Effects of Cevimeline on the Immunolocalization of Aquaporin-5 and the Ultrastructure of Salivary Glands in Sjögren’s Syndrome Model Mice

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Received 15 June 2009, accepted 21 October 2009

Edited by TADASHI NAKASHIMA

Summary: Sjögren’s syndrome (SS) is an autoimmune disorder whose main symptoms include xerostomia and dry eyes. It has been demonstrated that abnormal expression of aquaporin (AQP)-5 in the parotid and submandibular glands in SS model mice was corrected by cevimeline. In the present study, we orally administered cevimeline hydrochloride (cevimeline) to female MRL/l mice, which are widely used as a model for SS, to immunohistochemically investigate the localization of AQP-5 in the salivary glands. We also assessed the ultrastructure of acinar cells in the submandibular glands. AQP-5 was expressed in the apical and lateral cell membranes of acinar cells in the parotid and submandibular glands of normal mice, but not in the sublingual glands. In contrast, AQP-5 was expressed not only in the cell membranes in the apical domains but also in the cytoplasm in the SS model mice, indicating that the localization of AQP-5 was disordered in the SS model mice. After administration of cevimeline, AQP-5 was predominantly localized in the cellular apical domains of the acinar cells. Electron microscopy revealed that administration of cevimeline to the SS model mice and normal mice markedly reduced the number of secretory granules, increased the area of the rough endoplasmic reticulum, and expanded the intercellular gaps in the cells of the submandibular acini. Condensed vacuoles were also observed in the Golgi apparatuses, indicating that secondary enhancement of secretion and production of saliva had occurred. Consequently, the results of the present study demonstrate that the administration of cevimeline to the SS model mice increased salivary secretion in the submandibular glands. Furthermore, cevimeline transiently normalized the localization of AQP-5 expression in the parotid and submandibular glands.

Key words aquaporin-5, Sjögren’s syndrome model mice, cevimeline, immunolocalization, salivary glands

INTRODUCTION

Sjögren’s syndrome (SS) is an autoimmune disease whose main symptoms include xerostomia and dry eyes that are caused by dysfunctions of exocrine glands such as the salivary glands and lacrimal glands in the craniofacial region. Cevimeline hydrochloride (cevimeline) is commonly used by patients with SS to stimulate the secretion of saliva and tears. Cevimeline shows strong binding affinity for muscarinic acetylcholine receptors present on the membrane of salivary gland cells, and is an acetylcholine analog that functions as a muscarinic receptor agonist to stimulate salivary secretion from the salivary glands [1]. Numerous reports have indicated that the administration of cevimeline accelerates saliva secretion in patients with SS, and have corroborated its safety and efficacy [2,3]. In addition, many studies have investigated the administration of cevimeline in a murine model of SS, MRL/l mice, which show spontaneous autoim-
mune abnormalities [4-6], and resulted in dose-dependent increases in saliva secretion [7]. Similarly, the effects of cevimeline on saliva secretion have been investigated in rats and dogs [8,9], and the ultrastructure of the salivary glands of rats and mice have also been reported [10-12]. However, there are no reports describing the morphological changes in the salivary gland cells of cevimeline-treated SS model mice.

Aquaporin (AQP) is a six-transmembrane protein that was discovered in 1987 and is expressed in the cell membranes of a diverse range of animals [13]. It is a water channel that plays an important role in water transfer across cell membranes. Thirteen subtypes of AQP have been identified, AQP-0 to AQP-12; of these AQP, AQP-1, -5 and -8 are expressed in the salivary glands [14,15]. A marked reduction in saliva secretion has been reported in AQP-5-knockout mice, prompting the theory that AQP-5 plays a major role in water transfer during saliva secretion by the salivary glands [16,17]. Although several studies have reported the localization of AQP-5 in SS model mice and rats [18-20], there are no reports describing changes in AQP-5 localization in cevimeline-treated SS model mice. Therefore, in the present study we investigated changes in the localization of AQP-5 in the salivary glands of cevimeline-treated SS model mice by immunohistochemistry, and determined the ultrastructural changes in acinar cells in the submandibular glands.

