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Original article

Immunohistochemical expression of involucrin and filaggrin at the peri-implant epithelium implanted in the rat palate in early stage

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Abstract

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 made and hematoxylin-eosin and immunohistochemical staining was performed, using primary antibodies to involucrin and filaggrin. Further, the ratio of involucrin-positive length was calculated.

Results: Involucrin in the PIE immunoreacted with almost all epithelial cells except basal cells at 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 at 14 and 28 days after implantation no involucrin-positive cells were seen at the apical portion of the PIE. The measurement of the involucrin ratio decreased from 14 to 28 days. Filaggrin immunoreacted at the upper portion of the spinous layer of normal oral mucosal epithelium, but did not immunoreact in the all layer of PIE at 3, 7, 14 days or in the apical portion of PIE at 28 days after implantation.

Conclusion: Involucrin and filagrrin at the apical portion of the PIE at 28 days was not immunoreacted. So, the PIE in the apical portion is structuralized by basal cells to contact to the implant material and is on the differentiation, but the barrier function of the cornified layer does not function well in early stage.

Keywords: 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 health of PIE. This phenomenon is the same environment as a wound repair. So, although the peri-implant epithelium (PIE) plays an important role as a barrier against the outside, understanding the characteristics of the PIE is very important for dental implant treatment. The oral mucosa is always metabolizing, and functions in a homeostatic mechanism. The PIE is often compared with the gingival junctional epithelium. The junctional epithelium, a nonkeratinized 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 a few 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 dogs or monkeys[3, 4].

About PIE, in the same 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, they observed the epithelium at 14 and 28 days after implantation, the epithelial cells had spread further apically, and a non-keratinized PIE 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 methods

1. Implant materials and implantation

The method of Iizuka et al. (2009) was carried out to prepare implant materials and to implant them[5]. 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 pentobarbital sodium (Rabonal, 50 mg/kg), a mucosal incision was made in the 4th transverse palatine fold 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. This study protocol was used in accordance with the *Princiles of Laboratory Animal Care* (NIH publication no. 86-23, revised 1985) and relevant national laws. Further, all experiments were performed according to the laboratory animal guidance of the Tokyo Dental College.

2. Histochemical procedure

Five rats were sacrificed at each time period (3, 7, 14 and 28 days). Animals were anesthetized with pentobarbital 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 longituginally for 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 at1:1000, 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 for differentiation of wound healing around implant, and was calculated by the following formula:

(the length of involucrin-positive area of the PIE facing the implant) / (the length of the PIE facing the implant) x 100. Figure 2 shows the method for calculating method of the ratio.

Statistical analysis was performed as ANOVA multiple comparison Scheffe's test were used to compare ($P<0.05$)

Results

Histologically, 3 days after implantation, no epithelium migrated. Red blood cells and inflammatory cells could be observed. At 7 days, a new epithelium from the basal cells of the oral epitheium formed. At 14 and 28 days after implantation, keratinized PIE could be seen , but non-keratinized epithelium was observed at the apical portion of PIE (Fig. 3). 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. 4). Further, involucrin-positive cells could be observed at the apical portion of the PIE at 3 and 7 days after implantation, but at 14 and 28 days after implantation no involucrin-positive cells could be seen at the apical portion of the PIE. Figure 5 shows the mean ±standard deviation of the ratio of involucrin-positive length. The ratio of the involcrin-positive length decreased from 7 to 28 days (Fig. 5). The ratio of involcurin positive-length 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 was not immunoreactive in the all layer of PIE at 3, 7, 14days or in the apical portion of PIE at 28 days after implantation (Fig. 6).

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[3]. There are some reports about the healing of PIE. Suzuki et al. reported that the behavior of Merkel cell in PIE[7]. Red blood cells and inflammatory cells infiltration were shown in this experimental design, because the implant socket in rat maxilla was smaller than that in human. Further, healing in rat was generally earlier than in human. Therefore, investigation of the characteristics of the PIE is important for follow-up of dental implant treatment. Formation of an interface between the host tissue and the titanium implant occurs 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 was penetrated along the implant surface and, as a result, the PIE is created.

