J Oral Pathol Med (2004) 33: 23–9

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Cell death and cell proliferation in the regeneration of atrophied rat submandibular glands after duct ligation

S. Takahashi, K. Shinzato, S. Nakamura, T. Domon, T. Yamamoto, M. Wakita

Department of Oral Health Science, Hokkaido University Graduate School of Dental Medicine, Sapporo, Japan

BACKGROUND: The present study aimed to clarify the proliferation and apoptosis of parenchymal cells during regeneration of rat submandibular glands following atrophy.

METHODS: Atrophy of the right submandibular gland of rats was induced by excretory duct ligation at the hilum with metal clips, which were removed 1 week (day 0) after ligation. The right submandibular glands were collected from 0 to 14 days after removal of the clips and investigated using immunohistochemistry for proliferating cell nuclear antigen (PCNA) as a marker of proliferating cells, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) as a marker of apoptotic cells, and transmission electron microscopy (TEM).

RESULTS: After 1 week of ligation, there were many remaining ducts and a few acini in the atrophic glands. At day 3 after discontinuing the ligation, newly formed acini appeared and thereafter increased in number and maturity. Many residual and newly formed acinar cells showed positive reaction to PCNA especially at days 4 and 5. The PCNA-positive duct cells decreased in number with the regeneration. A few TUNEL-positive acinar and duct cells were identified during regeneration. Mitosis and apoptosis of parenchymal cells were also identified by TEM.

CONCLUSIONS: During regeneration of the submandibular gland after atrophy, both residual and newly formed acinar cells proliferate actively. There is also apoptosis of parenchymal cells; however, the significance of apoptosis is low.

J Oral Pathol Med (2004) **33**: 23–9

Keywords: apoptosis; proliferation; regeneration; submandibular gland

Correspondence: Dr Shigeru Takahashi, Department of Oral Health Science, Hokkaido University Graduate School of Dental Medicine, Kita 13, Nishi 7, Kita-ku, Sapporo, 060-8586, Japan. Tel./fax: +81 11 706 4225. E-mail: tshigeru@den.hokudai.ac.jp
Accepted for publication August 6, 2003

Introduction

Understanding of the regeneration of salivary glands after atrophy is clinically important and experimental investigations have shown that acinar cells, which disappear from atrophic salivary glands, re-differentiate from remaining duct cells (1–3). In addition, it has been reported that mitotic cell proliferation of the remaining acinar cells participates in increase of acini in the regenerative process of parotid glands where acinar cells remain (4, 5) and newly differentiated acinar cells proliferate actively in the regeneration of atrophic parotid glands without residual acinar cells (6). During regeneration of the submandibular gland which differs histologically from the parotid gland, proliferation has also been demonstrated (7), however, it is not fully understood how parenchymal cells proliferate.

Apoptosis is known to play an important role in many biological phenomena, complementary but opposite to mitotic proliferation in the regulation of cell populations (8). In salivary glands, acinar cells are removed by apoptosis in regressive changes such as experimental atrophy (9–13), chronic sialadenitis (14), and Sjögren syndrome (15). It has also been reported that apoptosis occurs in salivary gland development, which is a progressive change (16–19). The process of regeneration of salivary glands is considered to be similar to that of the development of salivary glands (20); however, the occurrence of apoptosis is unclear in regeneration of salivary glands. Apoptosis has been observed in regeneration of the liver (21, 22).

The present study was designed to determine how parenchymal cells proliferate and whether apoptosis of parenchymal cells occurs during regeneration of submandibular glands after atrophy. For this purpose, regeneration of duct-ligated atrophic rat submandibular glands was examined, using immunohistochemistry for proliferating cell nuclear antigen (PCNA), terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL), and transmission electron microscopy (TEM).

