Response of Merkel cells in the palatal rugae to the continuous mechanical stimulation by palatal plate.

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Abstract

(1) Objective: The aim of the present study was to investigate the behavior of Merkel cells which have been suggested to be mechanoreceptors that exist in extensive amounts in the palatal rugae, due to the continuous mechanical stimulation exerted by the palatal plate.

(2) Design: Forty golden 8-weeks-old hamsters were used in this experiment. The palatal plate was made of adhesive resin (4-META/MMA-TBB) and it was set on the palate of the animal for 1, 4 and 7 days. In order to achieve a continuous pressure over the mucosa underlying palatal plate, the palatal plates were pressed against the palatal mucosa and fixed to the posterior teeth using the same adhesive resin described above. To exert a continuous pressure, a 0.8 mm prominence on the internal surface of the palatal plate was created at the middle portion of the fourth palatal ruga. Thereafter, the number of Merkel cells in the mucosa was calculated by immunohistochemical observation. Furthermore, the electron microscopic features of Merkel cells were examined regarding the cell profile, continuity of the cell membrane, mitochondria, nerve tissues, the number of cell processes, neurosecretory granules in the cytoplasm and the nuclear-cytoplasmic (N/C) ratio. The data were analyzed using Bonferroni's multiple comparison procedure and Fisher's exact test. The level of significance was 0.05 in all analyses.

(3) Results: 1. There was significant deference among the control and any of the treated groups on the number of CK20 positive Merkel cells ($p<0.05$) and that numbers were decreased at the sites where continuous mechanical stimulation was exerted. 2. A degeneration of the cytoplasm mitochondria and nerve endings, and a decrease in both the number of neurosecretary granules and cell processes were observed. Furthermore, the presences of nuclear chromatin aggregation and fragmentation were recognized.

(4) Conclusion: The continuous mechanical stimulation by the palatal plate affected the behavior of Merkel cells and nerve endings, thus inducing a decrease in the number of Merkel cells. A portion of these changes were also associated with the expression of apoptosis.

I. INTRODUCTION

It is common practice to select removable dentures for the prosthodontic treatment of multiple missing teeth. However, the insertion of such a denture base sometimes gives formation of depression on the mucous membrane and/or reduction of palatal rugae. Many studies have explored the histologic effects on underlying soft tissues, demonstrating deformed epithelial ridges of the mucosa[1,2], degeneration of epithelial cells[1], changes in fibers of the connective tissues[3] and alveolar bone resorption[4-7]. Nevertheless, effects on the neurosensory system are still an open question.

The palate is known as an extremely sensitive region in the oral mucosa and is involved, along with the tongue, in food ingestion and mastication[8-10]. In the palatal mucosa, a variety of mechanoreceptors are distributed site-specifically, such as free nerve endings, simple corpuscles, Ruffini corpuscles, and Merkel cell-neurite complexes[11-13]. In particular, Merkel cell-neurite complexes are known to be densely distributed in the epithelial ridges of the palatal mucosa (palatal rugae)[14-17].

Merkel cells were found by F.S. Merkel in 1875 as chromophobic cells which occur together with Langerhans cells and melanocytes in human epithelium[18]; they are characterized morphologically by polymorphic profiles, cellular processes and intracytoplasmic special granules. One popular theory is that Merkel cells, in terms of physiological function, are slowly adapting type I mechanoreceptors[19, 20], but this is yet to be verified.

In prosthodontic treatment using a removable denture, the palate has to be covered with a denture base. Thus there is a possibility that sensory receptors mediated by such Merkel cell-neurite complexes in the palate may be affected either functionally or morphologically.

The purpose of this study was to investigate the behavior of Merkel cell-neurite complexes following continuous mechanical stimulation by a denture base inserted over the palate of hamsters, using immunohistochemistry, fluorescent microscopy and electron microscopy.

II. MATERIALS AND METHODS

1. Animals

All experiments were performed in compliance with the Tokyo Dental College Animal Experimental Guidelines. Forty male golden hamsters, 8 weeks old and weighing about 80 g, were used in this study. The hamsters were fed cow's milk *ad libitum*.

