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Mitotic proliferation of myoepithelial cells during regeneration of atrophied rat submandibular glands after duct ligation

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BACKGROUND: The purpose of the present study was to elucidate whether myoepithelial cells proliferate mitotically during regeneration of rat submandibular glands after atrophy.
METHODS: The excretory duct of the right submandibular gland of rats was doubly ligated near the hilum with metal clips, which were removed after 7 days of ligation (day 0). The regenerating right submandibular glands were removed from 0 to 14 days after removal of the clips. The removed tissue was examined with immunohistochemical double staining for proliferating cell nuclear antigen (PCNA) as a marker of proliferating cells and actin as a marker of myoepithelial cells, as well as with transmission electron microscopy (TEM).
RESULTS: The PCNA-positive myoepithelial cells were observed at the periphery of transitional duct-acinar structures, ducts and acini in the regenerating glands at every time-point, and the PCNA-labeling index of myoepithelial cells increased greatly especially between day 2 and 4. The mitosis of myoepithelial cell was also identified by TEM at day 4.
CONCLUSION: These findings suggest that myoepithelial cells are able to proliferate mitotically during regeneration of rat submandibular gland.
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Keywords: mitosis; myoepithelial cell; proliferation; regeneration; submandibular gland

Introduction

Salivary gland tumors are known to vary histologically (1), and this is speculated to be due to myoepithelial cell participation in the histogenesis of several kinds of salivary gland tumors (2). This gives rise to an important problem concerning myoepithelial cells. If myoepithelial cells participate in the histogenesis of salivary gland tumors, they must be able to proliferate.

It had been considered that myoepithelial cells have extremely low or absent proliferative activity, because they are terminally differentiated cells (3). However, Sapino et al. (4) used immunohistochemical double staining for actin as a marker of myoepithelial cells and 5-bromo-2′-deoxyuridine (BrdU) as a marker of proliferating cells to demonstrate that there were double-positive cells in mammary glands under some experimental conditions. In salivary glands, Burgess et al. (5) examined atrophy and regeneration of rat parotid glands, using double immunohistochemistry for actin and proliferating cell nuclear antigen (PCNA) instead of BrdU, and observed many double-positive cells. Following this, double-positive cells were also identified in atrophic submandibular (6) and sublingual glands (7). These findings suggest that myoepithelial cells in salivary glands are able to proliferate, however, no mitotic figure of myoepithelial cell in salivary glands has been observed under pathologic conditions yet except in the development (8–10). To confirm that mitotic proliferation of myoepithelial cells takes place, ultrastructural observations of the mitotic figure of myoepithelial cells is necessary.

The proliferative activity of myoepithelial cells has been investigated in the histologically different atrophic parotid (5), submandibular (6), and sublingual glands (7). In these atrophic glands, proliferative myoepithelial cells were identified, but the PCNA-labeling indices of myoepithelial cells were different. This suggests that there are differences in the behavior of myoepithelial cells in histologically different salivary glands during atrophy. However, it is still unclear how different the proliferative activity of myoepithelial cells is in the different salivary glands during regeneration.

The purpose of the present study was to demonstrate the mitosis of myoepithelial cells by transmission

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electron microscopy (TEM), to clarify the proliferative activity of myoepithelial cells during regeneration of submandibular glands, and to compare it with that of parotid glands (5). To achieve this, we examined the regeneration of atrophied rat submandibular glands after duct-ligation, using double immunohistochemistry for actin and PCNA, and TEM.

Materials and methods

Experimental procedures

Eighty male Wistar rats aged 7 weeks, weighing 190–220 g, were used in the present study. In 72 experimental rats, the right submandibular gland and its excretory duct were exposed. Then the excretory duct of the right submandibular gland was doubly ligated with metal clips near the hilum of the gland to induce atrophy, and the clips were removed after 1 week of ligation. The experimental rats were anesthetized with inhalation of ether and the skin was sutured at both operations. The right submandibular glands were taken from experimental animals at 0, 1, 2, 3, 4, 5, 7, 10, or 14 days after removal of the clips. Eight control animals were not subjected to any of the operations.

All animal experimentation was approved by Animal Experimental Committee and followed the Guide for the Care and Use of Laboratory Animals of Hokkaido University Graduate School of Dental Medicine.

