Localization of Hsp27 in the Rat Submandibular Gland Following the Application of Various Surgical Treatments

Kenichi Mizobe1,2, Yoshihiro Kawabe1,2, Yasuhiko Bando1, Koji Sakiyama1, Hisao Araki2 and Osamu Amano1

1Division of Anatomy, Department of Human Development and Fostering, Meikai University School of Dentistry, 1–1 Keyakidai, Sakado, Saitama 350–0283, Japan and 2Division of Oral Rehabilitation, Department of Restorative and Biomaterials Sciences, Meikai University School of Dentistry

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Salivary glands repair and regenerate following various types of injuries and surgical procedures. However, the tissue responses induced in the contralateral glands have yet to be elucidated in detail. Hsp27, a member of the heat-shock protein (Hsp) family, is strongly expressed in physiological environments, particularly during development. Hsp27 was previously shown to play a role in the regulation of acinar cell proliferation and differentiation in the rat submandibular gland.

The present study performed the following surgical treatments on the right submandibular glands of adult rats: 1) duct ligation followed by unligation after one week; 2) partial sialoadenectomy; and 3) total sialoadenectomy. Immunohistochemistry for Hsp27 and Ki67 was performed in the experimental and normal contralateral glands, and localization was histologically and morphometrically analyzed.

The results obtained revealed the localization of Hsp27 to the intercalated duct in the submandibular glands of non-treated rats. The expression of Hsp27 was strongly induced in both the uninjured contralateral control glands as well as treated glands of experimental rats regardless of the surgical procedure performed. The number of Hsp27-immunopositive cells increased rapidly following surgery, and subsequently returned to the same level as that in non-treated rats after 4 weeks. However, no marked changes were observed in the number of Ki67-immunopositive proliferating cells. Therefore, the change in the number of Hsp27-immunopositive cells may have contributed to compensatory hypertrophy. The results of the present study indicate that the expression of Hsp27 in the intercalated duct in the submandibular gland may play a role in the differentiation of acinar cells.

Key words: Hsp27, immunohistochemistry, intercalated duct, submandibular sialoadenectomy, rat

1. Introduction

The mucosal membrane lining the oral cavity is normally moistened by saliva secreted from the major and minor salivary glands. Saliva plays a role in various processes, such as digestion, taste, and lubrication, and also acts as an antibacterial as well as buffering agent. Previous studies demonstrated that saliva makes important and varied contributions to the efficient working and protection of the body [3]. Dysfunction in salivary secretion caused by various etiologies, including aging, inflammation, neoplasm, and autoimmune diseases, such as Sjögren’s syndrome as well as medical treatments including surgical excision, radiotherapy, and psychotropic drugs, has been shown to lead to severe diseases in teeth and mucosal mem-
branes, resulting in a marked reduction in Q.O.L. Therefore, elucidating the mechanisms responsible for restoring the function of the salivary glands in more detail will be beneficial for clinical sciences. Studies have been conducted on ductal occlusion [11, 14, 25–27, 31, 32, 34], surgical excision [9, 12, 16, 17, 19], irradiation [18, 20, 23], and laser irradiation [28, 29] using experimental animals to determine the mechanisms underlying recovery from salivary gland damage. Although compensatory hypertrophy was previously reported in the remaining glands with congenital salivary gland aplasia or following surgical extraction, very little is known about the mechanisms occurring in control or contralateral organs.

Heat shock proteins (Hsps) are molecular chaperones that protect against cell death in response to heat and other non-physiological stresses. They are also expressed in cells in a non-stress state and are thought to have various physiological roles in addition to their anti-apoptotic function [13]. Hsps have been classified into several families according to their molecular weights. Hsp27 is a low molecular weight Hsp of 25–27 kDa that is known to be involved in the regulation of cell kinetics, particularly during developmental processes, under physiological conditions [4–6, 10]. The progression of cellular proliferation and differentiation in rat submandibular glands has been reported in the postnatal development stage and tissue composition was shown to be mature 8 weeks after birth. Various undifferentiated acinar cells have been observed during proliferation in the submandibular glands of juvenile rats. However, proliferation is nearly completed 3 weeks after birth, followed by differentiation and maturity [1]. In the transition period from proliferation to differentiation, Hsp27 is temporarily expressed in terminal tubule cells, the most important among the remaining embryonic acinar cells, up to 3 weeks after birth [2]. Moreover, the strong temporary expression of Hsp27 in the acinar regeneration process has been reported in adult rats injured by duct ligation. Therefore, it may also be associated with the regulation of acinar cell growth and differentiation [27].