2. Preparation of the Palatal Plate

The method for preparation of the experimental palatal plate was as follows. Each animal was anesthetized with intraperitoneal pentobarbital (50 mg/kg i.p.; Nembutal, Abbott, USA), and a precision nonpressure impression of the upper jaw was taken using vinylsilicon impression material (EXAFINE INJECTION; G. C. Inc., Tokyo, Japan), after which a cast was made using ultrahard plaster (NEW FUJIROCK; G. C. Inc., Tokyo, Japan). For mechanical stimulation, a hemispherical indentation about 0.8 mm in depth was made at the center of the left transverse palatine fourth ruga of the cast using a round bur 1.0 mm in diameter. Palatal plates were made by 4-META/MMA-TBB adhesive resin (Super-Bond C & B; SUN MEDICAL Co., Shiga, Japan) on the casts, and covered from the area of the incisor to the distal region of the last posterior molar. Each palatal plate was set on the palatine mucosal surface and was fixed to the right and left molars using the same adhesive resin (Fig. 1). Animals were sacrificed by overdose of anesthetic solution 1, 4 and 7 days later. Hamsters without palatal plate insertion were used as

controls.

3. Observations

1) Immunohistochemical observations

For immunohistochemical observation, 6 animals at each of 1, 4, 7 days (and the controls) were sacrificed using pentobarbital sodium. The maxilla with palatal plate from each animal was removed and was fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.2) for 3 days at room temperature. After the fixation, the palatal plates were removed by mechanical means, then the tissues were decalcified with 10% EDTA, pH 7.2 for 10 days at room temperature. The specimens were then dehydrated through a graded series of ethanol before being embedded in paraffin. Sagittal paraffin sections, approximately 5 μm in thickness, were cut and deparaffinized sections were immersed in methanol containing 0.3% H_2O_2 for 30 min in order to block intrinsic peroxidase activity. The sections were preincubated with 0.01% citric acid for 20 min at 60°C. These sections were then incubated overnight at 4°C in 10% normal goat serum in a 1:30 dilution of monoclonal antibody against cytokeratin 20 (CK20, Dako, Denmark), which detects Merkel cells. All reaction products were developed by the streptavidin-biotin method using a commercially available SAB kit (HISTFINE SAB-PO, Nichirei, Japan). The presence of peroxidase was visualized after diaminobenzidine (DAB) reaction. Finally, the samples were counterstained with hematoxylin. Six serial sections were cut at 0.1 mm intervals from the center of the fourth palatine ruga.

2) Fluorescence microscopy

Merkel cells were marked by intraperitoneal injections of quinacrine (10-15 mg/kg) into the

palatal mucosa 12 to 24 hr before sacrifice of the animals. Two animals were sacrificed at each of the time periods using sodium pentobarbitone anesthesia (50 mg/kg i.p.) and the palatal mucosa were isolated. To facilitate separation of the epidermis from the dermis, the excised mucosa was treated in Krebs solution containing 3 mg/ml $_{DL}$-dithiothreitol at 31°C for 10 min. Using a dissecting microscope, the epidermal layer where Merkel cells are located was mechanically separated. Quinacrine fluorescent cells were identified as Merkel cells using a fluorescence microscope, according to the methods of Yamashita *et al.*[21] and Chan *et al.*[22].

3) Electron microscopy

For electron microscopic observation, 2 animals were sacrificed at each of the time periods and fixed by intracardiac perfusion with Karnovsky's fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde dissolved in 0.24 M Sorensen's phosphate buffer). The maxilla with palatal plate was removed from each animal and was immersed in the same solution for 3 days. The tissues were decalcified in 10% EDTA for 10 days at room temperature, then the palatal plates were removed by mechanical means. The tissues were post-fixed with 2% osmium tetroxide solution for 2 hr at 4°C. The tissues were then dehydrated through a graded series of ethanol before being embedded in epoxy resin (Epon 812, TAAB, England). Ultrathin sections, approximately 70 to 80 nm thick, were cut using an ultramicrotome (Reichert Ultracut UCT, Leica, Vienna, Austria) and were then stained with uranyl acetate and lead citrate. These sections were then observed using a transmission electron microscope (H-7100, Hitachi, Japan).