Materials and methods

Experimental procedures

Eighty male Wistar rats weighing 190–220 g were divided into experimental (72 rats) and control groups (8 rats). In the

experimental rats, the right submandibular gland and its excretory duct were exposed through a ventral incision in the neck. The right excretory duct was doubly ligated with metal clips near the hilum to induce atrophy, and the clips were removed after 7 days of duct ligation. Both operations were carried out under general anesthesia with ether, and the skin was closed with silk sutures. The animals were sacrificed at day 0, 1, 2, 3, 4, 5, 7, 10, or 14 after removal of the clips. The control rats were not operated. The body weight of rats was 220–250 g at day 0 and 270–330 g at day 14. Thirty-six experimental and four control animals were used for general histology and TEM. They were anesthetized using sodium pentobarbital given by intraperitoneal injection at a dose of 40 mg/kg and perfused with 2% paraformaldehyde−1.25% glutaraldehyde from the ascending aorta through the heart for 15 min. Here, the right submandibular glands were excised and placed in the above fixative. A small portion of each gland was set aside for TEM. After fixation, the tissue was routinely processed and embedded in paraffin. The sections were cut at 4 μm and stained with hematoxylin and eosin (H&E). Other 36 experimental and four control animals for immunohistochemistry and TUNEL were sacrificed with deep inhalation of ether. The fresh right submandibular glands were immediately removed and frozen in liquid nitrogen. Then, 5-μm thick frozen sections were prepared with a cryostat, air-dried, and fixed in 4% paraformaldehyde for 2 min. After rinsing with phosphate-buffered saline (PBS), the sections were immersed in 0.3% hydrogen peroxide to quench endogenous peroxidase activity and then made ready for immunohistochemical and TUNEL staining.

All animal experimentation followed the Guide for the Care and Use of Laboratory Animals, Hokkaido University Graduate School of Dental Medicine (Sapporo, Japan).

Immunohistochemistry

To examine the proliferating cells, the prepared sections were incubated with anti-PCNA mouse monoclonal antibody, PC-10 (DAKO JAPAN, Kyoto, Japan) (23). The secondary antibody was biotinylated anti-mouse rabbit polyclonal antibody (DAKO JAPAN, Kyoto, Japan). Then, the sections were reacted with streptavidin-biotin horse-radish peroxidase complex (DAKO JAPAN, Kyoto, Japan). After visualization of peroxidase activity by diaminobenzidine tetrahydrochloride (DAB), the sections were counterstained with hematoxylin.

Normal mouse serum was substituted for the primary antibody as the negative control.

TUNEL

Apoptotic cells were identified by the modified TUNEL method (24) using an Apop Tag Peroxidase In Situ Apoptosis Detection Kit (Intergen, New York, NY, USA). The prepared sections were incubated with TdT and dUTP-digoxigenin without proteinase K treatment. After rinsing with PBS, anti-digoxigenin-peroxidase sheep polyclonal antibody was applied and the reaction was visualized by DAB method, before the sections were counterstained with hematoxylin.

Negative control sections were incubated with distilled water in the absence of TdT.

Quantification

Five sections stained with PCNA and with TUNEL were randomly chosen for each animal. In each section, approximately 500 duct cells and 500 acinar cells were randomly counted at a magnification of ×200 (BH-2, OLYMPUS, Tokyo, Japan), and the percentages of PCNA- and TUNEL-positive cells in ducts and acini were calculated. The labeling indices of each animal were obtained by averaging the percentages of the five sections, and then the mean and standard error of the mean (SEM) were determined for four experimental animals at each time point and four control animals. Using StatView 4.5 statistical software (Abacus Concepts, Berkeley, CA, USA), the comparison between experimental data at each time point and control was made by one-way analysis of variance (ANOVA), followed by the Fisher's protected least significant difference (PLSD) post hoc test, with $P < 0.05$ as statistically significant.

Transmission electron microscopy

The tissue was post-fixed in 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. Then, the ultrathin sections were examined with a transmission electron microscope (H-7000 electron microscope, HITACHI, Tokyo, Japan).

Results

Histological observations

After 7 days of duct ligation (day of removal of clips, day 0), the ligated submandibular glands showed atrophy. Most acini had disappeared, but there were a few remaining small acini, although there were many residual ducts. The remaining parenchymal components were separated by increased amounts of connective tissue with inflammatory cell infiltration (Fig. 1A). At day 2 after the removal of the ligation, transitional duct-acinar structures appeared, and after day 2, these structures increased mainly at the peripheral region of lobules. The remaining acinar cells had recovered the original shapes. Newly formed acini comprising small immature acinar cells appeared from day 3 (Fig. 1B) and were common at day 5 (Fig. 1C). From day 7, many newly formed acini had matured and were indistinguishable from the original acini. Many ducts had also acquired the characteristics of intercalated or striated ducts. The connective tissue and inflammatory cell infiltration around acini and ducts had decreased (Fig. 1D).

