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<td>著者</td>
<td>伊藤 文敏、松坂 賢一、石田 昇平、懸田 明弘、井上 孝</td>
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Immunohistochemical Expression of Involucrin and Filaggrin at the Peri-Implant Epithelium Implanted in the Rat Palate in Early Stage

ITOU Fumitoshi*, MATSUZAKA Kenichi*, ISHIDA Shouhei, KAKETA Akihiro and INOUE Takashi

ラット口蓋に埋入したインプラント周囲粘膜上皮における初期の involucrin と filaggrin の発現

伊藤 文敏 松坂 賢一 石田 昇平 懸田 明弘 井上 孝

Purpose: The purpose of this study was to investigate the expression of involucrin and filaggrin in the peri-implant epithelium (PIE).

Materials and Methods: Titanium implants, 2 mm in diameter and 5 mm in length, were surgically implanted in the palatal region of rats. Animals were sacrificed at 3, 7, 14 and 28 days after the implantation. Paraffin sections were cut, and hematoxylin-eosin and immunohistochemical staining was performed using primary antibodies to involucrin and filaggrin.

Results: Involucrin in the PIE immunoreacted with almost all epithelial cells except basal cells 3 days after implantation, but was positive only in the upper area of the spinous layer at 7, 14 and 28 days after implantation. Further, involucrin-positive cells could be observed in the apical portion of the PIE at 3 and 7 days after implantation, but not at 14 and 28 days. The measurement of the involucrin ratio decreased day by day. Filaggrin immunoreacted at the upper portion of the spinous layer of normal oral mucosal epithelium, but not in the entire layer of PIE at 3, 7 and 14 days or in the apical portion of the PIE at 28 days after implantation.

Conclusion: The PIE in the apical portion is structured by basal cells which contact the implant material, but the barrier function of the cornified layer does not function well at an early stage.

Key words: peri-implant epithelium, involucrin, filaggrin, rat, immunohistochemistry

Introduction

It is well known that dental implant materials penetrate the oral mucosal epithelium, and the interior environment communicates with the exterior environment. At that interface, gingival soft tissue needs to attach well via an epithelial seal to the implant surface to maintain dental health. This process is similar to wound repair. So, although the peri-implant epithelium (PIE) plays an important role as a barrier against the outside, understanding the characteris-

*These authors contributed equally to this work.
Department of Clinical Pathophysiology, Tokyo Dental College
東京歯科大学臨床検査病理学講座
平成 25 年 12 月 26 日受付

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tics of the PIE is very important for dental implant treatment. The oral mucosa is continuously metabolizing, and functions by a homeostatic mechanism. The PIE is often compared with the gingival junctional epithelium. The junctional epithelium, a non-keratinized stratified epithelium, extends apically in apposition to the surface of the enamel to form a seal between the epithelium and the tooth\(^1\), and adheres to the enamel surface via hemidesmosomes.

A number of studies have examined the implant and tissue interface of dental implants, but investigations of the PIE are scarce and controversial. Gould et al. reported that the connection between the PIE and a titanium implant is similar to the connection to natural teeth, including a basal lamina that forms between the implant and the plasma membrane of PIE cells, and hemidesmosomes that connect cells to the basal lamina\(^2\). However, Inoue et al. and Fujiseki et al. reported that hemidesmosomes are not formed between titanium implants and the PIE in large animals such as dogs or monkeys\(^3\)\(^4\).

Regarding experimental design, Iizuka et al. obtained the results that no epithelium migration at 3 days after implantation could be seen along the dental implant, but at day 7, new epithelial cells from basal cells of the oral epithelium had begun to migrate along the implant surface\(^5\). Further, when they observed the epithelium at 14 and 28 days after implantation, the epithelial cells had spread further apically, and a non-keratinized peri-implant epithelium had formed consisting of a few cells in thickness\(^5\).

On the other hand, it is well known that the oral epithelium plays an important role as a barrier and that epithelial cells differentiate from the basal layer to the cornified layer through the spinous layer and the granular layer\(^6\). Involucrin, a component of the keratinocyte crosslinked envelope, is found in the cytoplasm and is crosslinked to membrane proteins by transglutaminase. Involucrin is synthesized in the spinous layer and is crosslinked in the granular layer by transglutaminase which makes it highly stable. Thus, involucrin provides structural support to cells, thereby allowing the tissue to resist invasion by micro-organisms. Further, filaggrin is synthesized in granular cells, and plays a role in the formation of crosslinks between keratin and involucrin and is an architectural component of the keratinous layer after it breaks down during maturation.

The purpose of this study was to investigate the expression of involucrin and filaggrin in the PIE.