4. Analytical Procedures

1) Rate of Merkel cells

The slides immunostained with the anti-CK20 antibody were microphotographed at a magnification of 50× with an AxioPhoto microscope (Carl Zeiss, Switzerland), and were then further magnified 4-times on a computer. On the computer-magnified photomicrograph, a tangent line was drawn which crossed the anterior lowest point (A) and the posterior lowest point (B) of the basal cell layer of stratified squamous epithelium in the ruga (Fig. 2). The rate of Merkel cells was calculated by dividing the number of CK20-positive cells by the total number of basal cells in the area [i.e., (No. of CK20-positive cells/total No. of basal cells)× 100].

For statistical analysis, one way ANOVA was used to assess whether significant inter group was noted or not, after which the data were analyzed using the Bonferroni multiple comparison test. The level of significance was 0.05 in these tests.

2) Morphology of Merkel cells

Electron microscopic features of Merkel cells were examined with respect to cell profile, continuity of cell membrane, mitochondria and nerve tissues. For such analysis, 20 Merkel cells were selected from each group. For statistical analysis, Fisher's exact test was used.

In addition, the number of cell processes, neurosecretory granules in the cytoplasm and the nuclear-cytoplasmic (N/C) ratio were determined. For statistical analysis, one way ANOVA was used to assess whether significant inter group differences were noted or not, after which the data were analyzed using Bonferroni's multiple comparison procedure. The level of significance was 0.05 in these tests.

III. RESULTS

1. Macroscopic Observations

Although during the experiment, cleansing of the palatal plate or intraoral cleaning were not carried out, no food residues were found between the palatal plate and the palatal mucosa in any of the animals examined. A hemispheric indentation measuring about 0.5 mm in diameter was observed in the palate of all animals due to the prominence created on the internal surface of the palatal plate.

2. Immunohistochemical Findings

1) Control group

The palatal ruga was comprised of keratinous, stratified squamous cell epithelium and lamina propria mucosae, where the apical epithelium of the ruga showed markedly protruding ridges toward the lamina propria mucosa, in contrast to the ruga trough epithelium.

CK20-positive cells were observed among the basal cells and were more frequently distributed in the basal cell layer of apical epithelial ridges of the ruga (Fig. 3a). The rate of Merkel cells in the basal cell population was 23.2 $\pm$ 2.1% (Fig. 4).

2) Day 1 after the palatal plate setting

Interpapillary epithelial ridges in the depressed apical portion of the palatal ruga were smaller, than seen in the control group. The stratum corneum, the stratum granulosum and the stratum spinosum showed no appreciable change. No morphologic alteration was observed in the subepithelial connective tissue or vascular tissues. There was no evidence of inflammatory cell infiltration.

CK20-positive cells were densely distributed at the apex of epithelial ridges in the apical regions of ruga; hence the same distribution as in the controls (Fig. 3b). The Merkel cell ratio of the basal cell population was 8.5 ± 1.0%, thus there was significant difference compared with the control (Fig. 4).

3) Day 4 after the palatal plate setting

Depressions due to the overlying palatal plate were noted in regions of the ruga apices as seen 1 day after insertion of the palatal base and the epithelial ridges inferior to the apices were contracted. No morphologic alteration was observed in the subepithelial connective tissue or vascular tissues. There was no evidence of inflammatory cell infiltration.

Distribution of CK20-positive cells was confined to the tips of epithelial ridges in the peak regions of the ruga. The Merkel cell ratio of the basal cell population was 4.6±0.5%, again, there was significant difference compared with the control group (Fig. 4).

4) Day 7 after the palatal plate setting

Depressions were still evident in the regions of ruga apices as seen 1 and 4 days after insertion of the palatal plate, but were lesser in degree than those seen on Day 4. Epithelial ridges were contracted while there were no morphologic alterations in the subepithelial connective tissue or vascular tissues. There was no evidence of inflammatory cell infiltration.

Sparsely distributed CK20-positive cells were observed in the peak regions of the ruga. The Merkel cell ratio of the basal cell population was 4.1 ± 0.2%, here again there was significant difference compared with the control (Fig. 4).

