Original

Distribution of aquaporin-5, transforming growth factor- β_1 and laminin during regeneration of atrophic rat submandibular glands after duct ligation

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Abstract: The aim of this study was to determine the localization of aquaporin-5 (AQP5), transforming growth factor- β_1 (TGF- β_1) and laminin during regeneration of the rat submandibular gland. After duct ligation for 7 days, the regenerating glands were collected on days 0, 1, 3, 7, and 14 after ligation release to study the process of regeneration. Immunohistochemical staining revealed apical expression of AQP5 in many acinar cells, strong expression in intercalated ducts (ICDs) of the normal submandibular gland at Day 14, and strong expression in duct-like structures (DLSs) during regeneration from Day 0 to 7. However, a few AQP5-negative acinar cells were detected during regeneration. At Day 0, immunopositivity for TGF- β_1 was detected in connective tissue. At Days 3 and 7 during regeneration, TGF- β_1 immunostaining was observed in DLSs, which were surrounded by α -smooth muscle actin-positive thickened myoepithelial cells. Laminin staining was strong in the thickened basement membrane of DLSs at Day

3 during regeneration, but weak around acinar cells at Day 14. These findings suggest that TGF- β_1 is involved in the environment around DLSs, myoepithelial cells and laminin, that DLSs have the same functional properties as ICDs, and that AQP5-negative acinar cells may be mucous cells.

Keywords: aquaporin-5; transforming growth factor- β_1 ; laminin; duct-like structures; rat submandibular gland; regeneration.

Introduction

The process of salivary secretion depends on the level of expression and localization of the water channel protein aquaporin-5 (AQP5) in the secretory epithelium and expression of α -smooth muscle actin (α -SMA) by the myoepithelium (1-3). Loss of these functional markers is associated with salivary hypofunction (4,5). In rats, AQP5 is localized at the apical membrane of acinar cells, and highly expressed only in the submandibular gland intercalated ducts (ICDs) (2,6). By contrast, myoepithelial cells have a stellate shape and form a basket-like structure around the acini, largely mediating the interaction between acinar cells and the basement membrane (7).

The basement membrane consists of two layers: the basal lamina (formed by epithelial cells) and the lamina fibroreticularis (formed by connective tissue). The basal

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lamina can be divided into two regions (8,9): a transparent plate directly below the epithelium, the lamina lucida, consisting of laminin and its receptor, integrin; and the lamina densa, which has a type IV collagen reticular structure. These extracellular matrix (ECM) components are critical to many cellular processes, including growth, survival, morphogenesis, and differentiation (10,11). Our previous study has shown that type IV collagen is expressed strongly in the thickened basement membrane around duct-like structures (DLSs), and that type IV collagen, but not type III collagen, is involved in the regeneration of acinar cells (12).

Transforming growth factor- β_1 (TGF- β_1) is a multi-functional cytokine that influences salivary gland development and homeostasis. In particular, TGF- β_1 regulates ECM deposition by inducing the biosynthesis of collagen and laminin as well as promoting the expression of protease inhibitors (13,14). Additionally, TGF- β_1 signaling promotes myoepithelial cell differentiation (15). Myoepithelial cells synthesize basement membrane components, such as collagen IV and laminin-1 (7). Although these findings suggest that these components interact, the role of TGF- β_1 during regeneration of the submandibular gland is unknown.

To investigate the association between salivary gland atrophy and regeneration, many studies have employed a duct ligation model (16-19). In this model, ligation causes acinar cells to disappear and increases the amount of connective tissue, while its release induces the appearance of DLSs and the proliferation of acinar cells, after which immature acinar cells appear. It has been reported previously that DLSs play an important role at the point of acinar regeneration (20-22). In the present study, the distributions of AQP5, TGF- β_1 , and laminin were investigated immunohistochemically to clarify the environment around DLSs during regeneration and the acinar cells that are functional in this setting.

Materials and Methods

Animals

Eight-week-old male Wistar rats were used in this study. All rats were maintained under pathogen-free conditions in the Animal Faculty of Nihon University School of Dentistry. Experimental protocols were conducted in accordance with ethical principles for the use of laboratory animals, as approved by the Committee on Animal Experimentation at Nihon University (approval number: AP14D027).