Immunohistochemistry

For immunohistochemistry, 36 experimental and four control rats were killed with deep inhalation of ether. The fresh submandibular glands were removed from four experimental animals at each time-point and four control animals and immediately frozen in liquid nitrogen. The 5 μm thick frozen sections were cut with a cryostat, air-dried, fixed in 4% paraformaldehyde for 2 min, and immersed in 0.3% hydrogen peroxide to inhibit endogenous peroxidase. The sections were in turn incubated with anti-PCNA mouse monoclonal antibody (PC-10; Dako Japan, Kyoto, Japan) as a marker for proliferating cells (11), biotinylated anti-mouse rabbit polyclonal antibody (Dako Japan), and streptavidin-biotin horseradish peroxidase (HRP) complex (Dako Japan). Then the immunoreaction of PCNA was visualized with 3-3′-diaminobenzidine. After the visualization, the sections were reacted with HRP-labeled anti-α smooth muscle actin (1A4; Dako Japan), a marker of myoepithelial cells (12, 13). The actin localization was developed with a VIP Substrate Kit (Vector Laboratories, Burlingame, CA, USA). In the immunohistochemical staining, the sections were rinsed thoroughly with phosphate-buffered saline after every incubation. Finally the immunostained sections were counter-stained with Mayer's hematoxylin. In this double immunohistochemistry, the PCNA-positive reaction was expressed as brown nuclei and the actin-positive reaction as purple cytoplasm.

Single actin immunohistochemistry was also performed omitting the part with PCNA from the above double staining. Normal mouse serum was substituted for the primary antibodies in the negative control sections.

After immunohistochemical double staining, the labeling index of PCNA in the myoepithelial cells were determined from the four experimental animals at each time-point and the four control animals as follows. Approximately 200 myoepithelial cells were counted at a magnification of ×400 (BH-2; Olympus, Tokyo, Japan) in randomly chosen fields, and the percentage of double-positive cells was calculated in each section. The mean of the percentages from five stained sections was used as the labeling index for that animal. Then the mean and SEM were calculated. These data were evaluated by one-way ANOVA, followed by the Fisher's protected least significant difference (PLSD) *post hoc* test, both with STATVIEW 4.5 statistical software (Abacus Concepts; Berkeley, CA, USA), and $P < 0.05$ was considered statistically significant.

Transmission electron microscopy

For TEM, other 36 experimental and four control rats were used. Four experimental rats at each time-point and four control rats were anesthetized using sodium pentobarbital given by intraperitoneal injection at a dose of 40 mg/kg. Then the animals were perfused with 2% paraformaldehyde, 1.25% glutaraldehyde from the ascending aorta through the heart for 15 min. After perfusion, the right submandibular glands were removed and immersed in the same fixative. The tissue was then post-fixed in 1% osmium tetraoxide and embedded in Epon 812. Ultrathin sections were made with a ultramicrotome, doubly stained with 4% uranyl acetate and lead citrate, and examined with a TEM (H-7000 electron microscope; Hitachi, Tokyo, Japan).

Results

One week of duct ligation led the submandibular glands to atrophy. Histologically, most acinar cells had disappeared, although many duct remained at day 0 (day of removal of clips). Transitional duct-acinar structures were identified at day 2 and the newly formed acini at day 3 after removal of the ligation. After 7 days, many newly formed acini were matured and the experimental glands were quite similar to the control (14).

Immunohistochemical observations

The single immunohistochemical staining for actin showed the myoepithelial cell distribution in the rat submandibular glands. At days 0 and 1, there were actin-positive myoepithelial cells around residual acini and ducts except at some larger ducts (Fig. 1a). The immature acini appearing after 3 days were also surrounded by myoepithelial cells (Fig. 1b). After 7 days, myoepithelial cells were observed at the periphery of acini and intercalated ducts, however, there were no myoepithelial cells around the striated ducts (Fig. 1c). This was the same distribution as in the control submandibular glands.

There were double-positive cells in the experimental glands at every time-point as well as in control glands.