3. Fluorescent Microscopic Findings

1) Control group

Fluorescent photomicrographs of the palatal rugae from the control animals demonstrated a zonal alignment of Merkel cells from the ruga apex (arrows) to the anterior trough and from the ruga apex to the posterior trough (Fig. 5a). The Merkel cell distribution close to the apex was high in density and diminished progressively toward the trough. However, Merkel cells were rarely observed at the apex.

2) Day 1 after the of palatal plate setting

Merkel cells were oriented irregularly and were decreased at the site of the palatal ruga that was depressed by the palatal plate prominence (arrows). In non-depressed, mesial areas of the ruga however, findings were comparable with those in the controls (Fig. 5b).

3) Day 4 after the palatal plate setting

Irregularly oriented Merkel cells, which were significantly decreased in number, were noted at the site of each depression (arrows) (Fig. 5c).

4) Day 7 after the palatal plate setting

There was a markedly irregular orientation of Merkel cells, with a conspicuous decrease in number, at the site of each depression (arrows) (Fig. 5d).

4. Electron Microscopic Findings

1) Control group

Merkel cells were situated within the basal cell layer and could be identified as electron-lucent, clear cells distinct from the surrounding keratinocytes. The cells showed desmosomal adherence to adjacent keratinocytes, with their cytoplasmic processes projecting toward the keratinocytes. Merkel cells were round or oval in profile and were observed to form Merkel cell-neurite complexes. In the cytoplasm, there were many Merkel granules about 100-150 nm in diameter which tended to aggregate in the regions synaptic with nerve fibers and in those adjoining the basement membrane. Mitochondria, rough endoplasmic reticulum and fiber bundles consisting of intermediate filaments were also observed. Most of the nuclei had profiles similar to those of the cells, but occasionally the nuclei were constricted or deeply notched, presenting complicated features. An abundance of mitochondria was noted in the presynaptic nerve endings (Fig. 6).

2) Day 1 after the palatal plate setting

Merkel cells were situated within the basal cell layer and mostly had oval profiles. Also noted were cell processes and desmosomes to adjacent keratinocytes. In the cytoplasm of Merkel cells, Merkel granules, mitochondria, rough endoplasmic reticulum and fiber bundles consisting of intermediate filaments were observed. The Merkel cells had nuclear profiles similar to those of the cells and showed no changes. Neurons synaptic to Merkel cells were noted to be vacuolated.

3) Day 4 after the palatal plate setting

Merkel cells had fewer cell processes compared with the controls, and showed cytoplasmic vacuoles, disappearance of mitochondrial cristae, and nuclear separation from the cytoplasm. Adjoining neurons exhibited degenerative changes such as marked swelling or vacuolation (Fig. 7).

4) Day 7 after the palatal plate setting

Merkel cells had non-oval cellular and nuclear profiles. Their cell membranes were discontinuous in places, and boundaries between the cytoplasm and nucleus were indiscrete. Merkel granules were present but were diminished in number. Adjoining neurons displayed pronounced degenerative changes as seen 4-days after treatment (Fig. 8).

Nuclear chromatin aggregation and fragmentation were also noted in other Merkel cells from animals sacrificed 7 days after the palatal plate application. The cytoplasm contained fewer secretory granules and was vacuolated (Fig. 9,10).

5) Morphological changes

The rate of cells with polygonal profiles was 5% in the control group, 10% in the 1-day group, 25% in the 4-day group, and 35% in the 7-day group. The cell membranes were continuous in all cells of the controls, and the incidence of cells with membrane discontinuity was 5% in the 1-day group, 10% in the 4-day group, and 10% in the 7-day group. Vacuolation or other degenerative changes in mitochondrial cristae were not seen in the control group, but vacuolated cells were frequent at 1 day (35%), 4 days (45%) and 7 days (85%); the number of vacuolated cells increased with the duration of palatal plate application.

