Chronic Changes in the Atrophied Submandibular Gland after Long-term Ligation of the Main Excretory Duct in Mice

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Abstract: To examine the pathology of salivary glands that have undergone atrophy or hypofunction due to old age or disease, the duct ligation model is used. This model has been used in studies on the course of glandular parenchyma atrophy and the potential for repair and regeneration. However, the period of ligation was short in most of these studies, and none have examined long-term progress. Therefore, we investigated long-term ligation of the submandibular gland in mice and examined the chronic changes in atrophied salivary glands. The ligation periods were 1, 2 and 3 months, and atrophied salivary glands were resected after each period. Resected glands were examined by histology, immunohistochemistry, RT-PCR, and transmission electron microscopy. Histologically, disappearance of acinar cells and increases in duct-like structures were observed over time in atrophied salivary glands, resulting from long-term submandibular gland main excretory duct ligation. Acinar cell markers (α-amylase, aquaporin 5) showed marked weakness in expression after ligation, but expression was still observed after 3 months of ligation. Stem cell markers (c-kit, Sca-1) showed greater expression at 1 month of ligation, compared with controls, but expression subsequently decreased with time. Expression of the precursor cell marker cytokeratin 5 was retained throughout long-term ligation. Atrophied salivary gland tissue resulting from long-term ligation showed increases in specific duct-like structures over time, and the expression of stem cell markers and progenitor cell markers in the area of these structures suggests that the repair capability remained intact.

Key words: Duct-like structure, Duct ligation, C-kit, Sca-1, Cytokeratin

Introduction

Atrophy or hypofunction of the salivary glands may occur for a variety of reasons, including old age and disease. This is accompanied by reduced salivation, leading to dryness of the mouth. Saliva has various functions, including secretion of digestive enzymes, moistening and repair of mucous membranes, antibacterial action and buffering action\(^1\). Consequently, a reduction in saliva secretion has a number of secondary effects on the intraoral environment, including an increase in caries, swallowing and articulation disorder, reduced immune function, halitosis, taste disorder and poor retention of dentures, thus leading to reduced quality of life\(^2\)\(^-\)\(^7\).

Currently, numerous studies have been carried out to clarify the mechanisms leading to salivary gland atrophy and hypofunction by ligating the main excretory duct of salivary glands. Changes in salivary gland tissue that have been found as a result of duct ligation include atrophy and disappearance of acinar cells above the ligation, an increase in duct-like structures, and an increase in fibrous connective tissue\(^8\)\(^-\)\(^10\). There are also reports noting residual salivary gland function, restorative function, and regenerative ability in atrophied salivary glands\(^10\)\(^-\)\(^23\). However, most of these studies had a short ligation period, and there have been no studies observing progress over the long term. Most of the pathological conditions brought on by salivary gland atrophy develop over the long term, and most studies do not examine salivary gland atrophy that resembles clinical pathologies. In this study, the main excretory duct of the submandibular gland in mice was ligated for longer periods of time and chronic changes in gland tissue and residual function of atrophied salivary gland were investigated using an acinar cell marker, markers for different types of stem cell, and a progenitor cell marker.

Materials and Methods

Animals

Experimental animals were 10-week-old female Jcl:ICR mice
Mice were kept in a rearing room under a 12-hour light and dark cycle with free access to pellets (MF; Oriental Yeast Co., Ltd. Tokyo, Japan) and tap water. This study was approved by the experimental animal ethics committee of The Nippon Dental University School of Life Dentistry at Niigata (approval no. 129).

**Ligation of main excretory duct of submandibular salivary gland and tissue collection**

Ligation of the main excretory duct of the submandibular salivary gland was carried out under general anesthesia (sodium pentobarbital, 50 mg/kg body wt ip). Each mouse was supported in the supine position, and a midline skin incision was made in the neck. The main excretory duct of the submandibular gland was exposed by detaching peripheral tissues such as nerves and blood vessels, with care taken not to damage them, exposing the main excretory duct of the submandibular gland. The duct was ligated approximately 2 mm above the salivary gland using 4-0 silk suture, and the wound was closed. Two groups were formed by ligating only the salivary gland on the right side in each individual (ligated group) and leaving the gland on the left side non-ligated (control group). The ligation periods were 1, 2 and 3 months, with 3 mice in each group. After the ligation period, mice were sacrificed in order to collect tissue. The chest was opened, blood and bodily fluids were cleared using phosphate buffered saline (PBS: 9.57 mM, pH 7.35–7.65), and perfusion fixation carried out with 4% paraformaldehyde (PFA). The submandibular glands were then excised under a stereomicroscope with care taken to ensure that the sublingual gland was not included.