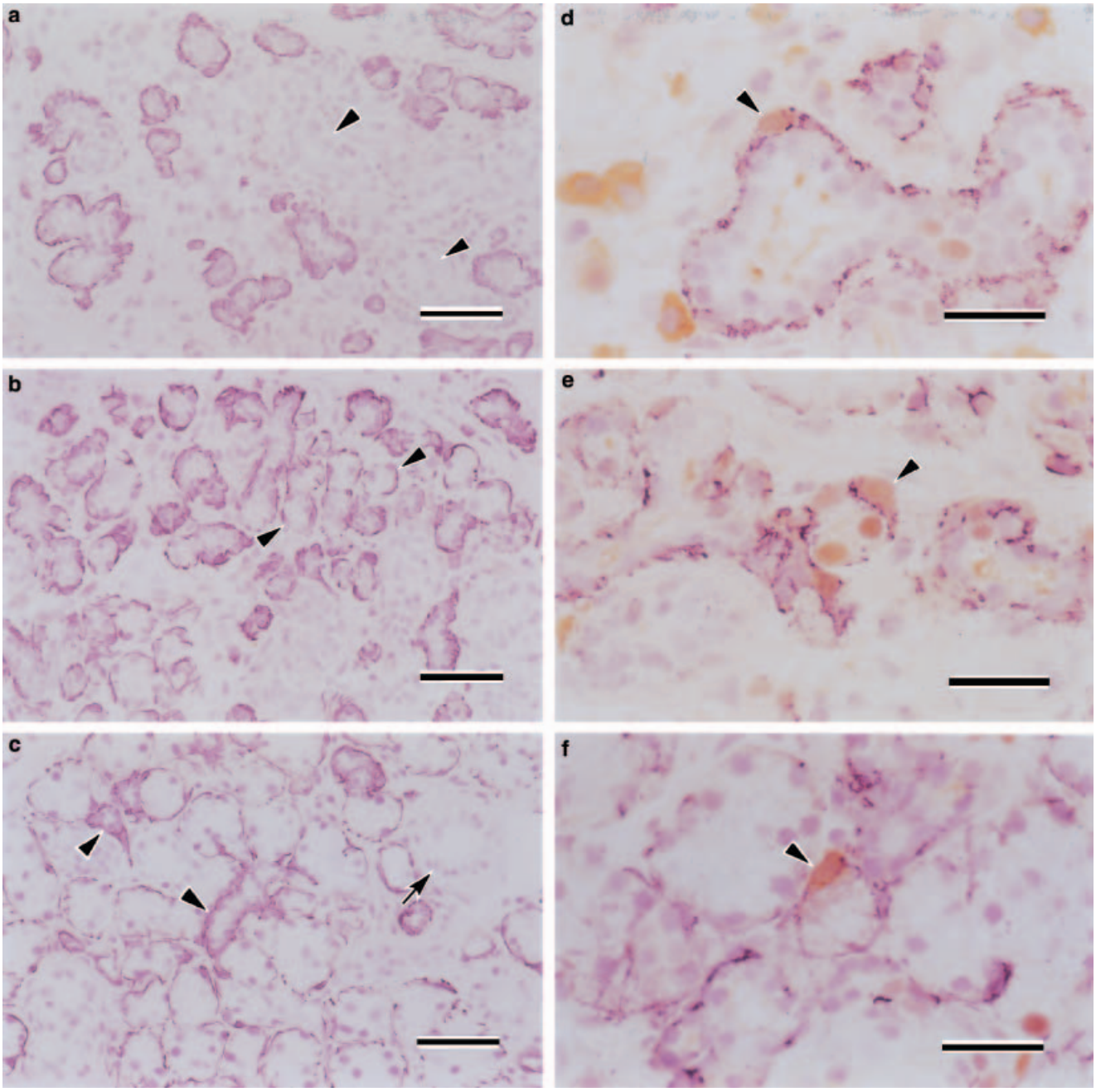

Figure 1 Single immunohistochemistry for actin (a–c) and double immunohistochemistry for proliferating cell nuclear antigen (PCNA) and actin (d–f). (a) Day 1: myoepithelial cells were seen around small ducts, but not large ones (arrowheads). (b) Day 3: immature acini were surrounded by myoepithelial cells (arrowheads). (c) Day 14: myoepithelial cells surround acini and intercalated ducts (arrowheads), but not striated ducts (arrow). (d–f) Double-positive cells (arrowheads) were observed around duct at day 4 (d), immature acinus at day 3 (e), and mature acinus at day 14 (f). Bars: a–c = 50 μm; d–f = 25 μm.

In the regenerating glands, there were double-positive cells at the periphery of ducts (Fig. 1d), immature (Fig. 1e) and mature acini (Fig. 1f). Negative control sections for single and double immunohistochemical staining showed no positive reaction.