In the control group, adjoining nerve fibers were observed in 50% of the Merkel cells. In the 1-day group this percentage was 55%, almost the same as the control group. However, this percentage then progressively reduced to 25% at 4 days and 20% at 7 days. Moreover, in the control group, degenerative changes of the adjoining nerve fibers were not observed, while in the experimental groups, the incidence of changes was 82% at 1 day, 100% at 4 days and 75% at 7 days. The average number of cell processes was 1.4 ± 1.1 for the 1-day group, which was similar to that of the control group, 1.9 ± 1.3. However, there was significant difference between 4 days group (0.8 ± 1.1) and 7 days group (0.6 ± 0.8) (Fig. 11). The number of secretory granules was 67.8 ± 32.5 for the 1-day group, which was comparable to that seen in the control group, 82.9 ± 29.1. However, there was significant difference between 4 days group (52.0 ± 24.3) and 7 days group (36.4 ± 20.4) (Fig. 12). There were no significant differences in the N/C ratio of Merkel cells among the control and any of the treated groups, viz., 51.6 ± 16.9% for the control group, 45.6 ± 22.6% for the 1-day group, 39.9 ± 25.7% for the 4-day group, and 45.5 ± 22.5% for the 7-day group.

IV. DISCUSSION

1. Experimental Procedures

The experimental palatal plate used in this study was prepared from 4-META/TBB-MMA and it is necessary to consider the toxicity to the oral epithelium. However, the palatal plate used had been molded by polymerization on a cast, and Inoue[23] reported that the components of a 4-META/TBB-MMA resin infiltrate the superficial horny layer but no degeneration of cells

occurred. This suggests that the 4-META/TBB-MMA resin has no appreciable chemical effect on these tissues.

The size of prominence on the internal surface of the palatal plate; 0.8mm in depth for continuous stimulation was selected by preliminary experiment (unpublished data).

The fourth ruga of the palate was selected for this study was based on our previous report that Merkel cells are most frequently distributed in the fourth ruga of the hamster palate[24].

2. Decrease of Merkel cells

It is known that there are two types of Merkel cells; round type which possessed adjoining neuron and dendric type which did not. Tachibana *et al.* reported that round type was mainly observed in the gerbil palatal rugae[25]. The results of the present study support those findings and these cells were located in the basal cell layer, particularly in the rete ridges. In this study, reduction of Merkel cells was seen in the experimental groups. Fluorescent microscopic observations revealed irregular alignments of Merkel cells with decreased numbers of these cells at each of the time periods. These findings are consistent with the light microscopic evidence of deformed, atrophied epithelial ridges noted in the experimental groups. Electron microscopic observations disclosed degeneration and decreases in the number of adjoining neurons, and furthermore, decreased secretory granules, mitochondrial degeneration and nuclear chromatin aggregation and fragmentation of Merkel cells were observed. However, no remarkable changes of other keratinocytes in the basal layer, in terms of morphology, were noted.

Kydd *et al.*[1] and Fleisch and Austin[2] reported that sustained mechanical stress posed by dentures on the palatal mucosa leads to contraction of epithelial ridges subjacent to the

pressure-loaded region. Kanabayashi reported that cells of the prickle cell layer and upper layers of the epithelium remained unchanged while cells of the basal layer were prone to atrophy in the pressure-loaded region of the epithelium of the palatal mucosa under an inserted experimental plate[26]. Ichihara also suggested that cells of the epithelial basal layer are more vulnerable to mechanical stress than are those of the overlying layers[27]. Inoue *et al.* reported that the internal pressure due to swelling in fibromas arising from the oral mucosa induced the disappearance of epithelial projections and reduced numbers of Merkel cells.[28] However, these mechanical pressures on the palatal mucosa did not induce any morphologic changes in the intra-corium connective tissue [29]. Taken together, continuous mechanical stimulation of the oral mucosa in this study converged on the basal cell layer, influenced morphologic changes of Merkel cells in the epithelial ridges, and led to Merkel cell death and a decrease in their number.

3. Death of Merkel cells

The death of cells can be roughly classify into apoptosis and/or necrosis, according to death mechanisms and morphologic characteristics. Apoptosis, generally recognized as spontaneous cellular death, represents biocybernatic functions such as morphogenesis and normal cell relay as well as biophylactic functions, such as removal of cancer cells and virus-infected cells. Apoptosis is characterized morphologically by a decrease in cell volume, a loss of cellular surface microvilli, an aggregation of chromatin and cell fragmentation. Necrosis, on the other hand, is a process of passive cellular disintegration due to excessive stimulation and is considered to be usually accompanied by inflammation.