In the present study, different types of surgical insults were performed in the unilateral submandibular glands of adult rats. The localization of Hsp27 on the operated and non-operated sides were then analyzed immunohistochemically, and the relationship between the roles of Hsp27 and compensatory hypertrophy in salivary glands was assessed.

II. Materials and Methods

Animals and submandibular gland surgery

Fifty male, 8-week-old Wistar rats weighing approximately 200 g were used in the present study. They were cared for in accordance with the Meikai University Experimental Animals Guidelines. They were given solid feed during the experimental period and had free access to water. These experiments were approved by the Meikai University School of Dentistry Animal Ethics Committee (A1223). Operations were performed under the general anesthesia via intraperitoneal injection of 1 ml/kg sodium pentobarbital (Somunopentyl, Kyoritsu Seiyaku, Tokyo, Japan).

Duct ligation and unligation

Operations for duct ligation and unligation were performed according to our previous study by Takahashi-Horiuchi et al. [27]. An incision was made along the right paramedian line on the skin of the ventral neck with care not to damage the gland itself as well as surrounding peripheral tissues including nerves and blood vessels, and the right submandibular (Wharton's) duct was exposed. A ligation was made on the right excretory duct using a surgical vascular ligation clip (Straight type, RU 3950-04, Rudolf Medizintechnik, Fridingen, Germany) at 5 mm distal to the glandular porta. At 7 days after ligation, the ligation clip was removed under general anesthesia.

Rats were then sacrificed 1, 3, and 7 days after ligation, or 3 days, 1 week and 2 weeks after unligation, and the submandibular glands on the operated (right) and non-operated (left) sides were removed.

Partial submandibular sialoadenectomy

The mid-portion of the submandibular gland was ligated with a silk suture and separated into distal and proximal sides. The distal side was resected and skin was immediately closed.

Unilateral submandibular sialadenectomy

The right submandibular gland including the sublingual gland was excised en bloc.

Histological procedures

Fixation and general staining

Rats were anesthetized with an intraperitoneal injection of 1 ml/kg pentobarbital sodium (Somunopentyl) and physiological saline was perfused through the left ventricle. Perfusion fixation was then performed with 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4). The remaining submandibular gland was excised and immersion-fixed overnight with the same fixative at 4°C. After fixation, the glands were immersed in 30% sucrose/phosphate buffer solution (PBS, pH 7.4) overnight, and cryosections of 20 μm in thickness were then prepared. Representative sections were stained with hematoxylin-eosin (H-E).

Immunohistochemistry

After being dried at room temperature, sections were immersed for 60 min in 0.1 M PBS (pH 7.4) containing 0.3% Triton-X-100. They were then washed with distilled water and PBS and treated for 10 min in methanol containing 0.3% H₂O₂ to inhibit endogenous peroxidase activity. These sections were washed again in PBS and treated for 30 min at room temperature in 10% normal goat serum (Nichirei, Tokyo, Japan). After washing with PBS, a rabbit
anti-mouse Hsp27 polyclonal antibody (dilution 1:500; Stressgen Biotechnologies, Victoria, Canada), which was used as the primary antibody, was reacted overnight at room temperature. After washing with PBS, sections were then reacted with a biotin-labeled goat anti-rabbit IgG antibody (Nichirei) as the secondary antibody for 60 min at room temperature. The immunoreaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Dojindo, Kumamoto, Japan) containing 0.01% \( \text{H}_2\text{O}_2 \). Sections were subsequently rinsed in water, dehydrated, mounted, and then observed under a light microscope. Negative control sections were reacted with PBS instead of the primary antibody, stained in the same manner, and were confirmed to have no non-specific immunoreactions. The application and specificity of these antibodies to rat tissue have already been confirmed in our previous studies [2, 3, 24].