**Submandibular gland measurement**

The size of the submandibular gland (major axis, minor axis) was measured using vernier calipers. Measurement was carried out after the mouse was sacrificed and prior to collection of tissue for RT-PCR and removal of blood and bodily fluids, as two samples of each tissue from each mouse were collected for analysis.

**General staining**

Following perfusion fixation, collected salivary glands were immersion fixed in 4% PFA and then embedded in paraffin according to standard protocols. Sections (5 μm) were prepared and used for staining. Sections were stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS), and histological examination was carried out under an optical microscope (Olympus BX51, Tokyo, Japan).

The number of characteristic duct-like structures appearing as a result of ligation was counted in 15 randomly selected 0.3 × 0.3 mm visual fields.

**Immunohistochemistry analysis (DAB method)**

Paraffin was removed from the sections according to standard protocols, and sections were treated to block endogenous peroxidase (Dako REAL™ Peroxidase-Blocking Solution, Agilent Technologies, CA, USA) and activate antigens (HistoVT One, nacalai tesque, Kyoto, Japan). Sections were then reacted with primary antibody and secondary antibody (EnVision™+ Dual Link System-HRP, Agilent Technologies, CA, USA), and were then stained (ImmPACT™DAB, Vector Laboratories, CA, USA), nuclear stained (hematoxylin), cleared, and embedded. Primary antibodies are shown in Table 1.

**Gene expression analysis by RT-PCR (Reverse transcription-polymerase chain reaction)**

Tissue for RT-PCR analysis was collected immediately after the glands were measured. Collected tissue was promptly immersed in RNAlater RNA Stabilization Reagent (QIAGEN, Hilden, Germany) for storage until use. Total RNA was extracted by homogenization and purified using ISOGEN II (Wako Pure Chemical Industries, Osaka, Japan), and using 1 μg of mRNA from this, cDNA was synthesized with a high-capacity cDNA Reverse Transcription kit (Life Technologies, CA, USA). PCR amplification of cDNA was carried out using Platinum PCR SuperMix (Life Technologies) and a 2720 Thermal Cycler (Life Technologies). PCR conditions were as follows; an initial denaturing step of 95 °C for 2 minutes, 35 repetitive cycles of denaturing at 95 °C for 30 seconds, primer annealing at 54–58 °C for 30 seconds and an extension at 72 °C for 1 minute, and then a final extension at 72 °C for 10 minutes.

PCR products were all separated via gel electrophoresis (1.8% agarose gel) and visualized via UV detection (FAS-III) using

<table>
<thead>
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<th>Antibody</th>
<th>Source (catalog no.)</th>
<th>Dilution</th>
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<tr>
<td>Anti-α-amylase</td>
<td>Sigma-Aldrich(A8273)</td>
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<td>Abcam(ab104751)</td>
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<tr>
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<tr>
<td>Anti-cytokeratin5</td>
<td>Covancee(PRB-160P)</td>
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Table 1. List of primary antibodies
etidium bromide. Sequences of primers used in the present study are shown in Table 2. Glyceraldehyde-3-phosphate (GAPDH) was used as an endogenous control. Experimental assay kits noted above were all used in accordance with the manufacturer’s instructions.

**Electron microscopic observation**

Part of the tissue collected for histological analysis was examined using a transmission electron microscope. Samples were from the 1- and 3-month ligation groups. Collected tissue was immersion fixed in 2.5% glutaraldehyde (Nisshin EM Corporation, Tokyo, Japan) and post-fixed with 1% osmium tetroxide (Nisshin EM Corporation). After ethanol dehydration, samples were embedded in resin (Quetol 812: Nisshin EM Corporation). Slices (90–100 nm) were prepared, and after staining with uranyl acetate and lead citrate, were examined with a transmission electron microscope (JEOL JEM-1200EX-II).

**Statistical analysis**

Measurement of submandibular gland size and number of positive cells for immunohistochemistry analysis statistical analysis were carried out using two-way repeated measures ANOVA and Bonferroni multiple comparison procedure. The number of characteristic duct-like structure statistical analysis was carried out using one-way ANOVA and Bonferroni multiple comparison procedure. The significance level was set at p < 0.05.