Involucrin is a useful marker for an early stage in the pathway of terminal differentiation, and is a soluble protein precursor of the cross-linked envelope [8]. 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[9]. About the report of involucrin related with wound healing of epithelium, Vriens et al. reported that comparison of autologous gingiva and skin for wound healing[10]. In this study, involucrin was immunohistochemically positive in almost all epithelial cells of the PIE except for basal cells in the early days following

implantation. However, at later times, involucrin-positive cells were observed only in the upper area of the spinous layer, and could not be observed in the apical portion of the PIE at 14 and 28 days after implantation. It is considerable 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 from 7 to 28 days.

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[11]. Murakami et al. reported about the filaggrin expression of oral mucosa. They obtained that keratinization is closely associated with expression of filaggrin[12]. 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 for filaggrin at any experimental time.

Implant creates the lack of continuous epithelium because the oral mucosa is penetrated along the implant surface, and as a result, an epithelium-implant inter face 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[13]. It is recognized that the PIE sealing ability is weak[14]. Further, there are reports that PIE plays an important role in the prevention against initial stage of imflammtion[4, 13, 15, 16]. So, this study indicated the differentiation of PIE using the antibodies of involucrin and filaggrin. PIE in the apical portion in early stage of implantation is nearly constructed by basal cells, so the apical portion is on the differentiation, and barrier function is still not accomplished in this experimental stage of day 28.

In conclusion, involucrin and filagrrin at the apical portion of the PIE at 28 days was not immunoreacted in this experimental design. So, tthe PIE in the apical portion is structuralized by basal cells to contact to the implant material, but the barrier function of the cornified layer does not function well in early stage.

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Figure Legends

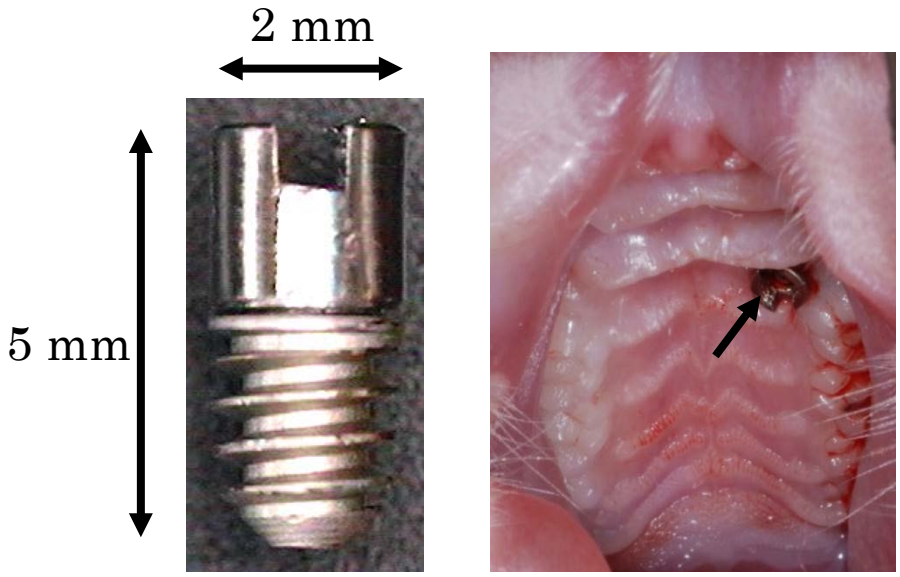
Fig. 1. The dental implant and implant site. Dental implant (a) and the dental implant in the cavity (b) are shown. Arrow: implant

Fig. 2. The schema of the method for calculating of ratio of involucrin positive length at the PIE
The ratio is B / A X100

Fig. 3. 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 7, 14 and 28 days after implantation. Bar=0.5mm

Fig. 4. The ratio of involucrin-positive length.
The involucrin-positive ratio decreased from 7 to 28 days.