In this study, degeneration of adjoining neurons, reduction of neurosecretory granules, nuclear chromatin aggregation and fragmentation and indiscrete boundaries between the

cytoplasm and the nuclei of Merkel cells were observed in the pressure-loaded region, but there was no evidence of inflammation in the surrounding tissues. It has been reported that diminution of secretory granules[30] and cell disappearance[31] in Merkel cells occurred after synapsing nerve fibers had been cut or removed. The results of the present study support those findings. Agar *et al.* reported that pressure gives rise to neuronal apoptosis *in vitro*[32], and Takeda *et al.*[33] have reported that taste buds present in the mouse circumvallate papillae undergo a slight apoptosis under normal conditions but the number of apoptotic cells increases 3 to 5 times following denervation. The present results suggest that sustained mechanical stimulation causes degeneration of adjoining neurons and consequent degeneration and apoptosis of Merkel cells, which results in a decrease in the number of those cells. We found the chromatin aggregation and fragmentation of nucleus which might is the sign of apoptosis. However, macrophages were not found in the intercellular spaces of the stratified squamous epithelium in this study, thus we speculate that these apoptotic Merkel cells might be undergone a prosess of epithelial metabolism and move to the surface.

Merkel cells have been postulated to be of neural crest origin[34] or to be of epithelial origin[35], however, no conclusion has been reached up to now. Merkel cells proliferate in the elongated rete ridges of the oral epithelium when inflammation occurs in the underlying connective tissue[28], while another report purports to demonstrate that Merkel cells are incapable of undergoing mitotic division and regeneration[36]. Thus, Merkel cells diminished in number due to excessive pressure stress by a inappropriate denture base may not be restored, and must change during oral functions such as the sensory system and swallowing.

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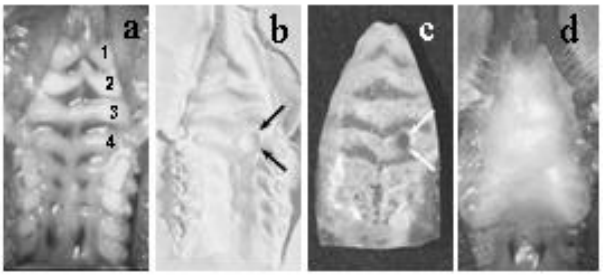
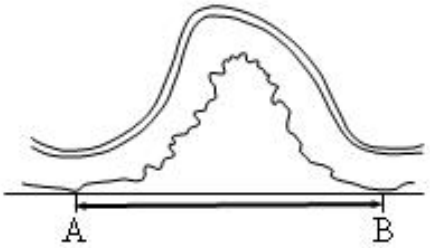


Fig. 1



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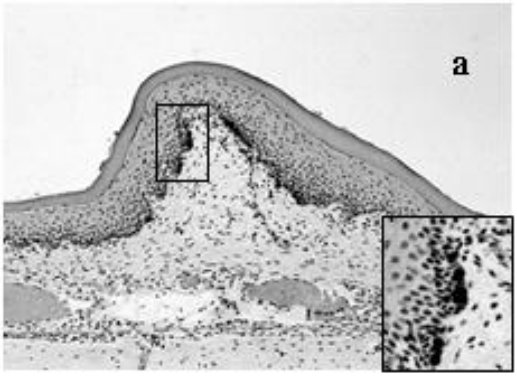


Fig. 3a

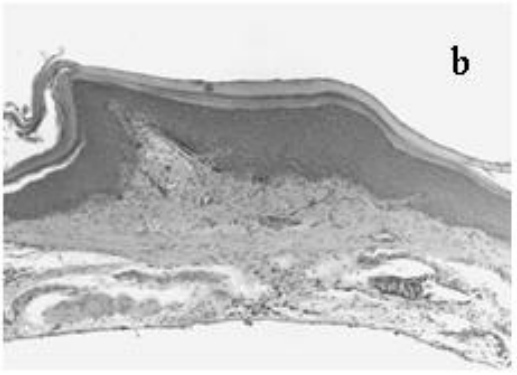
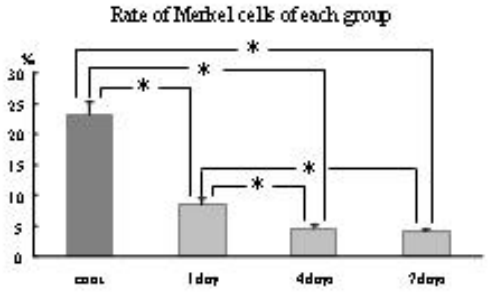