PCNA and TUNEL observations

Some PCNA-positive cells were observed in both the ducts and acini of submandibular glands of control animals. In experimental glands, there were several PCNA-positive cells in ducts at day 0, but a few after day 2. There were few PCNA-positive cells in residual acini at day 0. Between days 2 and 7, there were many acinar cells showing positive reaction to PCNA (Fig. 2A), and thereafter PCNA-positive acinar cells decreased in number (Fig. 2B). TUNEL-positive cells were common (Fig. 2C) at day 0 – however, later there were a few TUNEL-positive cells (Fig. 2D) – in experimental and control animals. Negative control sections for PCNA and TUNEL showed no reaction.

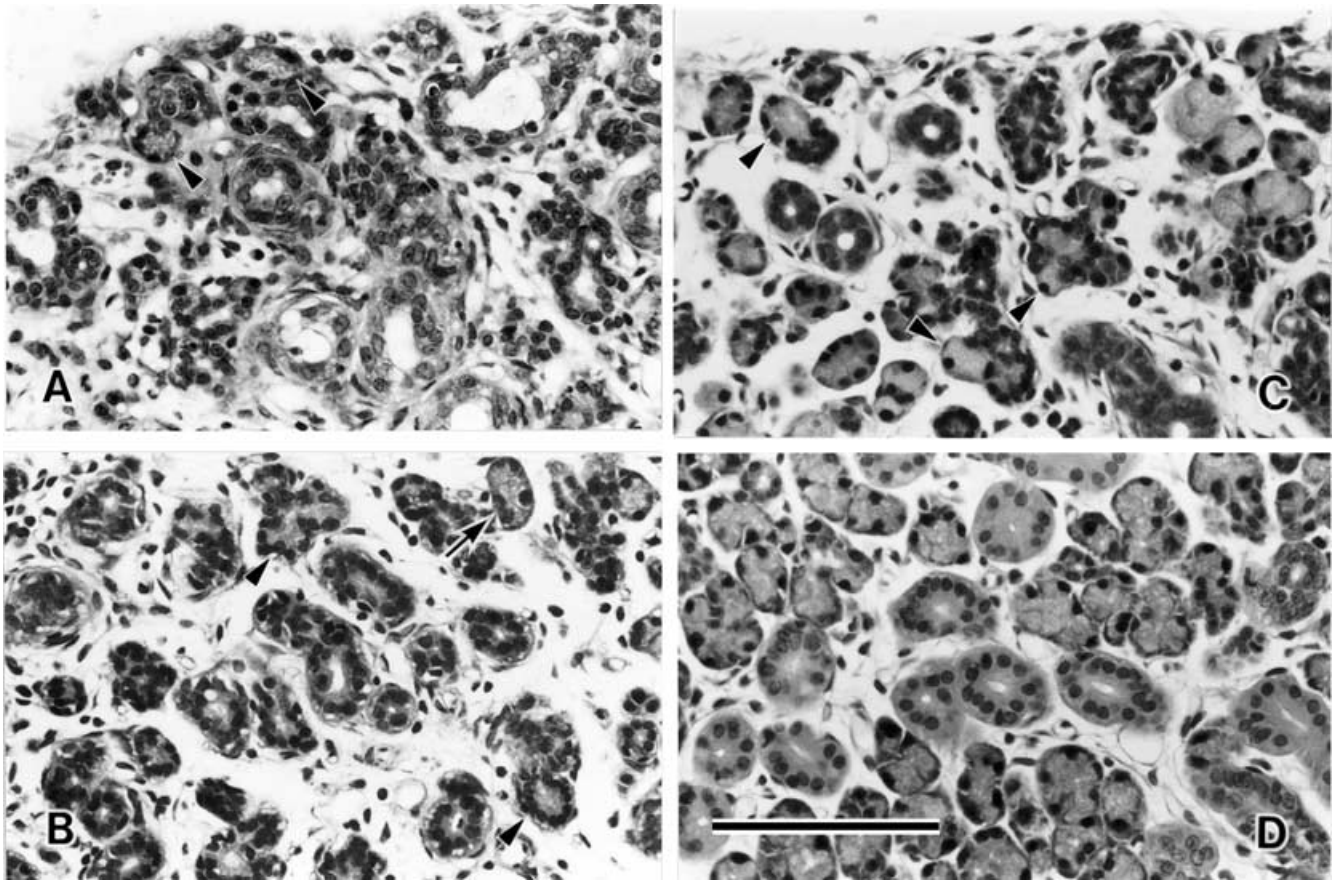

Figure 1 Histological micrographs (H&E). Bar = 100 μm. (A) Day 0, many ducts and a few acini (arrowheads) remaining in the atrophic submandibular gland. (B) Day 3, transitional duct-acinar structures (arrowheads) and immature acinus (arrow). (C) Day 5, immature acini (arrowheads). (D) Day 14, the experimental submandibular gland appearing similar to the normal.

The labeling indices of PCNA and TUNEL in the experimental glands are shown in Figs. 3 and 4, respectively. The PCNA index for acinar cells in experimental animals increased after the removal of ligation and was the highest, 21.9% at day 4, after which it declined. The PCNA indices for acinar cells from day 2 to day 10 were significantly different from that of the control (1.0%). The PCNA index for duct cells at day 0 was 8.3%, which declined thereafter. After day 2, the PCNA indices for duct cells were not significantly different from that of control (1.0%). The TUNEL indices for acinar and duct cells at day 0 were high, 5.9 and 4.9%, respectively. However, both TUNEL indices remained low after the removal of ligation and were not significantly different from those of the control (acinar cells, 0.07%; duct cells, 0.17%).