Fig. 5. Immunohistochemical staining with anti-filaggrin.
Filaggrin was immunoreactive in the upper portion of the spinous layer of the normal oral mucosal epithelium, but was not immunoreactive in the PIE at 3, 7, 14 and 28 days after implantation. Bar=0.5mm



a b

Fig. 1

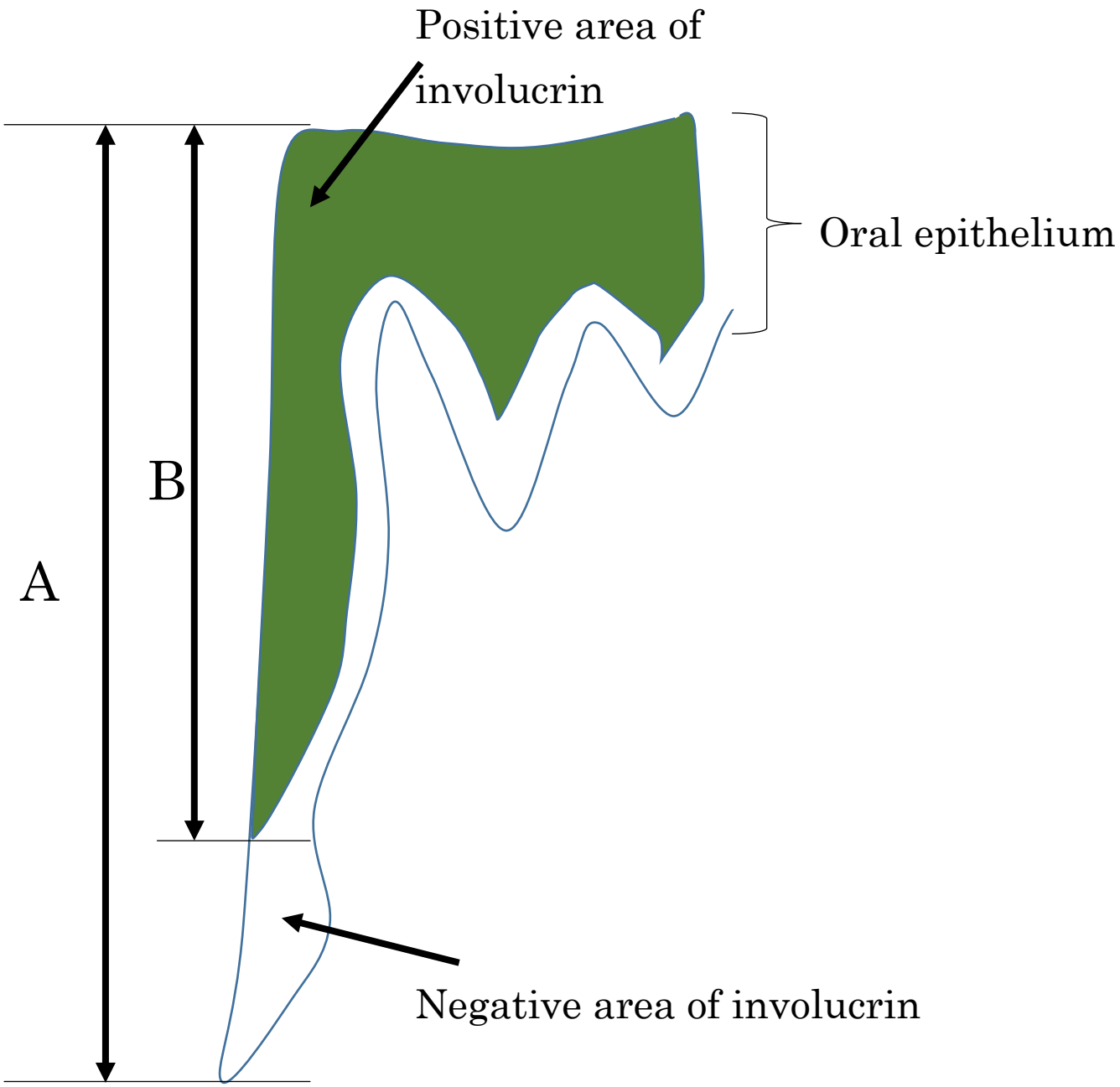
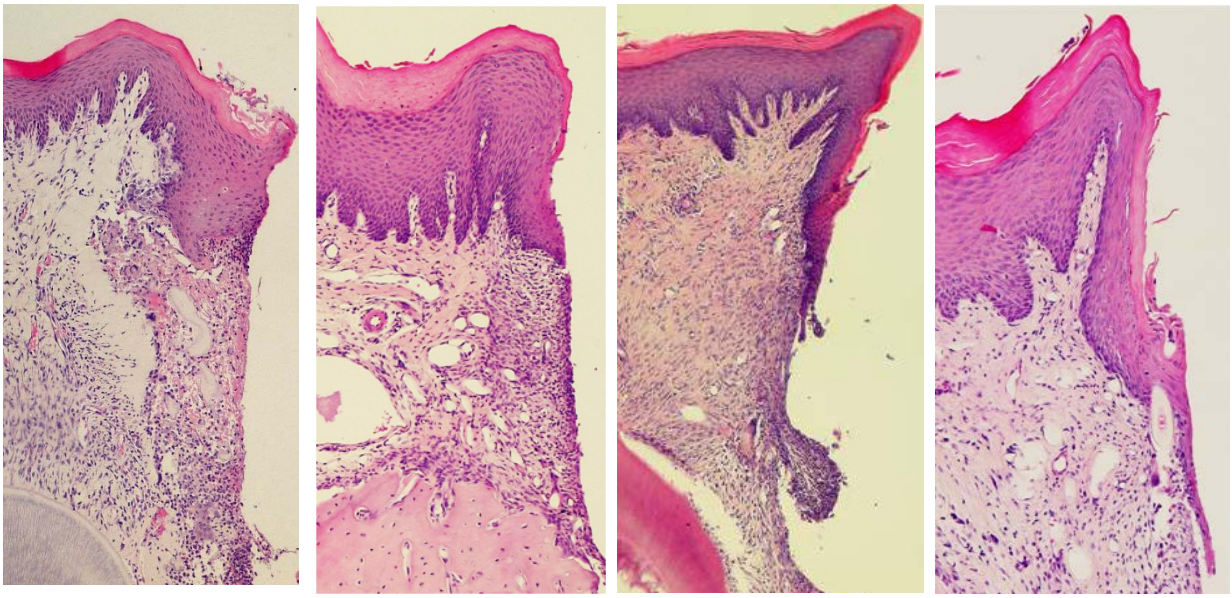