### Double immunostaining
Using a mixture of the rabbit anti-mouse Hsp27 polyclonal antibody (dilution 1:250; Stressgen Biotechnologies) and goat anti-mouse Ki67 polyclonal antibody (dilution 1:125; Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies, sections were reacted overnight at room temperature. An immunoreaction was then performed for 60 min at room temperature using a mixture of fluorescein-labeled donkey anti-rabbit IgG (dilution 1:50; Millipore, Billerica, MA, USA) and Cy3-labeled donkey anti-goat IgG (dilution 1:250; Millipore) as secondary antibodies. After washing with PBS and mounting with glycerol vinyl alcohol aqueous mounting solution (Nichirei), sections were examined under a confocal scanning laser microscope (LSM-510; Carl Zeiss, Jena, Germany).

### Statistical analysis
Following immunohistochemical staining, Hsp27-immunopositive epithelial cells or Ki67-immunopositive cells were counted in visual fields of \( 330 \times 410 \, \mu\text{m}^2 \) at 10 locations, and statistically analyzed using the Mann-Whitney U test for between-group comparisons. \( P < 0.01 \) was considered to indicate significance.

### III. Results

#### Histological structure and immunohistochemistry

##### Normal submandibular glands

The submandibular gland tissue of 8-week-old rats was composed of the acinus, intercalated duct (ID), granular duct (GD), and striated duct (SD). The ID, which was long and narrow in shape, was observed between the acinus, consisting of numerous serous cells, and the GD (Fig. 1a). Regarding location and orientation in the ID, we referred to sites in contact with acini as “the distal end” and sites in contact with the GD as “the proximal end”. The interstitium in the glandular tissue was filled with intra-lobular connective tissue including blood vessels and nerves [27].

Immunohistochemistry for Hsp27 revealed small masses of Hsp27-positive epithelial cells at the distal end of the ID (Fig. 1b). No Hsp27-immunopositive cells were observed in the remaining portions of the ID, acini, GD, or SD. An intense immunoreaction was localized in the cytoplasm of Hsp27-positive cells, but was not noted in the nucleus (Fig. 1b). Immunoreactivity for Hsp27 was also constitutively detected in the vascular endothelium and nerve fibers, which is consistent with the findings of previous studies [27].

##### Duct ligation

No marked changes were observed in the tissue structure of operated (right) submandibular glands 1 day after surgery (not shown). However, acinar structures had atrophied and were reduced markedly in size after 3 days (Fig. 2a), and finally disappeared on the operated side after 1 week (not shown). Although duct-like structures were detected, no structures that could be identified as normal acini were found, as has been reported previously [27] (Fig. 2a). Immunohistochemistry for Hsp27 revealed a few small immunopositive epithelial cells in atrophied acini 3 days after surgery (Fig. 2b), whereas almost none were observed after 1 week (not shown). As noted previously, blood vessels and nerves were Hsp27-immunopositive constitutively in the rat submandibular gland [2, 27].

No marked changes were observed in H-E-stained
specimens obtained from non-operated contralateral (NC, left side) glands throughout the experimental period after surgery (Fig. 2c). Immunohistochemistry for Hsp27 showed that small masses of immunopositive cells were localized in the distal end of the ID 3 days after ligation (Fig. 2d). A detailed observation of sites including longitudinal sections of the ID revealed the localization of Hsp27-immunopositive cells in 2–3 groups at the distal end of the ID. No apparent differences were noted in H-E-stained histology between 3 days and 1 week after surgery. However, a slight increase was noted in the number of positive cells at the distal end of the ID on high magnification images of longitudinal sections immunostained with Hsp27 (Fig. 2e).

