their biologic seal. In natural teeth, the non-keratinized junctional epithelium attaches to the enamel surface via the internal basal lamina and hemidesmosomes along the entire length of the junctional epithelium. However, the attachment of the non-keratinized peri-implant epithelium to the implant surface is confined at the apical
region only. A further key difference is the orientation of the connective tissue collagen fibers around implants compared to the teeth. In teeth, Sharpey’s fibers attach perpendicularly to the cementum, whereas the peri-implant connective tissue fibers are oriented in a circular arrangement at the inner zone and run in different directions at the outer zone.

The lack of attachment structures such as hemidesmosomes and connective tissue attachment on the implant surface could contribute to a weaker biologic seal around implants than that of the natural tooth.

There is increased interest among researchers to investigate the factors that influence the soft tissue attachment to the implant. Most of these studies are carried out in animal models, whereas limited studies are conducted in human subjects. Alternatively, in studies involving cell-culture models, primary or cell lines of keratinocytes and/or fibroblasts are used for the investigation of cell responses on different implant surfaces. Based on these in vitro models, information such as histomorphologic features of cell attachment, cell proliferation, activity, and cell adhesive strength are reported. Most cell-culture models are based on monolayer systems that have the disadvantages of: 1) a lack of polarized cell phenotype, 2) poor cell differentiation, and 3) the lack of cell-to-cell contact.

The importance of cell-to-cell interactions and modulation is highlighted. Thus, a tissue-engineered three-dimensional oral mucosal model (3D OMM), which consists of both keratinocytes and fibroblasts, may provide better evidence than the monolayer cell-culture system. A full-thickness, tissue-engineered oral mucosal model was previously developed for the biologic assessment of dental materials and oral health products. To our knowledge, there is no study published in the literature that indicates the use of a tissue-engineered human oral mucosa for evaluation of the implant–soft tissue interface. Therefore, this study further developed the 3D OMM into a relevant in vitro model for the assessment of implant–soft tissue interactions.

The primary oral keratinocytes were isolated from the oral mucosal biopsy and cultured using an explant technique. The biopsy was first washed three times in freshly prepared 10 ml Dulbecco’s modified Eagle’s medium (DMEM) that contained 50 U/ml penicillin, 50 U/ml streptomycin, and 625 mg/ml fungizone. The biopsy was placed in 2.5 mg/ml dispase overnight in the fridge. Just before isolation, the biopsy was incubated in a CO2 incubator at 37°C for 30 minutes. After the enzyme reaction, the epithelium was easily separated from the connective tissue layer. The separated epithelium was cut into tiny pieces using a scalpel blade #22. Subsequently, the explants were transferred into a T-75 collagen-coated flask and submerged in a minimal volume of fresh Green’s medium (3 to 5 ml) and left incubated for 3 to 5 hours in a CO2 incubator to ensure explants were attached on the flask surface. Green’s medium consisted of DMEM and Ham’s F-12 medium in a 3:1 ratio and supplemented with 10% fetal calf serum (FCS), 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, 10⁻⁴ mol/l adenine, 5 μg/ml insulin, 5 μg/ml transferrin, 2 × 10⁻⁷ mol/l triiodothyronine, 2.5 mM/l glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin. After the attachment, about 7 ml Green’s medium was gently added to the flask without disturbing the explants. The Green’s medium was changed every 2 to 3 days until the keratinocytes became 80% confluent.

To isolate the HF, the connective tissue of the biopsy was incubated in 10 ml 0.05% (weight/volume) collagenase type IV at 37°C overnight. After a 24-hour incubation, the tissue-containing medium was centrifuged at 1,500 rpm for 4 minutes to form HF pellets in a universal vial. The HF pellets were resuspended in DMEM that contained 10% FCS and plated in a T75 flask. The medium was changed every 2 to 3 days until confluent.

The 3D OMMs were constructed inside 12-mm diameter inserts with a 0.4-μm pore size and a 1 × 10⁸ pore density/cm² in a six-well plate. An acellular human-cadaveric dermis (about 0.9-mm thick) was expected to provide a relevant in vitro model for the assessment of implant–soft tissue interactions.