### Materials and Method

#### 1. Implant materials and implantation

The method of Iizuka et al. (2009) was carried out to prepare implant materials and to implant them\(^6\). Briefly, titanium implants (Platon, Tokyo, Japan) 2 mm in diameter and 5 mm in length were used in this study (Fig. 1). The diameter of the implant was determined according to the space of the palate of the rat. Before implantation, the titanium implants were sterilized with acetone and ethanol, and then were washed with distilled water and were finally autoclaved. Twenty six-week-old male Sprague-Dawley rats, weighing approximately 180 g each, were used in this study. Under general anesthesia with pento-barbital sodium (Rabonal, 50 mg/kg), a mucosal incision was made in the 4th palatal rugae of each rat. Before the implantation, the recipient site was thoroughly disinfected with 10% iodine and was prepared by drilling with a dental reamer, after which the implant body was screwed into the cavity with a micro driver. The rats were housed and given water and a powdered diet until sacrificed. Each rat received one implant. The study protocol complied with the Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985) and relevant national laws.

#### 2. Histochemical procedure

Five rats were sacrificed at each time period (3, 7, 14 and 28 days). Animals were anesthetized with pento-barbital sodium (Rabonal, 100 mg/kg) and tissues were fixed by intercardiac perfusion with 10%
neutral-buffered formalin. The maxillary jaw with the implant was removed from each animal and was fixed in neutral-buffered formalin (10%) for 3 days, after which it was decalcified in formic acid for 1 week before being embedded in paraffin. After the decalcification, each implant body was removed meticulously by mechanical means. Paraffin sections were cut in the sagittal plane, and sections were stained using hematoxylin and eosin (HE). All implants had osseointegration without peri-implantitis and mobilization except one implant which had no osseointegration. Paraffin sections were cut parallel to the longitudinal axis of the implant.

3. Immunohistochemical staining
Paraffin sections were deparaffinized with xylol and were incubated in 3% hydrogen peroxide with methanol for 13 min at room temperature to block endogenous peroxidase activity. For antigen retrieval, sections were treated with 3% bovine serum albumin (BSA) or 10% goat serum for 30 min at room temperature.

Anti-involucrin (diluted at 1:100, mouse monoclonal, Abcam, Cambridge, UK) and anti-filaggrin (diluted at 1:1,000, rabbit polyclonal, Abcam, Cambridge, UK) were used as primary antibodies. The sections were incubated at room temperature with the primary antibody for 60 min, and then were incubated with a biotinylated secondary antibody: NICHIREI-Histofine simple-stain MAX-PO® (NICHIREI, Tokyo, Japan, http://www.nichirei.co.jp/bio/english/tech_info/pap/414191f.html) for 30 min at room temperature. Thereafter the sections were rinsed with PBS and were stained with NICHIREI-Histofine simple-stain DAB® (NICHIREI, Tokyo, Japan) and counterstained with hematoxylin and observed using light microscopy. Further, the normal epithelium of oral mucosa, which can be observed in the same section, was also observed as a control.

4. Ratio of involucrin-positive length at the PIE
The ratio of involucrin-positive length was calculated by the following formula: (length of involucrin-positive area of the PIE facing the implant) / (length of the PIE facing the implant) × 100.

Statistical analysis was performed as ANOVA multiple comparison Scheffe’s test were used to compare (p<0.05)

Results

Involucrin was positive in almost all epithelial cells in the PIE except for basal cells 3 days after implantation, but was positive only in the upper area of the spinous layer at 7, 14 and 28 days after implantation (Fig. 2). Further, involucrin-positive cells could be observed at the apical portion of the PIE at 3 and 7 days after implantation, but not at 14 and 28 days. The ratio of the involucrin-positive length to the total area of epithelium was 86.8±6.7 at 3 days, 89.2±5.6 at 7 days, 78.5±9.5 at 14 days, and 727±1.3 at 28 days after implantation. The measurement of the involucrin ratio decreased day by day (Fig. 3). The ratio of involucrin-positive area at 28 days after implantation was significantly lower than at 3 and 7 days.

Filaggrin immunoreacted at the upper portion of the spinous layer of the normal oral mucosal epithelium, but not in the entire layer of the PIE at 3, 7 and 14 days or in the apical portion of the PIE at 28 days after implantation (Fig. 4).