Duct unligation

Epithelial buds arose from the duct-like structures in operated (right) submandibular glands 3 days after unligation, and the localization of Hsp27-immunopositive cells were detected in epithelial bulging at the distal end of duct-like structures (not shown), as has been reported previously by Takahashi-Horiuchi et al. [27]. The architecture of the tissue was normal 2 weeks after surgery. However, it was slightly smaller than that on the contralateral side, and a duct system that included the ID and acini was observed (Fig. 3a). Immunohistochemistry for Hsp27 showed the localization of small, round Hsp27-positive cells in the ID (Fig. 3b).

In the NC submandibular gland, no apparent differences were observed in the tissue structure 3 days after unligation from that in normal submandibular glands (Fig. 3c). Immunostaining for Hsp27 revealed the presence of small round cells with immunoreactivity in groups in the ID. A detailed observation with high magnification images, including longitudinal sections of the ID, showed that Hsp27-immunopositive cells were distributed throughout from the distal end to the proximal end of the ID (Fig. 3d). Immunohistochemical findings 2 weeks after unligation were the same as those 3 days and 1 week after ligation (Fig. 3e).

Unilateral partial submandibular sialoadenectomy

Inflammatory cell invasion was noted in the interlobular connective tissue of operated (right) submandibular glands 1 and 3 days after partial resection. However, no marked changes were observed in the epithelial tissue architecture in H-E-stained sections 3 days after surgery (not shown). Inflammatory cell invasion had disappeared 1 week after surgery. An epithelium consisting of duct-like structures without acini near the resection stump closely resembled the histology observed after duct ligation (Fig. 4a). Hsp27-immunopositive cells were found in the periphery of these duct-like structures (Fig. 4b).

The distally located tissues from the resection stump had a normal architecture (Fig. 4c). Hsp27-immunopositive cells were detected in the ID. Higher magnification images including longitudinal sections of the ID revealed numerous masses of Hsp27-immunopositive cells from the distal end to the midportion of the ID, whereas no positive cells were located at the proximal end (Fig. 4d). The tissue in the resection stump had almost healed 4 weeks after surgery, and normal structures were found in every portion of H-E stained sections. Hsp27-positive cells were seen in a small number of masses at the distal ends of the IDs as found in the normal submandibular gland (Fig. 4e).
Photomicrographs of duct-unligated (a, b) and contralateral (c–e) submandibular glands of adult rats stained with H-E (a, c) or anti-Hsp27 antibody (b, d, e) at 3 days (c, d) or 2 weeks (a, b, e) after unligation surgery. Hsp27-immunopositive cells (arrows) were located in the intercalated duct of operated glands, which represented regenerating acini, 2 weeks after surgery (b). In contralateral glands, Hsp27-immunopositive cells were located in the intercalated duct (arrowheads) from the distal to proximal end 3 days (c, d) and 2 weeks (e) after unligation. Acini (asterisks) are immunonegative for Hsp27. Bars=500 μm.

Fig. 3.

Photomicrographs of partially-resected (a–e) and contralateral (f–h) submandibular glands of adult rats stained with H-E (a, c, f) or anti-Hsp27 antibody (b, d, e, g, h). Duct-like (a and b, resection stump) and normal (c and d, distal portion) structures were observed in operated glands 1 week after surgery. Duct-like structures (◆) contained small numbers of Hsp27-immunoreactive cells (arrows, b). In the distal portion, Hsp27-immunoreactive cells were observed in the intercalated duct (arrowheads, d) 1 week after surgery. Many Hsp27 immunoreactive cells in the contralateral glands were located at the distal end of the intercalated duct (arrowheads) 1 week after surgery (g). However, a small number of immunopositive cells were found 4 weeks after surgery (h). Asterisks indicate the immunonegative acini. Bars=500 μm.

Fig. 4.
In NC (left) glands, a normal tissue architecture was maintained from 1 day to 4 weeks after surgery, and no marked changes from normal sections were detected using H-E staining (Fig. 4f). Immunohistochemistry for Hsp27 showed the presence of numerous immunopositive cell masses at the distal end of the ID until 1 week after surgery (Fig. 4g). However, a small number of immunopositive cells were confined to the distal end of the ID 4 weeks after surgery (Fig. 4h), which was similar to that observed in untreated healthy submandibular glands.