Positive area of
involucrin
Oral epithelium
B
A
Negative area of involucrin

Fig. 2



3 days 7 days 14 days 28 days

Fig. 3

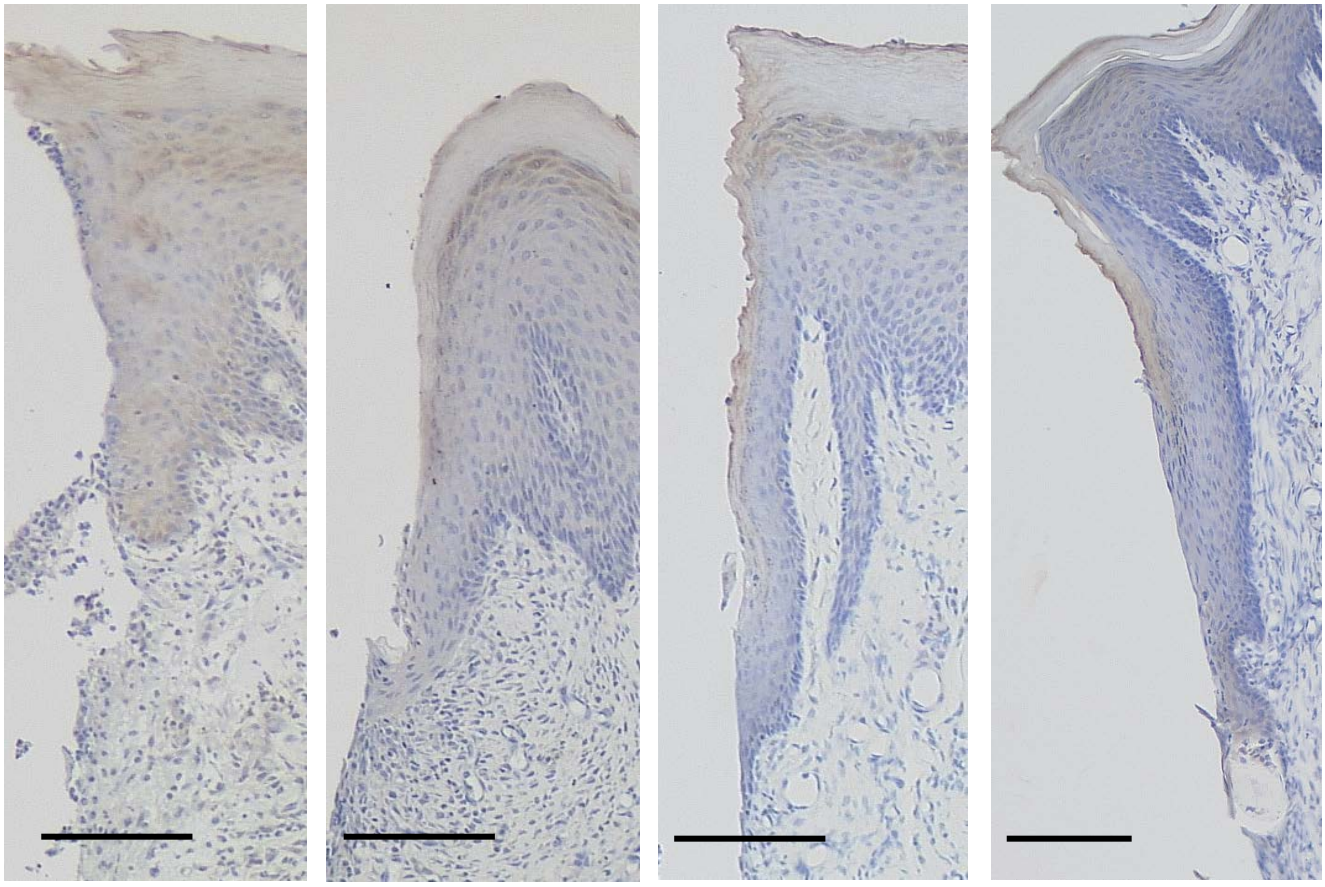
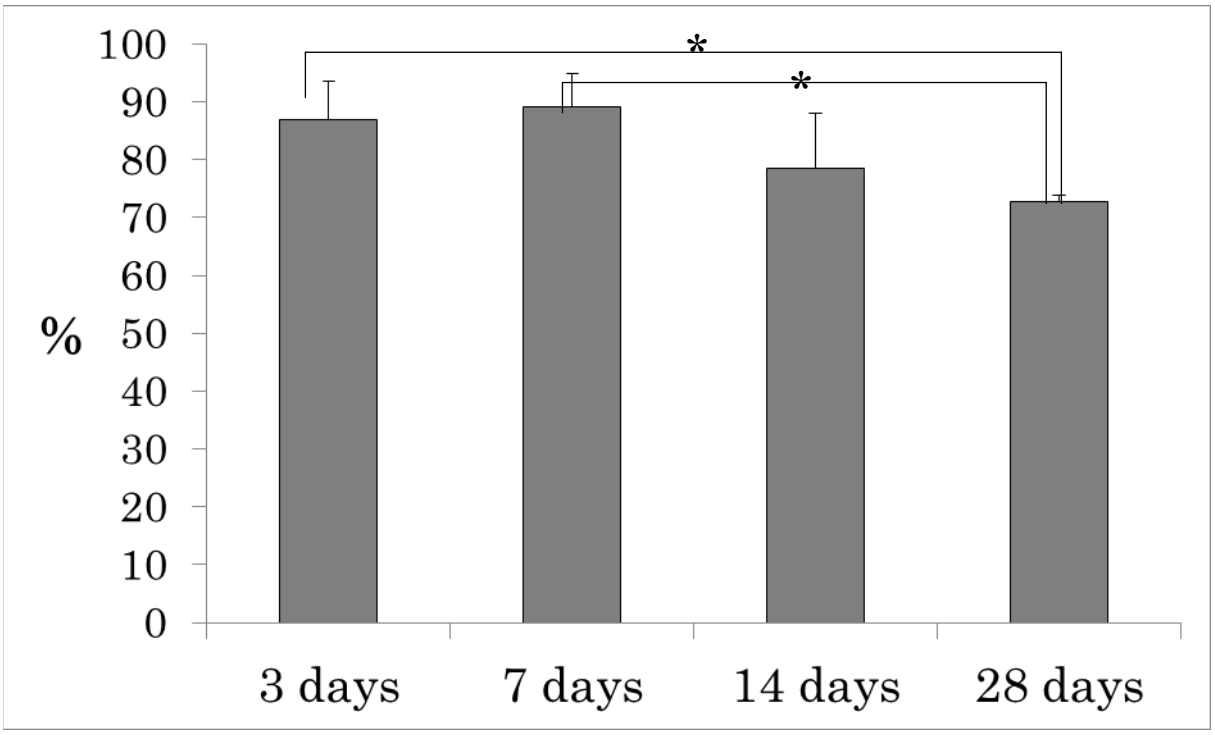