**Results**

**Measurement of submandibular gland size after ligation**

Both the major axis and the minor axis of the submandibular gland measured at the time of collection were significantly smaller in all ligation groups than in the control group. Comparing the size of the submandibular gland between the different ligation groups, a significant reduction in size was only observed in the major axis after 1 to 2 months of ligation, and no large reduction in size was observed after 2 to 3 months of ligation (Fig. 1).

**Histological analysis**

![Image of results](image-url)
Histological examination showed that in the control submandibular glands there were no atrophic changes of acinar cells, and the duct tissue including identifiable intercalated duct, granular convoluted tubule, and striated duct (Fig. 2A). In addition, PAS-positive secretory granules were found in the cytoplasm (Fig. 2E). In the 1-, 2- and 3-month ligation groups, acinar cells had atrophied or disappeared in comparison to the controls. In addition, and contrary to these results, a tendency toward increased duct-like structures and interlobular connective tissue over time was observed in the ligation groups (Fig. 2B-D). There was difference in the number of duct-like structure between 1-month and 3-month ligation groups, and the duct-like structure number significantly increased in 3-month (Fig. 3). No PAS-positive cells were seen from 1 month of ligation onward (Fig. 2F-H).

**Immunohistochemistry analysis**

In the normal submandibular glands of the control group, strong positive reactions were observed for α-amylase in the cytoplasm of acinar cells and for aquaporin 5 (AQP5) in the cellular membrane (Fig. 4A, E). In the tissue of submandibular glands after ligation, the acinar cells had disappeared and there were chronic increases in duct-like structures and interlobular connective tissue, accompanied by reductions in positive reactions for α-amylase and AQP5. α-Amylase was detected in the vicinity
Figure 3. Changes in the number of duct-like structures. Ligation for 1, 2 and 3 months. Mean ± SD. (*p < 0.05; other differences not significant).

Figure 4. Immunohistochemical analysis. Immunostaining for acinar cell markers, α-amylase and AQP5.
(A-D) α-amylase: A, control; B, 1-month ligation; C, 2-month ligation; D, 3-month ligation. (E-H) AQP5: E, control; F, 1-month ligation; G, 2-months ligation; H, 3-month ligation. Scale bars = 100 μm
Figure 5. Immunohistochemical analysis. Immunostaining for stem cell and progenitor cell markers c-kit, Sca-1, CK5.

(A-D) c-kit: A, control; B, 1-month ligation; C, 2-month ligation; D, 3-month ligation. (E-H) Sca-1: E, control; F, 1-month ligation; G, 2-month ligation; H, 3-month ligation. (I-L) CK5: I, control; J, 1-month ligation; K, 2-month ligation; L, 3-month ligation. Scale bars = 100 μm

of the duct-like structures (Fig. 4B-D), and AQP5 was detected in clusters of small cells characterized by less cytoplasm than acinar cells (Fig. 4F-H). In addition, there were significantly fewer cells positive for either α-amylase or AQP5 in the experimental groups than in the controls following ligation (Fig. 6A, B).

The stem cell markers c-kit and Sca-1 were investigated, and while positive cells were found in some ducts in the control group submandibular glands (Fig. 5A, E), positive cells were observed in the surroundings of the duct-like structures in submandibular glands after ligation (Fig. 5B-D, F-H). There were significantly greater numbers of cells positive for c-kit in the 1-month and 2-month ligation groups than in controls, and the 1-month ligation group showed a particularly clear increase, after which the 2-month ligation group was significantly lower (Fig. 6C). A greater number of Sca-1 positive cells were found in all ligation groups than in controls, and a comparison of the number of positive cells in each ligation group showed significantly fewer in the 3-month ligation group than in the 1- and 2-month ligation groups (Fig. 6D).

Investigation of cytokeratin 5 (CK5) showed that positive cells were present in only some ducts in the control group (Fig. 5I), while positive cells were observed in the duct-like structures in the experimental groups in the ligation groups (Fig. 5J-L). All the ligation groups possessed significantly more CK5 positive cells than the controls. The 2-month ligation group was higher than the 1-month ligation group in the number of the positive cells and there were significant difference between the groups (Fig. 6E).