MATERIALS AND METHODS

Construction of a 3D OMM

The methods used to develop the new 3D OMM were a modification of a previously reported model. Ethical approval was obtained from the North Sheffield Research Ethics Committee for using waste oral tissues of patients. The primary cells, i.e., human oral keratinocytes (HK) and human oral fibroblasts (HF) were harvested from an oral mucosal biopsy of a 20-year-old woman who underwent surgical removal of her wisdom tooth. Written informed consent was obtained for this study.
was cut into a 12-mm-diameter disk. It was rehydrated in 5 ml phosphate buffered saline (PBS) for 30 seconds and followed by immersion in 5 ml DMEM for 15 minutes before placing it onto the insert. A coculture technique was conducted, in which a mixture of $5 \times 10^5$ HKs and $5 \times 10^5$ HFs were seeded onto the basement-membrane site of the dermis. The tissue was submerged in Green’s medium for 3 days. On day 4, a 4-mm-diameter hole was punched in the middle of the tissue using a sterile disposable tissue-biopsy punch.†††† Four types of titanium (Ti) surfaces, i.e., polished, machined, sandblasted and TiUnite‡‡‡‡ in disk form, were tested (5-mm diameter × 2.5-mm height; a 5-mm grade IV–diameter Ti rod was donated by the manufacturer, and polished, machined, sandblasted Ti disks were prepared from the donated Ti rod using in-house equipment). A Ti disk was inserted into the prepared hole (Fig. 1). This model was covered with medium and incubated for another 5 days. The level of the medium was reduced to create an air–liquid interface (ALI) and to promote epithelial differentiation over a period of 3 to 5 days.

The 4-mm-diameter punched tissue was not discarded but was further cultured in a 24-well plate in parallel condition with the 3D OMM. At the end of the culture, the punch tissue was processed for wax sectioning, stained with hematoxylin and eosin, and immunostained with cytokeratin (CK) 10 and CK13. A modified immunostaining protocol was carried out.††††† The punch tissues and normal gingival tissue (positive control) were fixed in 10% formalin before being embedded in wax. The sections were dewaxed in xylene and rehydrated through 100%- and 70%-alcohol series for 5 minutes each. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol for 20 minutes and washed in PBS. The antigens were retrieved with 0.1% trypsin§§§§ at 37°C for 20 minutes. Unspecific proteins were blocked with horse normal serumiiii for 40 minutes. The sections were then incubated overnight at 4°C with mouse monoclonal primary antibodies¶¶¶¶ at a concentration of 1:50 and 1:200 for CK10 and CK13, respectively. Subsequently, the sections were treated with biotinylated anti-mouse secondary antibodies#### for 1 hour at room temperature and followed by incubation in avidin-biotinylated peroxidase complex***** for 30 minutes at room temperature. The reactions were visualized using 0.05% diaminobenzidine (DAB)††††† and 0.005% H$_2$O$_2$ in 0.05M Tris buffer, pH 7.6, for 10 minutes. The sections were counterstained with hematoxylin for light-microscopy (LM) examination. The negative control was similarly processed except without the primary-antibody incubation.

**Interface Examination**

To examine the 3D OMM–Ti disk interface under LM, two techniques were used: 1) a ground-sectioning technique (the Ti disk was retained in the sections) and 2) an electropolishing technique (the Ti disk was dissolved electrolytically). In addition, cells attached to the Ti disk were examined under a scanning electron microscope (SEM).

**Ground sections.** At the end of the incubation period, the specimens were fixed with 2.5% glutaraldehyde for 2 to 3 days and post-fixed with 1% OsO$_4$ for 2 hours. Dehydration was carried out in serial-ascending ethanol concentrations at 50%, 70%, 90%, 95%, and 100% and with 1,2-propylene oxide for 30 minutes each with two changes for each solution. The specimens were preinfiltrated in 1,2-propylene oxide: epoxy resin‡‡‡‡‡ (1:1) for 2 hours followed by infiltration in pure epoxy resin overnight. After that, the specimens were embedded in new epoxy resin.
and polymerized at 40°C for 15 hours and finally at 60°C for 48 hours. The embedded specimens were cut in half using a diamond-band saw.§§§§§ One-half was re-embedded in acrylic resiniiiii for the preparation of ground sections.