Experimental procedures

Atrophy was induced following the duct ligation proce-

dures described previously (12). The main excretory duct of both the right and left submandibular glands was exposed via a small incision in the neck. After 7 days of ligation, the metal clips were removed, and the glands were collected after a further 0, 3, 7, and 14 days ($n = 6$ respectively) to study their regeneration. The glands were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections 4 μ m thick were cut and stained with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) (23).

Immunohistochemistry

Antibodies against AQP5 (0.2 μ g/mL, Alomone, Israel), TGF- β_1 (2 μ g/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA), α -SMA (0.5 μ g/mL, Dako, Carpinteria, CA, USA), and laminin (2 μ g/mL, Progen, Heidelberg, Germany) were used. The sections were preconditioned using a Tris/borate/EDTA-based buffer (pH 8 and 98°C, Takara, Tokyo, Japan) for 40 min to activate the antigen, and incubated for 30 min with 1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) to suppress any nonspecific reaction. For enzyme antibody staining, the sections were incubated overnight at 4°C with primary antibodies diluted in 1% BSA-PBS. The horseradish peroxidase (HRP)-conjugated secondary antibodies were reacted for 60 min at room temperature. After washing with PBS, incubation for 10 min with 0.42 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., Saint Louis, MO, USA)-0.3% H_2O_2 solution was carried out to visualize the immunoreaction sites. The sections were counterstained with hematoxylin. For indirect immunofluorescence antibody staining, the sections were incubated overnight at 4°C with one or two primary antibodies diluted in 1% BSA-PBS. After washing with PBS, the secondary fluorescein isothiocyanate (FITC)- and/or tetramethylrhodamine (TRITC)-conjugated antibodies were applied for 60 min at room temperature. The stained sections were embedded in mounting medium for fluorescence microscopy (Kirkgaard & Perry Laboratories, Gaithersburg, MD, USA) and examined using an epifluorescence microscope (Nikon, Tokyo, Japan). Negative controls for immunostaining were prepared by substituting the primary antibodies with 4 μ g/mL normal rabbit or goat IgG (Santa Cruz Biotechnology).

Results

HE and PAS staining

HE-stained preparations of regenerating submandibular glands are shown in Fig. 1A-E. The ICDs were observed in the normal submandibular gland (Fig. 1A). At Day 0,

Fig. 1 HE (A-E) and PAS (F) staining, immunostaining for AQP5 (G-L) and TGF- β_1 (M-R) and double immunostaining for TGF- β_1 and α -SMA, as well as merged images (S-U), in normal submandibular glands (A, G, M, F, L, R), and in regenerating glands at Day 0 (B, H, N), Day 3 (C, I, O, S, T, U), 7 (D, J, P), and Day 14 (E, K, Q) after release from duct ligation. In the normal gland (A) and Day 14 gland (E), thin ICDs (black arrowheads) are evident. At Day 0 (B), transitional DLSS (yellow arrow) and connective tissue (green arrow) are evident in HE-stained sections. At Days 3 (C) and 7 (D), NFACs (red arrow) and DLSS (yellow arrows) are evident. Normal submandibular glands (F) show strong PAS-positive acinar cells (black arrows). Normal submandibular glands (G) and Day 14 glands (K) showed moderate expression of AQP5 at the apical membrane of acinar cells and strong expression in ICDs (black arrowheads). AQP5 immunopositivity was not observed in several acini (G, K, black arrows). Lingual mucous acinar cells showing absence of immunostaining for AQP5 are shown in the inset of (G). At Day 0 (H), AQP5 showed strong apical expression in DLSS (black arrowheads). At Days 3 (I) and 7 (J) during regeneration, moderate AQP5 expression was detected apically in NFACs (red arrowheads) and strong expression was detected in DLSS (black arrowheads). Acinar cells during regeneration showed no AQP5 expression (black arrows in I, J). In normal submandibular glands (M) and glands at Day 14 after release of duct ligation (O), TGF- β_1 expression was detected in GCTs (red double arrows) and SDs (yellow arrowheads). At Day 0 (N), TGF- β_1 immunopositivity was detected in the connective tissue (green arrows) and DLSS (yellow arrow). At Days 3 (O) and 7 (P) during regeneration, TGF- β_1 was detected in DLSS (yellow arrows). Negative controls are shown in (L) and (R). Immunofluorescence staining for TGF- β_1 (S) and α -SMA (T), and the merged image (U). Double immunostaining demonstrated positivity for TGF- β_1 in DLSS surrounded by α -SMA (yellow arrows). Scale bar: 100 μ m.