TEM observations

Mitotic figures were occasionally observed in the ducts from 0 to 7 days (Fig. 5A) and acini from 3 to 7 days (Fig. 5B) in the experimental submandibular glands. The chromosomes had condensed in the dividing cells. Some of the mitotic acinar cells contained secretory granules characterized by internal structures such as circular arrays, internal rods, or lamellar structures (Fig. 5B).

Apoptosis was rarely identified in duct epithelium during regeneration. Apoptotic bodies with characteristic nuclear fragments (Fig. 5C) were few in number, and other apoptotic bodies contained degraded nuclear fragments (Fig. 5D). There was no necrosis of duct and acinar cells during the regeneration of the glands.

Discussion

The ligation and release of the excretory duct of rat submandibular glands has frequently been used to investigate regeneration of atrophic submandibular glands because of convenient and reproducible method (1–3). In this model, the corda tympani would be included in the ligation of the excretory duct because it is on the excretory duct of the submandibular gland in rats. Therefore, it is understood that the atrophy of submandibular glands is induced by duct obstruction and damage to the corda tympani (25, 26).

Detection of proliferating cells commonly employs PCNA immunohistochemistry, as pre-treatment is unnecessary and species specificity is low. The PCNA expression is also identified in DNA repair (27); however, it has been suggested that DNA repair is not important in the atrophy and regeneration of salivary glands (4, 28), and mitotic figures were observed by TEM in the present study. Therefore, it is considered that PCNA is an appropriate marker for proliferating cells in this study.