The data of the PCNA-labeling index of the myoepithelial cells is demonstrated in Fig. 2. After removal of the clips, the index rose significantly between days 1 and 2 ($P = 0.015$). Between days 2 and 4, the index was high, over 4%, and it fell significantly from days 4 to 5 ($P = 0.002$). The maximum of the PCNA-labeling index was 6.31% at day 4 and the minimum was 0.91% at day 14. The index of the control rats was 1.18%.

TEM observations

At day 4, after removal of the ligation, mitotic cell was observed between the duct cell and basal lamina

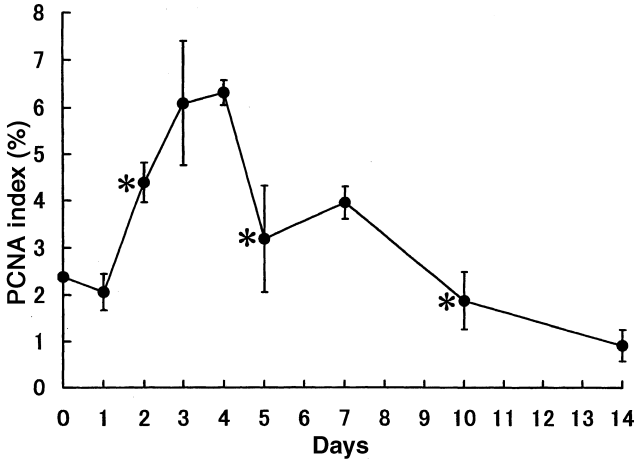

Figure 2 Labeling index of proliferating cell nuclear antigen (PCNA)-positive myoepithelial cells during regeneration of experimental submandibular glands. The results are expressed as mean ± SEM. The value of the control submandibular glands is 1.18 ± 0.13%. The values with asterisks are statistically significantly different from the adjacent previous time-point ($P < 0.05$).

(Fig. 3a). In the cytoplasm of this cell, there were microfilaments, some of which formed dense bodies, and caveolae were identified along the plasma membrane of this cell (Fig. 3b).

Discussion

In the present study, the excretory duct of the submandibular gland was ligated for 1 week to induce atrophy in this gland. This experimental method has been often used, and it is considered that this atrophy is caused by duct obstruction and damage to the corda tympani running on the excretory duct (15, 16).

Double-positive cells have been identified in previous studies examining salivary glands under physiologic and pathologic conditions with immunohistochemical double staining for actin and PCNA (5–7). However, no mitotic figures of myoepithelial cells have been identified ultrastructurally in such salivary glands. The reason for this may be that the M-phase is shorter than other phases, and that the number of myoepithelial cells is fewer than acinar and duct cells in salivary glands. The regenerating submandibular glands were thoroughly examined using TEM in the present study, and a mitotic cell, which was located between the duct cell and basement membrane, and which had microfilaments and caveolae was identified. This finding directly demonstrates that a mature myoepithelial cell is able to divide. Previously mitosis of immature (8, 9, 17) and mature (10) myoepithelial cells were observed only in developing salivary glands soon after birth. The present study is the first to identify the mitotic figure of mature myoepithelial cell in the submandibular gland of young adult rats. This suggests that myoepithelial cells proliferate mitotically in mature salivary glands under certain conditions at least in young adult rats and it supports the idea that myoepithelial cells as well as all other cell types should be considered potential progenitor cells for salivary gland tumors (5, 10).

Although PCNA has often been employed as a marker for proliferating cells, it has been pointed out