acinar cells had disappeared and the connective tissue had increased around the large ducts in comparison to normal glands (Fig. 1B). Transitional DLSS and newly formed acinar cells (NFACs) were observed at Day 3 (Fig. 1C). The NFACs had matured by Day 7 (Fig. 1D), and the

treated submandibular glands appeared to be similar to the normal submandibular glands by Day 14 (Fig. 1E). A preparation of a normal submandibular gland stained by PAS is shown in Fig. 1F. Acinar cells were weakly stained by PAS, but some were strongly PAS-positive.

Fig. 2 Immunofluorescence staining for laminin (A, B) and α -SMA (C, D), and the merged image (E, F) at Day 3 (A, C, E) and Day 14 (B, D, F). At Day 3 during regeneration, laminin (A) was strongly expressed in the basement membrane around DLSSs (white arrows). DLSSs were surrounded by thickened myoepithelial cells. At Day 14, laminin was strongly expressed around ICDs (white arrowheads). ICDs were surrounded by thickened cells that were strongly positive for α -SMA. Scale bar: 100 μ m.

Immunostaining

AQP5

In the normal submandibular gland, strong apical AQP5 staining was observed in the ICDs and moderate staining of acinar cells was evident (Fig. 1G). AQP5 was less abundant in acini than in ICDs. Several acini lacked AQP5 immunopositivity. The inset of Fig. 1G shows lack of AQP5 immunopositivity in sublingual glands. At Day 0 (Fig. 1H), AQP5 showed strong apical localization in DLSSs. At Day 3 (Fig. 1I), immunostaining for AQP5 was observed in DLSSs and NFACs. However, a few AQP5-negative NFACs were evident. At Day 7 (Fig. 1J), AQP5-positive acinar cells were increased and strongly positive ICDs had appeared. At Day 14 (Fig. 1K), many moderately AQP5-positive cells and strongly AQP5-positive ICDs were observed. However, a few AQP5-negative acinar cells were also evident. No positive immunoreaction was observed in the negative control (Fig. 1L).

TGF- β 1

In the normal submandibular gland (Fig. 1M), immunopositivity for TGF- β 1 was observed in granular convoluted tubules (GCTs) and striated ducts (SDs). No immunoreactivity was observed in acinar cells or ICDs. At Day 0 (Fig. 1N), immunopositivity for TGF- β 1 was detected in connective tissue and DLSSs. From Day 3 to Day 7 (Fig. 1O, P), TGF- β 1 immunopositivity was observed in DLSSs during regeneration. At Day 14 (Fig. 1Q), the staining pattern was similar to that in normal submandibular gland tissues. No positive immunoreaction was observed in the negative control (Fig. 1R). Double immunofluorescence staining of TGF- β 1 (Fig. 1S) and α -SMA (Fig. 1T) at Day 3 demonstrated TGF- β 1 immunopositivity in DLSSs surrounded by α -SMA-positive myoepithelial cells (Fig. 1U).

Laminin and α -SMA

At Day 3 during regeneration (Fig. 2A), laminin was strongly expressed in the thickened basement membrane of DLSSs, but showed only weak expression around large ducts. DLSSs were surrounded by thickened cells that were strongly positive for α -SMA (Fig. 2C, E). At Day 14, laminin was strongly expressed in the thickened basement membrane of ICDs. Laminin staining was detected at a consistent level around acinar cells (Fig. 2B). ICDs were surrounded by thickened cells that were strongly positive for α -SMA (Fig. 2D, F). Strong α -SMA staining was detected on the inner side of the laminin-stained basement membrane (Fig. 2E, F).