**Gene expression analysis**

In control groups, gene expression was observed for all primers. In the ligation groups, similar trends to those found with immunostaining were found for all ligation periods. Expression of the acinar cell markers α-amylase and AQP5 was observed from 1 month ligation onward, but showed a tendency to decrease over time at 2 months and 3 months. Expression of c-kit and Sca-1 was markedly greater in the 1 month ligation group than in controls,
Figure 6. Changes in number of positive immunostained cells. Number of positive cells in controls and in 1-, 2- and 3-month ligation groups. Means ± SD. Blue: control group; White: ligation group A: α-amylase; B: AQP5; C: c-kit; D: Sca-1; E: CK5 (**) p < 0.01, *p < 0.05; other differences not significant)

Figure 7. Gene expression analysis by RT-PCR (reverse transcription-polymerase chain reaction).
RT-PCR for GAPDH, α-amylase, AQP5, c-kit, Sca-1, CK5 after duct ligation for 1, 2 or 3 months.

and expression decreased over time with continued ligation in the 2-month and 3-month ligation groups. CK5 expression was found in both the control and ligation groups (Fig. 7, 8).

Electron microscopy analysis
In comparison to the control group (Fig. 9A), in the 1-month (Fig. 9C) and 3-month (Fig. 9D) ligation groups, greater atrophy of acinar cells was observed, nuclei were flattened and cells had a polygonal shape, and duct-like structures characterized by fewer secretory granules in the cytoplasm and enlarged lumens were observed. These were clearly different from the intercalated ducts in the control group (Fig. 9B) as they had little cytoplasm and thus a greater nucleus/cytoplasm (N/C) ratio. Apoptosis findings such as concentration and fragmentation of the nucleus were not observed (Fig. 9C, D). The 3-month ligation group tended to have larger lumens than the 1-month group.

Discussion
Ligation of the main excretory duct of the submandibular gland is a model for obstructive functional disorder of the salivary gland. Clinically, obstructive functional disorder of the salivary gland is a pathology that develops over a long period of time, due to
increases in salivary calculi or other substances. To date, reports on submandibular gland main excretory duct ligation models have mostly been short ligation periods of around 1 week\(^{19-23}\), and the longest have been approximately 1 month\(^{8,11}\). Thus, there is a need for studies investigating the tendency toward atrophy and residual regeneration function in salivary glands in conditions closer to actual clinical pathology. In this study, ligation of the main excretory duct of the mice submandibular gland were long-term. As a result, we were examined for changes in marker expression related to the residual functional and regenerative capacity of atrophy salivary glands.

Reduction in size of the glandular parenchyma over time was observed as a result of long-term ligation of the main excretory duct of the submandibular gland. Histologically, atrophy and disappearance of acinar cells, and gradual increases in duct-like structures and fibrous connective tissue were observed. Changes in salivary gland tissue after short-term ligation that have been reported include enlargement of duct lumens, atrophy and disappearance of acinar cells above the ligation, increases in duct-like structures and proliferation of fibrous connective tissue, degranulation and vacuolar degeneration of granular duct cells and acinar cells, and disappearance of basal striations from the striated ducts\(^{8-18}\). This was similar to findings in the long-term ligation.

Studies of residual salivary gland function and regeneration function in atrophied salivary glands expression of the acinar cell markers \(\alpha\)-amylose\(^{24-26}\) and AQP5\(^{23,26-28}\), and stem-cell markers such as c-kit\(^{19,23}\) are investigated. However, all of these results are from short-term ligation. After long-term ligation in the present study, \(\alpha\)-amylose was observed in the surroundings of duct-like structures and AQP5-positive cells were observed in groups of small cells that had less cytoplasm than acinar cells. This suggests that marker proteins are retained and have functions in acinar cells, even when cell morphology has changed due to atrophy. There is a need for detailed investigation of chronic changes in the localization of cells in atrophied salivary glands that test positive for acinar cell markers, which should also include morphological examination of acinar cells that have undergone degenerative changes.

Histological studies of atrophied salivary glands have reported the occurrence of duct-like structures\(^{17,18,29-31}\). Duct-like structures resulting from ligation were also observed in the present study, and it was shown that these increase over time with long-term ligation. However, almost no studies to date have examined the component cells or the nature of duct-like structures. In the present study, duct-like structures were examined from the perspective of evaluating the regenerative capacity of atrophied tissue, using the stem cell markers c-kit and Sca-1. Cells that are positive for c-kit and Sca-1 have been reported in salivary gland ducts and their surroundings\(^{19-22}\). In the present study, localization of cells positive for stem cell markers was observed in duct-like structures and their surroundings. This suggests that restorative or regenerative capacity still remains in the cells comprising these duct-like structures.