Unilateral total submandibular sialoadenectomy

No morphological changes were observed in the NC (left) glands with H-E staining 1 to 4 weeks after surgery (Fig. 5a). Immunohistochemistry for Hsp27 revealed small, round immunopositive cells in the ID (Fig. 5b). Higher magnification images of longitudinal sections of the ID revealed that several masses of Hsp27-positive cells had formed at the distal end and midportion of the ID, and continued for 2 and 4 weeks after surgery (Fig. 5c, d).

Changes in Hsp27-immunopositive ID cell number

Ligation and unligation of the right submandibular duct

Changes in the operated side included a significant decrease in the number of Hsp27-positive cells due to atrophy, that is, the disappearance of acini and ID due to ligation, which recovered after the release of ligation. A significant increase in Hsp27-positive cell number over time 1 week after duct ligation was observed on the non-operated contralateral side, and a larger number of positive cells than on the recovered operated side or in normal submandibular glands was maintained even 2 weeks after the release of ligation (Fig. 6a).

Partial submandibular sialoadenectomy

A significant decrease was observed in the number of Hsp27-positive cells on the operated side 1 day after partial resection. However, this number recovered to presurgery value after 3 days and was followed by a significant increase after 1 week. This number then decreased up to the 4th week. A significant increase was also observed in Hsp27-positive cell number on the non-operated contralateral side immediately after partial resection up to 1 week after, and was followed by a decrease up to the 4th week, similar to that on the operated side. No significant differences were observed from the changes observed in a normal 12-week-old submandibular gland (Fig. 6b).

Total submandibular sialoadenectomy

The number of Hsp27-immunopositive cells 1, 2, and 4 weeks after surgery was approximately twice the average number in normal untreated submandibular glands at each of these time points, and this difference was significant (Fig. 6c).

Cell proliferation and Hsp27

Double immunofluorescent staining for Hsp27 and Ki67, a proliferating cell marker, was performed to examine the relationship between proliferating and Hsp27-expressing cells (Fig. 7a–d). The colocalization of Ki67 and Hsp27 was not detected at any period in operated (Fig. 7b) and non-operated contralateral submandibular glands following partial resection (Fig. 7c). A very small number of nuclei that were immunopositive for Ki67 were detected in the SD and acini of the submandibular glands of rats that had undergone unilateral total submandibular sialoadenectomy (Fig. 7d). However, there were no such cells coexisting with Hsp27 in the ID (Fig. 7a–d). No significant
The above results indicated that there were no apparent changes in the proliferative activity of submandibular glands on either the operated or non-operated contralateral side. No proliferating cells coexisted with Hsp27, especially in the ID.

difference in the number of Ki67-immunopositive cells was evident between the normal, operated and NC glands (Fig. 7e).

The above results indicated that there were no apparent changes in the proliferative activity of submandibular glands on either the operated or non-operated contralateral side. No proliferating cells coexisted with Hsp27, especially in the ID.

**Fig. 6.** Graphs showing changes in Hsp27-immunopositive intercalated duct cells (330×410 μm² visual field) in rat submandibular glands following ligation/unligation (a), partial resection (b) or total excision (c). Asterisks indicate the presence of statistical significance (P<0.01).
IV. Discussion

The results obtained in the ligation and unligation experiments performed on submandibular ducts in our present and previous study [27] revealed that Hsp27 was temporarily expressed at sites that developed from duct-like structures when acini regenerated after unligation. During duct ligation, Hsp27-immunopositive epithelial cells decreased progressively in number and were almost extinguished before unligation. This suggests that Hsp27-immunopositive ID cells were atrophied together with acini since there was no sign of regeneration of acinar structures from ID when the duct was obstructed. Further observations were made in the present study on the NC submandibular gland that underwent duct ligation, partial resection, and total resection, and the mass of Hsp27-positive cells at the distal end was observed to be enhanced toward the proximal end after surgery without cell proliferation.