The embedded specimens were sectioned into thin sections of about 100 µm using a diamond-band saw. The thickness of the sections was further reduced to 25 to 30 µm by grinding and polishing with silicon carbide papers¶¶¶¶¶ of P800, P1200, and P2400 roughness using a grinding machine.##### Subsequently, the ground sections were immersed in 10% H2O2 for 10 minutes and stained with Richardson’s solution32 (equal parts of 1% azure II and 1% methylene blue in 1% borax) for 30 minutes before LM examination. Electropolishing. The other half of the epoxy resin–embedded specimen was processed with an electropolishing technique to remove the bulk of Ti.33 Briefly, the surface opposite to the cut Ti surface was exposed by grinding with silicon carbide paper (P80). The specimen was immersed in an electrolyte solution, which consisted of a mixture of 5% perchloric acid, 35% n-butanol, and 60% methanol, that was cooled to –30°C. The specimen served as the anode, and a platinum ring placed around the specimen served as the cathode. The electropolishing process was performed at 200 mA/cm² and 24 V for about 4 to 5 hours. This process dissolved the bulk of the Ti metal and allowed the preparation of 1- to 1.5-µm-thick semi-thin sections using glass knives on a microtome.****** These sections were stained with toluidine blue and examined under LM.†††††† SEM. In a machined Ti surface specimen, after the fixation in 2.5% glutaraldehyde, a pie-shaped section of the oral mucosa was removed to expose the interface. The specimen was further post-fixed in 1% OSO4 for 2 hours, dehydrated with serial ethanol, and critical-point dried. It was sputter coated with gold before being examined under the SEM.

RESULTS

Characterization of the 3D OMM

Figure 2 shows the histologic appearance of a punch tissue stained with hematoxylin and eosin. A well-differentiated stratified squamous oral epithelium, which mimicked that of the normal oral mucosa, was observed. The epithelium consisted of four distinct layers that included the basal, spinous, granular, and superficial keratinized layers. The epithelial cells show evidence of terminal differentiation toward the surface of the epithelium. HFs were present within the connective tissue layer.

The immunostained 3D OMM and normal oral mucosa are compared in Figure 3. The suprabasal cell layer of the 3D OMM showed a strong expression of CK13 but a weak expression of CK10, which are the differentiation markers of the non-keratinized and keratinized epithelium, respectively. This suggests that the 3D OMM reveals features closer to that of the non-keratinized stratified epithelium than the keratinized normal oral mucosa.

The ground sections and semi-thin sections, which were obtained from the same specimens are compared in Figure 4. The interfacial soft tissue appeared to be intact on the Ti disk. In a semi-thin section, more details were revealed; e.g., the apical migration of the cells along the surface of the Ti disk was more obvious compared to the corresponding ground sections. Some cells had noticeably migrated upward along the Ti disk. HFs were sparsely scattered in the connective tissue layer.

Two types of epithelial attachment were identified: pocket and non-pocket types. The pocket type of attachment manifests as a gap between the epithelial layer and the Ti disk. The non-pocket type attachment refers to the lack of gap between epithelium and the Ti disk. Clinically, the non-pocket type of attachment could indicate a more favorable attachment than the pocket type, as the latter has a higher risk of plaque retention, which may result in peri-implantitis. In this study, the examination of the ground sections or semi-thin sections showed both types of epithelial attachment were present in all types of Ti surfaces (Fig. 4). This suggested that the Ti surface topography did not significantly affect the soft tissue attachment.
In the SEM specimen, the soft tissue appeared to be detached from the Ti surface (Fig. 5A) either during the sectioning of the soft tissue or during fixation and dehydration procedures. Nevertheless, there was evidence of cells attached on the machined Ti surface at the exposed area. This indicated that the cells from the 3D OMM formed attachments on the Ti surface (Figs. 5B and 5C).

**DISCUSSION**

Because the biologic seal of the soft tissue around the implant surface is an important factor in determining the long-term success of implant treatment, many researchers use different study models to investigate the implant–soft tissue interface. Animal and human models have been the reliable study models but are constrained and governed by strict ethical procedures. Although in vitro cell-culture models are simpler, they consist of monolayers that limit the ability to extrapolate information to the in vivo situation. To overcome these limitations, models of tissue-engineered oral mucosa, consisting of multiple layers of epithelial cells, were used in various in vitro investigations such as cytotoxicity testing and micro-organism infection testing. There are some commercially available oral mucosal models which are cultured from normal oral keratinocytes, and others which are cultured from a squamous cell carcinoma of the buccal mucosa. However, these commercially available oral mucosal models consist of an epithelial layer only and lack the connective tissue component. Locke et al. reported that fibroblasts play an important role in modulating epithelial cell differentiation. In the present study, a full-thickness model that contained both types of cells (i.e., keratinocytes and fibroblasts) was used to construct the 3D OMM. To our knowledge, this is the first study that used a tissue-engineered 3D OMM for the investigation of the implant–soft tissue interface.