Fig. 3a


Rate of Merkel cells of each group
%
30
25
20
15
10
5
0
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1day
4days
7days

Fig. 4

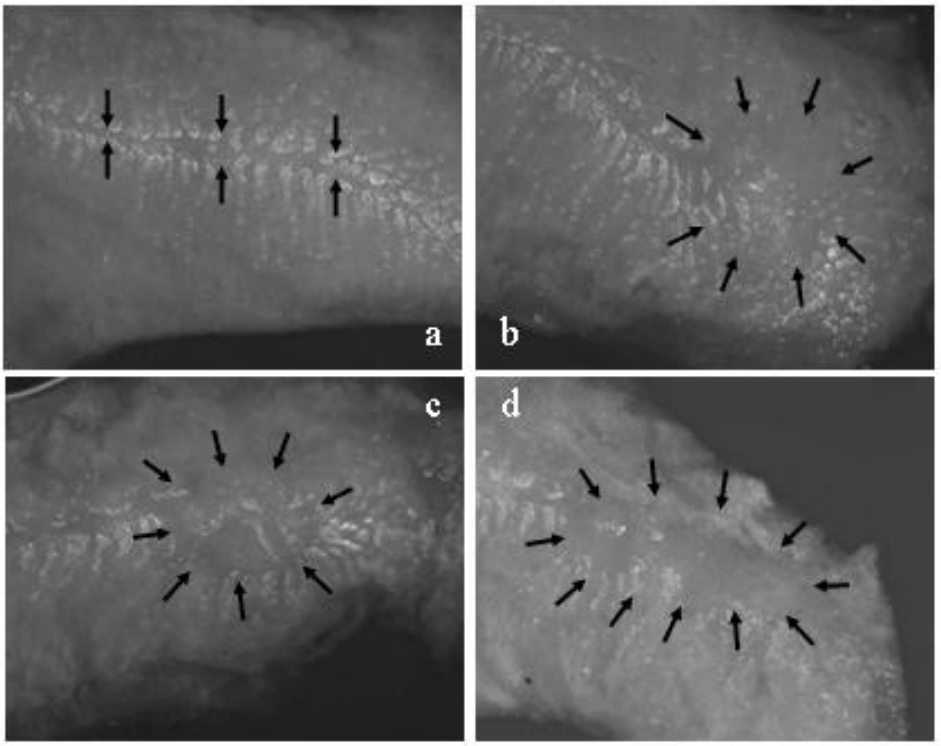
a
b
c
d

Fig.5

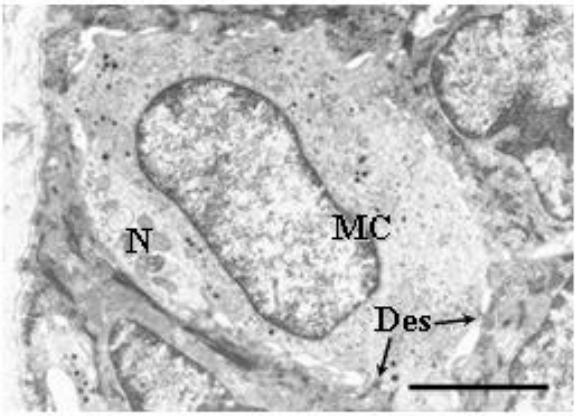

Fig. 6:

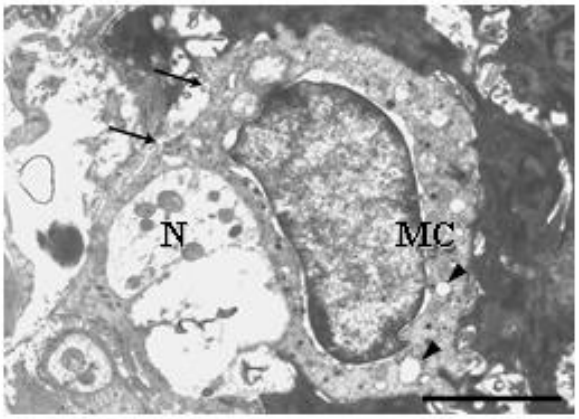

Fig. 7:

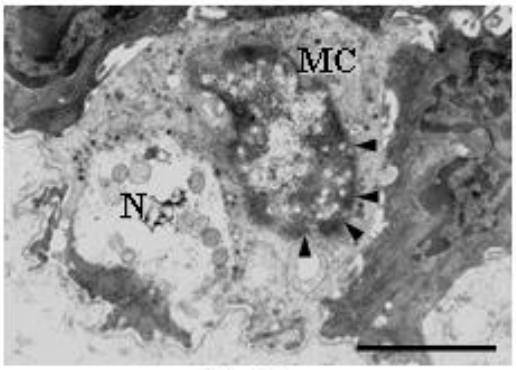

Fig. 8:

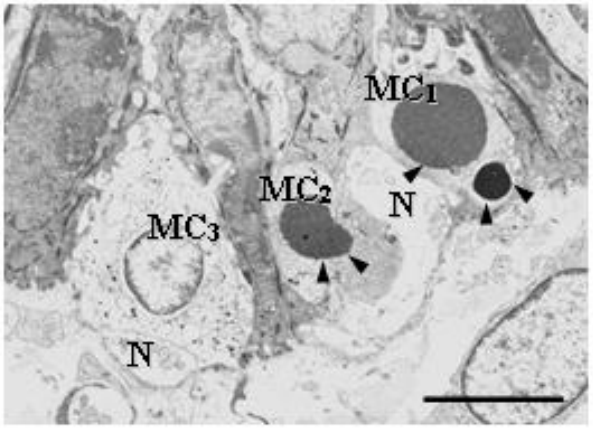

Fig. 9:

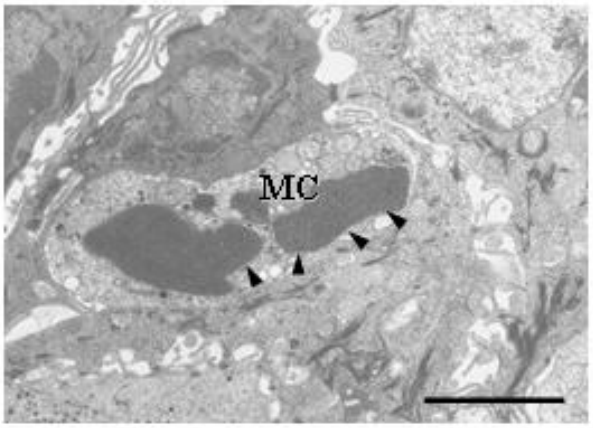

Fig. 10:

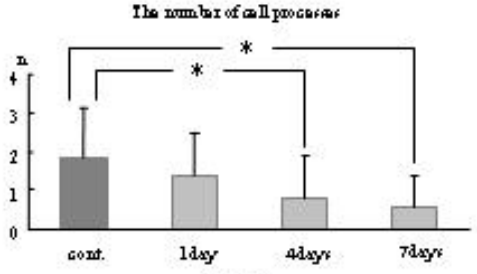

Fig.11

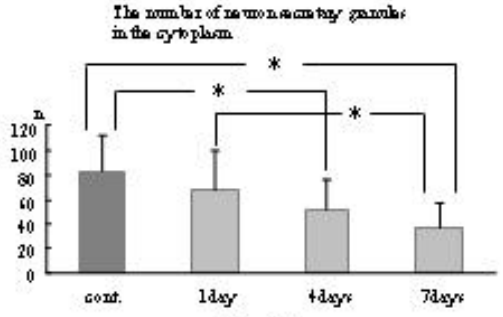

Fig.12