Discussion

The present study revealed the temporal-spatial pattern of AQP5 expression during regeneration of the rat submandibular gland. In the normal gland, AQP5 immunopositivity was observed at the apical membrane of acinar cells, and expression was particularly high in the ICDs, as reported previously (2,6). In the present study, NFACs became morphologically evident at Day 3, and many of them were AQP5-positive. AQP5 became detectable in proacinar cells, although AQP5-negative cells were observed in the terminal buds at the early canalicular stage of salivary gland development (1,24). Therefore, expression of AQP5 protein in NFACs during regeneration appears similar to that during normal development. On the other hand, there were a few AQP5-negative acinar cells in normal glands, and in glands between Days 3 and 14 after release of duct ligation. AQP5 was absent in mucous acinar cells in the rat submandibular gland, as mucous cells secrete mainly mucin (25). The present results indicate that AQP5 is absent in mucous cells of the sublingual gland. Additionally, the strongly PAS-positive acinar cells observed in

the present study were thought to be mucous cells (26). These findings suggest that AQP5-negative acinar cells, including NFACs, may be mucous cells.

Interestingly, the DLSs in glands on Days 0 to 3 after release of duct ligation and ICDs of normal glands showed strong apical AQP5 positivity. Although the reason for this is unclear, abundance of AQP5 in ICDs suggests that the latter may participate in transcellular water movement more effectively than acinar cells (6). Our previous study showed that F-actin was distributed and assembled at apical sites in the DLSs and ICDs of normal glands (18). Therefore, the strong AQP5 positivity observed in the present study suggests that the cells of DLSs may have functional properties similar to those of ICDs.

In the present study, the localization of TGF- β_1 was analyzed during regeneration of the rat submandibular gland. In the normal gland, a previous study has demonstrated localization of TGF- β_1 at the GCTs and SDs (27). Similarly, the present study revealed TGF- β_1 immunoreactivity at the GCTs and SDs in normal submandibular glands and at Day 14 after release of duct ligation. Ligation caused most acinar cells to disappear and led to a morphologically evident increase of connective tissue at Day 0. Furthermore, cells positive for TGF- β_1 were observed in the connective tissue. TGF- β_1 is considered to be a master switch for the program of fibrosis (28,29). Additionally, it has been shown that overexpression of TGF- β_1 induces normal salivary gland parenchyma with connective tissue in the submandibular glands of β_1^{tglo} /MC mice (30). Therefore, TGF- β_1 plays an important role in augmenting connective tissue during atrophy induced by ligation.

Our previous study showed that DLSs were surrounded by thickened myoepithelial cells during regeneration from Day 0 to Day 7 (18). The present study revealed strongly positive laminin staining around DLSs. The localization of laminin was similar to that of type IV collagen, but not that of type III collagen (12). These findings revealed that the lamina lucida and lamina densa become thickened around DLSs during regeneration. The results of double staining showed that TGF- β_1 was present in the DLSs surrounded by thickened myoepithelial cells. Additionally, in glands on Day 3 to Day 7 after release of duct ligation, a thickened ECM positive for laminin was observed around DLSs, with a temporo-spatial distribution similar to that of TGF- β_1 in myoepithelial cells. It has been reported that α -SMA expression is increased in the submandibular glands of β_1^{tglo} /MC mice, and that α -SMA staining is periductal as a result of myofibroblast recruitment by TGF- β_1 (30). Additionally, TGF- β_1 stimulates the synthesis of ECM proteins (13,14) and promotes

myoepithelial differentiation (15). Laminin-1 may regulate the polarity of myoepithelial cells (31). In addition, exogenously applied TGF- β_1 has been reported to rescue cell morphogenesis and differentiation, indicating that TGF- β_1 promotes cross-talk to ensure environmental homeostasis (32). Although the specific relationship between myoepithelial cell thickening and TGF- β_1 is unclear, the expression patterns observed in the present study suggest a stage-specific functional correlation between the two.

Although thickened myoepithelial cells were detected around DLSs, thin myoepithelial cells were observed around maturing acinar cells (18). It was noteworthy that myoepithelial cells around NFACs were thin, and that TGF- β_1 was not expressed in maturing acinar cells. These findings suggest that as acinar cells were generated from DLSs they exhibited a thick-to-thin shift and began to express TGF- β_1 , a transition that is likely to be important for the initiation and maintenance of their differentiated phenotype. Therefore, the thickening of the basement membrane and myoepithelial cells was not merely a result of rapid shrinkage (33). The present findings suggest that TGF- β_1 from DLSs constitutes and maintains the thickened basement membrane and myoepithelial cells in the rat submandibular gland.

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Conflict of interest

The authors declare no conflict of interest.

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