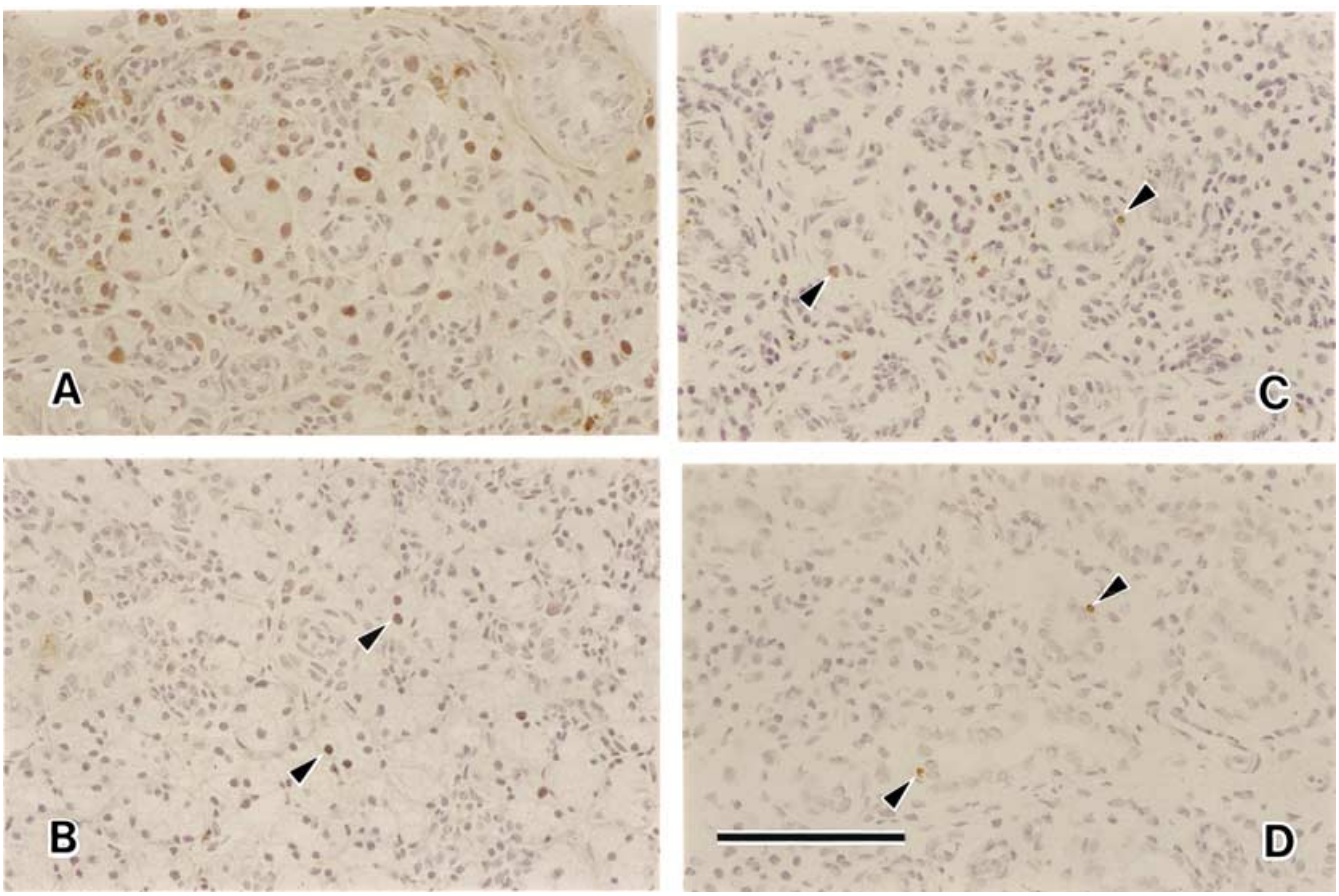

Figure 2 PCNA (A,B) and TUNEL (C,D) to show proliferating cells and apoptotic cells, respectively. Bar = 100 μm. (A) Day 5, many PCNA-positive acinar cells. (B) Day 14, a few PCNA-positive acinar cells (arrowheads). (C) Day 0, some TUNEL-positive duct cells (arrowheads). (D) Day 1, a few TUNEL-positive duct cells (arrowheads).

In the present study, the proliferative activity of acinar cells was high during the regeneration of the submandibular glands. This shows that acinar cell proliferation is significant for the glandular tissue regeneration. Two types of acinar cell proliferation are considered in this study. One is the proliferation of residual acinar cells, and as newly formed acinar cells appeared after day 3 of regeneration, at least the PCNA-positive acinar cells at days 1 and 2 are

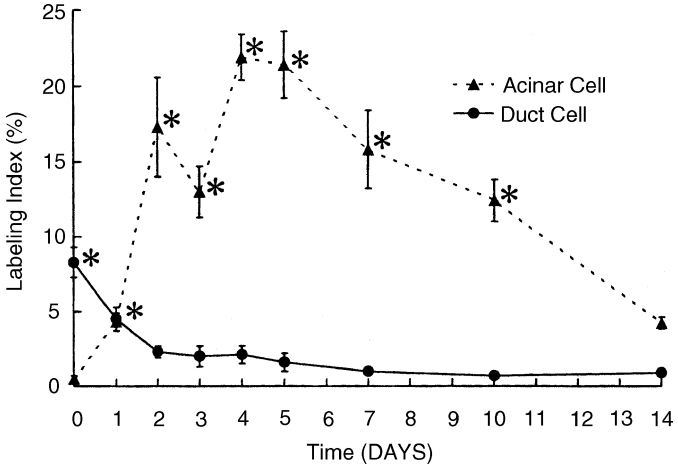

Figure 3 PCNA labeling indices of acinar (broken line) and duct (solid line) cells in experimental submandibular glands. The results are expressed as mean ± SEM. The values with asterisks are statistically significantly different from the control ($P < 0.05$).