The present 3D OMM was modified from an earlier in-house model. The previous model was used for the investigation of the biocompatibility for dental materials. However, the basement membrane matrix attached to the Ti surface in the absence of any cells. Thus, a modification of this model by replacing the basement membrane matrix with an acellular dermis had to be made for the investigation of the implant–soft tissue interface.

‡‡‡‡‡‡ EpiOral, EpiGingival, MaTek Corporation, Ashland, MA.
§§§§§§ SkinEthic HGE, SkinEthic HOE, SkinEthic Laboratories, Nice, France.
Matrigel, BD Biosciences, San Jose, CA.
Using acellular dermis as a scaffold and cocultured with HK and HF, the tissue-engineered oral mucosa had features that mimicked those seen in normal oral mucosa (Fig. 3). The primary cell models were cultured for 2 weeks with 3 to 5 days of ALI toward the end of culture. An optimal duration of ALI facilitates the differentiation of the epithelial layer, as was evident in the immunostaining of the tissue in this study. The main disadvantage of primary cells is that there can be batch-to-batch variations. Thus, it is suggested that the same pool of primary cells should be used in the same experiment for different test surface comparisons.

In the present model, Green’s medium enriched with FCS was used. However, the influence of different culture media on the structures and adhesion molecules involved in the interface was not obvious. Because the oral mucosal models produced in this study were not transplanted to patients, the use of a serum-containing culture medium was of less concern. One study suggested the addition of 0.5 mM calcium into the medium to enhance the differentiation of the epithelial cells.

Several techniques have been explored to examine the implant–soft tissue interface. Ground sectioning, which involves cutting through and grinding with the Ti in situ, is the most commonly used technique. This technique is a highly labor-intensive procedure. The advantage of ground sections is that they provide information about the position of the peri-implant tissue in relation to the implant surface at the interface.

**Figure 4.**
A comparison of the soft tissue attachment on four different types of Ti surfaces using ground section and semi-thin section techniques. Two types of soft tissue attachment were identified, i.e., ‘pocket’ and ‘non-pocket’ (original magnification x20).
In contrast, semi-thin sections do not provide the relationship between the soft tissue and Ti surface as do ground sections. However, semi-thin sections showed more detail of the structure (Fig. 4). Apical epithelial migration was more noticeable in the semi-thin section compared to the corresponding ground section because of their lower thickness.

Two types of epithelial attachment were observed (i.e., pocket and non-pocket) on all Ti surfaces, but the pattern of attachment could not be correlated to the type of surface topography. In sandblasted and TiUnite specimens, the ground sections showed a pocket type of attachment but appeared as a non-pocket type of attachment in semi-thin sections. It seems that the 3D OMM formed different types of epithelial attachment on the Ti surface.

SEM is another method that allows in situ examination of the specimens without cutting through the implant. The advantage of SEM is that it provides a three-dimensional architecture of the implant–tissue interface compared to the LM and TEM, which only show two-dimensional features of the interface. We found that there were residual cells still attached to the Ti disks after the soft tissue was removed (Fig. 5). This observation was in accordance with another study and suggested that the bond strength of the epithelial cells to the implant surface was greater than the cell–cell bonding within the epithelial layer. However, a major limitation of an SEM examination is that the detailed information on the nature of the cell organelles involved in the interface between epithelial cells and the implant material is not available.

Based on several techniques used in the present study to examine the implant–soft tissue interface, it was found that the 3D OMM formed an epithelial attachment on Ti surfaces. Thus, the model can be used to test different implant surfaces. The histomorphometric analysis of the interface provides information on the nature of the soft tissue response to different surfaces. For example, if there is a gap between the epithelial layer and test surface, it indicates that the biologic seal is not as favorable compared to surfaces that have a close epithelial attachment. This new 3D OMM provides more detailed information of the soft tissue response to implant surfaces than can be obtained using a monolayer cell-culture model. Therefore, this model is more clinically relevant than monolayer cultures and could reduce the need for animal testing. Further studies on quantitative approaches to assess soft tissue attachment to Ti surfaces using this 3D OMM are in progress.

CONCLUSION

The 3D OMM provides peri-implant features that mimic those seen in vivo and therefore, has the potential to be used as a relevant alternative model to assess implant–soft tissue interactions.

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