Discussion

The PIE is different from the natural periodontal epithelium, and dental implant therapy creates an open wound. An implant-epithelium interface is formed which is always exposed to the possibility of inflammation. Only a little bleeding and inflammatory cell infiltration were observed in this experimental design, because the implant socket in rat maxilla was smaller than that in human. Further, the healing ability in rat was generally was faster than in human. Therefore, investigation of the characteristics of the PIE is important for the follow-up of dental implant
Involucrin and Filaggrin in Peri-Implant Epithelium

An interface forms between the host tissue and the titanium implant during the process of wound healing. First, the space between the implant and the peri-implant soft tissue fills with coagulation after which leukocytes clean up the damaged tissue and cells and purge bacteria that have invaded the wound area. Capillaries and fibroblasts then appear and prepare the stroma for tissue restoration and, at the same time, the oral mucosa penetrates along the implant surface and, as a result, the PIE is created.

Involucrin is a useful marker at an early stage in the pathway of terminal differentiation, and is a soluble protein precursor of the cross-linked envelope. Involucrin is produced as a soluble cytosolic protein and is subsequently assembled through the action of transglutaminase to form part of the protective surface epithelial structure. In this study, involucrin was immunohistochemically positive in almost all epithelial cells except for basal cells 3 days after implantation, but was positive only in the upper area of the spinous layer at 7, 14 and 28 days after implantation. Bar = 0.5 mm

Fig. 1 The dental implant and implant site. Dental implant (a) and the dental implant in the cavity (b) are shown. Arrow: implant

Fig. 2 Immunohistochemical staining with anti-involucrin. Involucrin was immunoreactive in the PIE in almost all epithelial cells except for basal cells 3 days after implantation, but was positive only in the upper area of the spinous layer at 7, 14 and 28 days after implantation. Bar = 0.5 mm

Fig. 3 The ratio of involucrin-positive length. The involucrin-positive ratio decreased day by day.

Fig. 4 Immunohistochemical staining with anti-filagrin. Filaggrin was immunoreactive in the upper portion of the spinous layer of the normal oral mucosal epithelium, but not in the PIE at 3, 7, 14 and 28 days after implantation. Bar = 0.5 mm
28 days after implantation. This means that 3 days after implantation is an early stage of wound healing, and the orientation of epithelial cell differentiation is not yet decided. Further, the ratio of involucrin-positive cells decreased day by day. The implant material is structured in the apical portion of the PIE.

Filaggrin is a filament associated protein that binds to keratin fibers in epithelial cells, and is essential for the regulation of epithelial homeostasis. Further, filaggrin plays an important role in the barrier function of the cornified layer and is strongly predisposed to a severe form of dry skin. In this study, although filaggrin immunoreacted in the upper portion of the spinous layer of the normal oral mucosal epithelium, the PIE did not react with filaggrin at any time during the experiment.

Implantation creates a lack of continuous epithelium because the oral mucosa is penetrated along the implant surface, and as a result, an epithelium-implant interface is formed. Therefore, elucidation of the defense mechanism, including those of the epithelium itself, is important because the peri-implant tissue is always exposed to the possibility of inflammation. It is known that the sealing ability of peri-implant epithelium is weak. Further, there are reports that peri-implant epithelium plays an important role in preventing the initial stage of inflammation. So, this study indicated the differentiation of peri-implant epithelium using the antibodies of involucrin and filaggrin. PIE in the apical portion in early stage of implantation is constructed by basal cells, so the apical portion is on the differentiation, and barrier function is still not accomplished in this experiment stage of day 28.

In conclusion, the PIE in the apical portion is structured by basal cells which contact the implant material, but the barrier function of the cornified layer does not function well at an early stage.

References

目的：本研究の目的は、インプラント周囲上皮のinvulucrinとfilaggrinの発現の局在を検索することである。

材料および方法：直径2 mm、長さ5 mmのチタン製インプラントをラット口蓋に埋入した。実験動物はインプラント埋入後3、7、14、28日に屠殺した。パラフィン切片を作製し、HE染色およびinvulucrinあるいはfilaggrinを用いた免疫組織化学的染色を行った。

結果：インプラント埋入3日例におけるインプラント周囲上皮におけるinvulucrinは上皮層のほぼ全層に陽性反応を示したが、7、14、28日例では有棘細胞層の上層のみ陽性を示した。さらに、インプラント埋入3および7日例ではinvulucrin陽性細胞がインプラント周囲上皮のインプラント体先端側で観察されたが、14および28日例ではインプラント周囲上皮の根尖側に陽性細胞はみられなかった。また、invulucrinの陽性範囲は経時的に減少していた。filaggrinは正常な上皮棘細胞層上層に陽性を示していたが、インプラント周囲上皮では3、7、14日例および28日例の根尖側で陰性を示した。

結論：インプラント体根尖側におけるインプラント周囲上皮は、インプラントに接する部において基底細胞が接しており、分化途中であり、角質層のバリアとしての機能が低いことが示唆された。