Marked increases were observed in the expression of Hsp27 in the ID on the operated and NC sides following duct ligation, partial resection, and total resection of the rat submandibular gland. The rapid increase in the number of Hsp27-positive cells in submandibular glands on the NC side, which received no direct stimulation, may have been a response produced by an indirectly transmitted stimulation from the operated side. This result strongly suggests that Hsp27 may be involved in compensatory tissue responses.

Compensatory hypertrophy and Hsp27

When tissue or organ dysfunction occurs due to congenital malformations, acquired diseases, such as inflammation or tumors, or surgical treatments, compensatory hypertrophy has been reported in the remaining tissues or organs as well as the recovery of damaged tissues. These responses have been shown to prevent hypofunction in the damaged tissues [22]. The compensatory hypertrophy of paired organs was previously demonstrated in the remaining kidney following unilateral nephrectomy as well as in the remaining lung after total pneumonectomy [36]. Compensatory hypertrophy is also known to occur in unpaired organs such as the heart and liver in response to damage to cardiac muscle or hepatic tissues [22]. Regarding the salivary glands, marked compensatory hypertrophy in the contralateral parotid gland was previously reported in a case of unilateral parotid gland aplasia [15]. However, no established hypothesis has been proposed for the mechanisms responsible for inducing compensatory hypertrophy within the same organ or in distant organs.

Hypertrophy is a phenomenon that is characterized by cell volume expansion in response to a physiological request for greater bioactivity than the cardinal condition. However, it may include hyperplasia, an increase in cell number [22]. The absence of hyperplasia was confirmed during the present experimental period because marked increases were not observed in the number of cells that were immunopositive for Ki67, a marker of cell proliferation. Marked compensatory hypertrophy was previously reported 200 to 440 days after the excretory ducts of the parotid or submandibular gland were ligated [7] and also 90 to 250 days after resection of the bilateral submandibular glands [30]. Therefore, a long-term analysis is needed to more accurately evaluate increases in salivary gland acini or duct cell volume and/or number.

Although the mechanisms responsible for the induc-
tion of salivary gland compensatory hypertrophy remain unclear, the hypertrophy of salivary gland acinar cells in animal experiments was previously shown to be induced by the continuous administration of the β receptor agonist isoproterenol [35]. The same stimulation also facilitated the differentiation of ID cells to acinar cells [33]. Therefore, the NC submandibular gland may receive chronic sympathetic nerve stimulation as a result of surgical invasion on the operated side, which may induce acinar cell hypertrophy and ID differentiation. The expression of Hsp27 is known to be confined to the beginning of acinar cell differentiation, which strongly suggests that the increase in Hsp27-immunopositive ID cells in the NC submandibular glands may be induced in response to compensatory hypertrophy.

**Relationship between ID cell differentiation and Hsp27**

ID cells have been speculated to be latent stem cells. However, this has yet to be established. Evidence for the ID stem cell theory has been based on the location of the ID between the acinus and duct system as well as the higher synthesis of DNA in ID cells than in other portions in the gland [8, 9]. However, the expression of Hsp27 [2, 27] and stem cell-related factors [21] was recently shown to be strong in the ID following the initiation of regeneration after an injury.

Two cell types have been identified in the ID of the rat submandibular gland: cells containing small secretory granules (granular ID cells) and cells that do not contain these granules (nongranular ID cells). The properties of small secretory granules closely resemble those of secretory cells in the terminal tubule cells comprising the acinar portion of the fetal submandibular gland. Therefore, it is possible that granular ID cells may be derived from terminal tubule cells and have the capacity to proliferate and differentiate when stimulated [2]. Although terminal tubule cells that remain in the central portion of acini after birth strongly express Hsp27, they disappear due to apoptosis or differentiate into immature acinar cells and granular ID cells that express Hsp27 [2]. The expression of Hsp27 was shown to be absent in differentiated acinar cells and ID cells without secretory granules [2]. Thus, an enhancement in the expression of Hsp27 in the ID may be associated with a transition/differentiation from a nongranular to granular type. Therefore, compensation for the excised submandibular gland may be achieved by the differentiation of ID cells to acinar cells in the remaining glandular tissues.

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