The component cells of duct-like structures are reported to be morphologically different from acinar cells, and are reported to have ultrastructural characteristics similar to terminal tubule cells\(^{20}\). Embryologically, acinar cells are known to differentiate from terminal tubule cells in the early development of salivary glands\(^{32,33}\), and terminal tubule cells and connective tissue are known to be significantly present in the immediate postnatal salivary glands. While terminal tubule cells undergo vigorous proliferation for a time with the development of salivary gland tissue after birth, acinar cells subsequently increase and become predominant in mature salivary gland tissue, and the terminal tubule cells almost completely disappear. This suggests that terminal tubule cells have the nature of precursor cells in their differentiation into acinar cells. In the present study, we made chronic observations of duct-like structures by electron microscopy. These structures were only found in atrophied salivary glands, and had different tissue morphology from the acinar cells and intercalated ducts in the control groups. Changes such as enlargement of the lumens resulting from the ligation period were observed. Furthermore, as immunohistological analysis showed positive reactions for stem cell markers localized in the duct-like structures and their surroundings, it appears that the component cells of duct-like structures have the same nature as terminal tubule cells. We therefore examined the component cells of the duct-like structures using the epithelial basal cell marker CK5 to determine
whether they have the nature of progenitor cells. CK5 is a marker for stem cell of exocrine glands such as the mammary glands, and has been used a marker for progenitor cells. The results showed that CK5-positive cells were present in the ducts of the control group and in the duct-like structures of the ligation groups. Consequently, it appears that in addition to having epithelial characteristics, the duct-like structures also have characteristics similar to those of stem cell and progenitor cells. In the chronic long-term changes, the above-mentioned acinar cells and stem cell markers tended to decrease over time. However, CK5 is no substantial change, it was suggested that progenitor cell-like nature is maintained.

To date, it has generally been inferred that the duct-like structures are a simple degenerative variation of acinar cells. From the results of the present study, however, duct-like structures can be regarded as a variation of acinar cells that have taken on characteristics similar to progenitor cells and retained their residual repair or regenerative capabilities. In other words, the results suggest that the salivary gland repair potential is to some extent retained as a result of the appearance of duct-like structures. Thus, it may be possible to treat atrophied salivary glands by activating a progenitor with repair capability.

At the present, the therapeutic methods for dry mouth used in clinical settings include symptomatic therapies, such as moistening the mouth by gargling or artificial saliva, and the administration of drugs to stimulate the parasympathetic nervous system, which have many side effects. Therefore, there is a need for an effective, basic therapeutic method to be established. If cells that have the characteristics of progenitor cells and are able to differentiate remain in the atrophied salivary gland itself, functional recovery could be expected through an approach that activates these cells, thereby allowing the salivary gland to repair or regenerate itself. Treatments have recently been developed in which tissue regeneration is activated using autologous conditioned medium (CM). CM contains different types of low molecular weight cytokines that are expressed when differentiation from stem cell into different cell types is induced. Therapies using CM are advantageous, as they involve direct cell transplantation and there is little risk of harmful effects on the body, such as carcinogenesis or immune rejection, and these therapies may be readily introduced into clinical settings. If the potential for salivary gland repair is to some extent retained in salivary glands that have atrophied due to duct obstruction, the introduction of this therapy is expected to be remarkably effective. To date, salivary duct obstructive diseases such as salivary calculus have been treated with resection of the salivary glands in cases of advanced obstruction or findings of gland atrophy. However, the present study shows that if the obstruction causing the problem is removed, the salivary gland may be able to repair itself. To investigate this, we need to carry out long-term salivary gland ligation and then remove the ligature, in order to study the degree of tissue repair and the cell kinetics. There is also a need to study residual repair capacity in atrophied salivary glands after irradiation.

In conclusion, the main excretory duct of the submandibular gland in mice was ligated over a long period of time, and the atrophied glands were subjected to immunohistological and gene expression analysis. Specific duct-like structures increased over time in the tissue of salivary glands that atrophied due to long-term ligation, and markers for stem cell and progenitor cells were expressed in these areas. These results suggest that the atrophied glands have residual repair capacity.

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Conflict of Interest

The authors have declared that no COI exists.

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