Fig. 4



*: significant difference (P<0.05)

Fig. 5

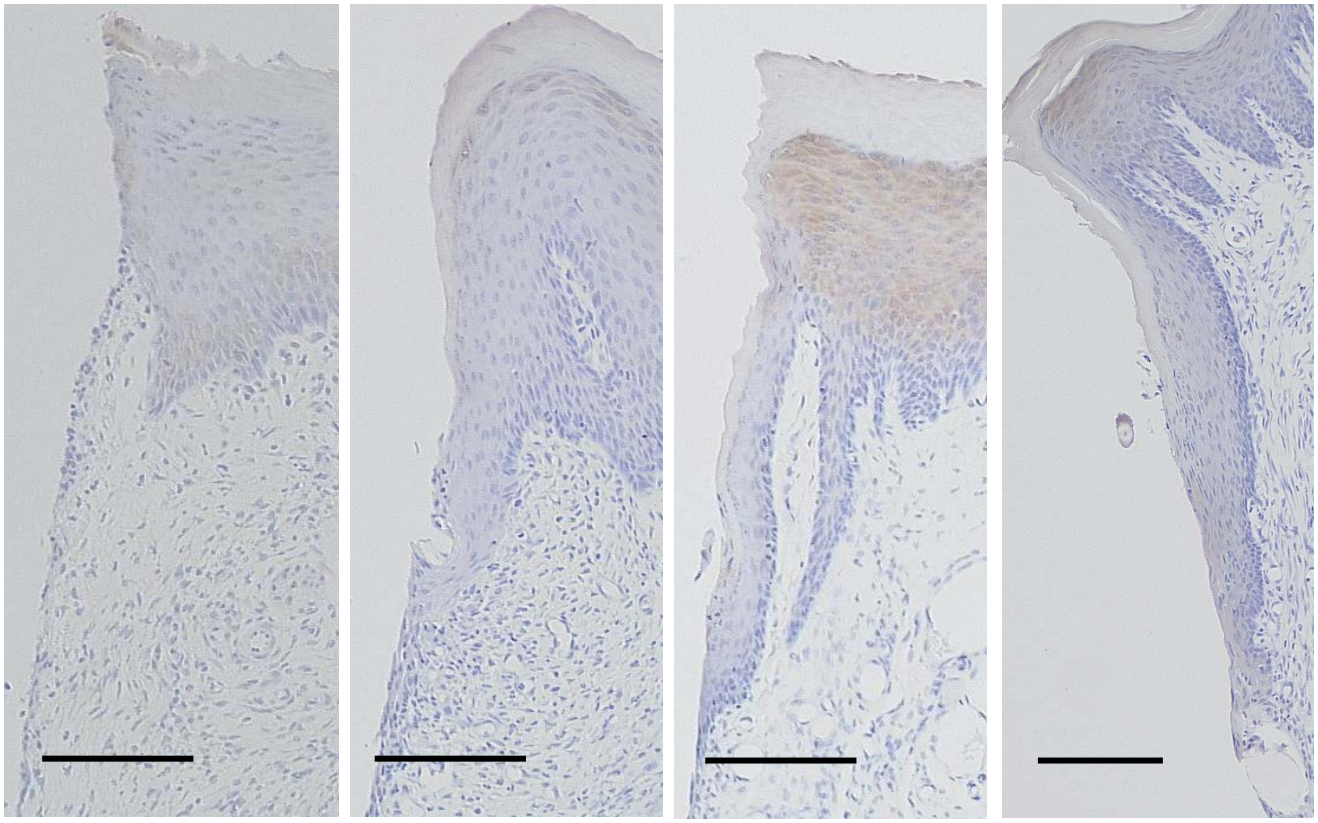


Fig. 6