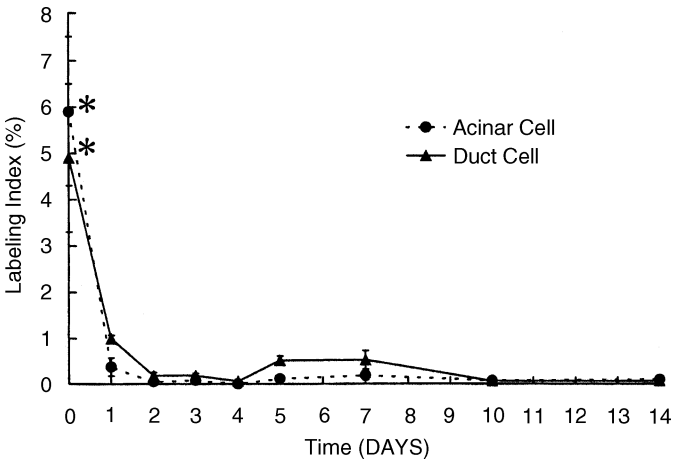

Figure 4 TUNEL labeling indices of acinar (broken line) and duct (solid line) cells in experimental glands. The error bars indicate SEM, and the asterisks stand for statistically significant differences from the control value ($P < 0.05$).