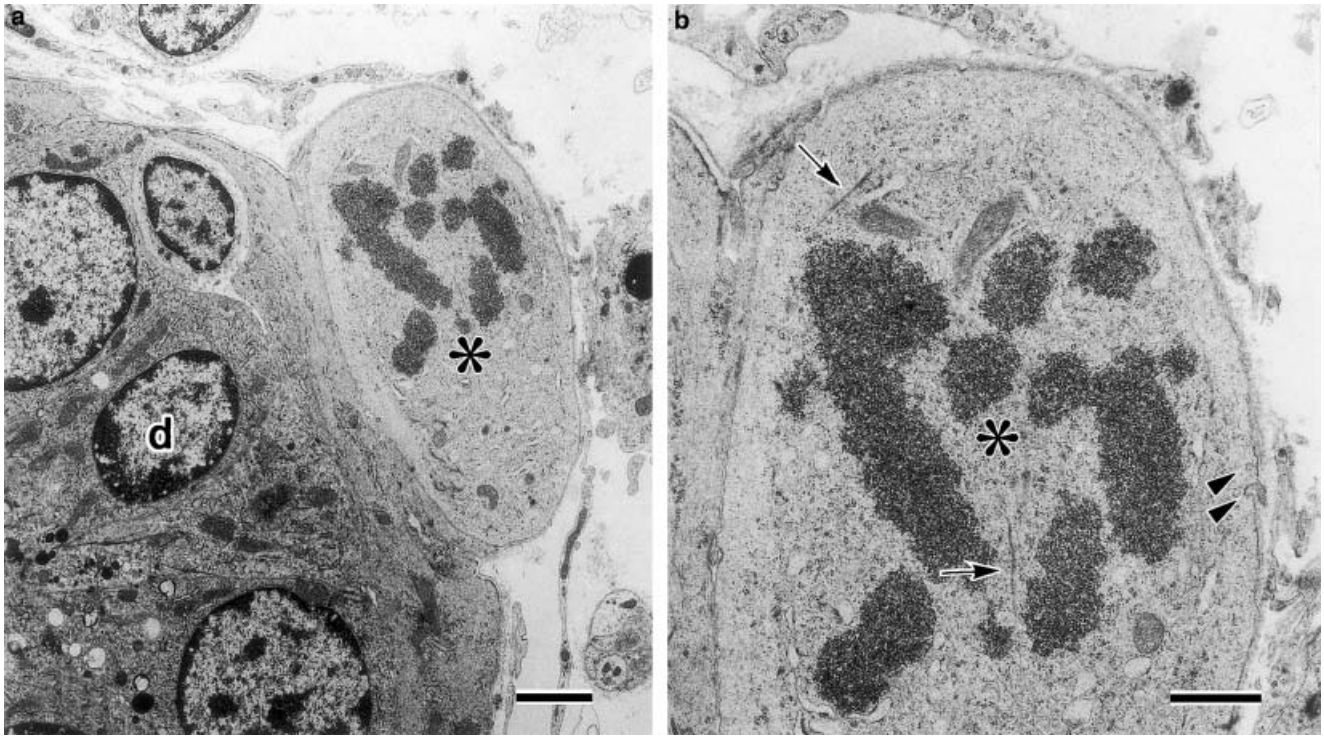

Figure 3 Transmission electron microscopy. (a) Day 4: mitotic cell (asterisk) between duct cell ('d') and basal lamina. (b) Higher magnification of mitotic cell (asterisk) in (a). Caveolae (arrowheads) and dense bodies (arrows) are observed. Bars: a = 2 μm; b = 1 μm.

that PCNA is also expressed in DNA repair (18). However, in atrophy and regeneration of salivary glands, it has been understood that DNA repair is not important and PCNA shows cell proliferation because of the observation of several mitotic figures (7, 19). In the present study, the labeling index of PCNA for myoepithelial cells was significantly high from 2 to 4 days and the maximum of the index was 6.31% at 4 days. Burgess et al. (5) reported that the peak PCNA-labeling index for myoepithelial cells was 3.1% and that there was no statistically significant difference between different time-points in the regeneration of atrophied parotid glands of rats after duct ligation. It is possible to consider that this disagreement with the present study is due to differences in the distribution of myoepithelial cells in both salivary glands. As myoepithelial cells do not surround the acini and were situated only at the periphery of intercalated ducts in the normal rat parotid glands (10, 20–22), the increase of myoepithelial cell numbers is not critically important in the regeneration of rat parotid glands. In rat submandibular glands, there were myoepithelial cells around the residual ducts and a few acini at the starting time-point of regeneration, and myoepithelial cells surround the intercalated ducts and many newly formed acini at the final time-point of the regeneration. Therefore, proliferation of myoepithelial cells is necessary as regeneration. This idea is also supported by the fact that the active proliferation phase of myoepithelial cells was almost coincident with that of acinar cells (14).

In conclusion, mitotic proliferation of myoepithelial cells during regeneration of rat submandibular glands was demonstrated by double immunohistochemistry and TEM, and it was shown that myoepithelial cells proliferate more actively during regeneration of rat submandibular gland than of parotid one.

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