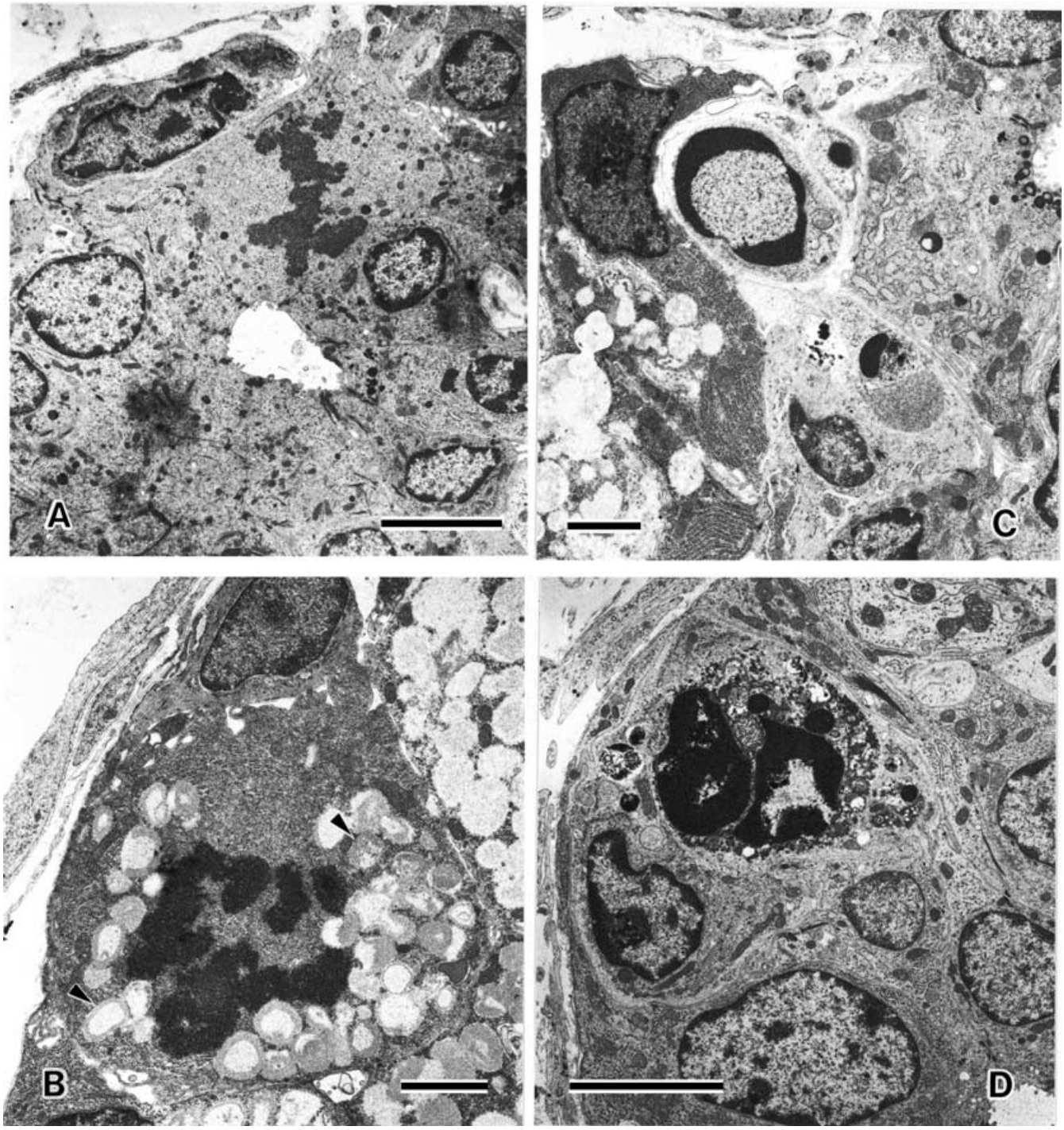

Figure 5 TEM micrographs. (A) Day 5, mitotic figure of duct cell. Bar = 5 μm. (B) Day 5, mitosis of immature acinar cell containing secretory granules characterized by internal structures (arrowheads). Bar = 2 μm. (C) Day 10, apoptotic bodies with characteristic nuclear fragments. Bar = 2 μm. (D) Day 10, degraded apoptotic bodies. Bar = 5 μm.

residual ones. This indicates that residual acinar cells start proliferation immediately after removal of the ligation and that they contribute to the early stage of regeneration. This type of acinar cell proliferation has been reported in the regeneration of atrophic parotid glands where acinar cells remain (4, 5). The other type considered is the proliferation of newly formed acinar cells. Electron microscopically, some mitotic acinar cells containing secretory granules with internal structures were observed in the present study. Similar secretory granules have also been observed in acinar cells in regeneration after partial extirpation (20) and development (29) in salivary glands, and such acinar cells are thought to be immature (30). Accordingly, such acinar cells observed in this study are newly formed

immature cells, and these newly formed immature acinar cells also proliferate. This type of acinar cell proliferation has been reported in the regeneration of atrophic parotid glands without remaining acinar cells (6). The present study suggests that both residual and newly formed acinar cells proliferate in the regeneration of submandibular glands after atrophy and that the PCNA labeling index for acinar cells may be very high at days 4 and 5 when both types of acinar cells proliferate.

The PCNA labeling index for duct cells gradually declined from the maximum at day 0. This agrees with the index in regeneration of parotid glands after atrophy under the same experimental conditions (6). The above suggests that active proliferation of duct cells is unnecessary in regeneration of salivary glands after atrophy. The probable reason for this is that duct cells proliferate and are residual during atrophy (11).

Apoptosis is a part in progressive as well as regressive processes. In the development of rat submandibular glands, apoptosis contributes to the deletion of terminal tubule cells (18) and lumen formation of ducts (16). Functionally insufficient cells or structures produced in rat liver regeneration after partial hepatectomy are removed by apoptosis in the remodeling process (21, 22). By TUNEL and TEM, the present study demonstrates that apoptosis is involved in the regeneration of submandibular glands after atrophy. However, the TUNEL labeling index of acinar and duct cells were low and there was no significant difference between the experimental and control groups, suggesting that the importance of apoptosis in the regeneration of submandibular glands after atrophy is lower than the importance in submandibular gland development and liver regeneration. The reason for this difference may be that the atrophic glandular tissue in the present study recovers while wholly new tissue is produced in submandibular gland development and liver regeneration after hepatectomy.

In the present study, the excretory duct was unilaterally ligated and reopened to understand the regeneration of submandibular gland. However, it is unknown whether the outcome of this study is general to all situations such as salivary gland regeneration following bilateral ligation and release of excretory ducts. Further investigation under different conditions will be needed.

In conclusion, during regeneration of submandibular glands after atrophy, both residual and newly formed acinar cells proliferate actively. Apoptosis of parenchymal cells also occurs; however, the significance of apoptosis is low.

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Acknowledgements

This study was supported by a Grant for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (No.15591990).