MATERIALS AND METHODS

This study was approved by the Kurume University Committee for Animal Experimentation. We used 11 female MRL/MpJ-Tnfrsf6f0pr/Crlj (MRL/l) mice (Japan SLC, Inc., Shizuoka, Japan) (23-26 g) as SS model mice, and 11 female BALB/C mice (Japan SLC, Inc., Shizuoka, Japan) (16-18 g) as control mice. The mice were provided solid feed and water ad libitum in a breeding cage with a 12-hour light/dark cycle (lights off at 7 pm). All mice were fasted for 24 hours before the day of the experiment. The SS model and control mice were each divided into four groups: a cevimeline non-administered group and an administered group, a normal saline solution administered group, and a group which was given solid feed from the morning of the experiment day. Cevimeline solution was prepared by dissolving 30 mg of powdered (+)-cis-2-methyl-spiro[[(1,3-oxathiolaen-5,3) quinuclidine] hydrochloride (cevimeline) 1/2 hydrate (SNI-2011; NIPPON KAYAKU Co., Ltd., Tokyo, Japan) into 30 ml of normal saline solution, and was orally administered at a dose of 30 mg/kg mouse weight using an oral zonde. Mice in the physiological saline-treated group were given 0.5 ml of physiological saline via an oral zonde. Mice in the postprandial group were given solid feed ad libitum from 8 am on the day of the experiment. All mice were anesthetized with diethyl ether 20 minutes after the administration of cevimeline, physiological saline, or provision of food. The mice in all groups were perfusion-fixed with 2.0% paraformaldehyde/2.5% glutaraldehyde in PBS solution (pH 7.3) via the heart.

For electron microscopy, the submandibular glands were excised, fragmented and fixed in 2.0% paraformaldehyde/2.5% glutaraldehyde in PBS solution (pH 7.3) for 2 hours. Tissue specimens were then fixed with 2% osmium for 1 hour and dehydrated in an acetone gradient before embedding in Epon resin. Ultrathin sections were prepared and subjected to uranium-lead double staining using conventional methods. A Hitachi Transmission Electron Microscope H-7000 (Hitachi, Ltd., Tokyo, Japan) was used to observe the specimens.

For immunohistochemical evaluation of aquaporin expression, we fixed the parotid, submandibular and sublingual glands obtained from some of the normal and SS model mice from each group in Bouin’s fixative for 24 hours or longer. The fixed salivary glands were embedded in paraffin and 5-μm-thick serial sections were prepared and subjected to hematoxylin/eosin (H/E) staining and AQP-5 immunostaining. Immunocytochemical staining was performed with a Histofine kit (Nichirei Co., Tokyo, Japan). In brief, sections were deparaffinized in xylene, hydrated in a graded ethanol series, and washed in phosphate-buffered saline (PBS) (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.5; PBS). All procedures were performed at room temperature (average temperature 20°C), and incubations were performed in closed humid chambers. Endogenous peroxidase activity was blocked with 3% H2O2/methanol solution. Next, 10% normal goat serum (Nichirei Co., Tokyo, Japan) was applied to block non-specific reactions with secondary antibodies. The sections were incubated with the primary antibody (anti-AQP-5, Santa Cruz Biotechnology, Inc., California, USA) overnight (approximately 15 hours), and washed in PBS. Biotin-labeled anti-rabbit IgG antibodies (Nichirei Co., Tokyo, Japan) were added as the secondary antibody, and peroxidase-labeled streptavidin (Nichirei Co., Tokyo, Japan) was added as the enzyme reagent. The final reactive products were visualized with 3,3‘-diaminobenzidine tetrahydrochloride (Nichirei Co., Tokyo, Japan) in 50 mM Tris-HCI buffer (pH 7.6) containing 0.05% hy-
hydrogen peroxide and the specimens were observed with a light microscope.

RESULTS

Evaluation of H/E staining and immunohistochemical staining by light microscopy

H/E and AQP-5 immunohistochemical staining were performed on the parotid, submandibular and sublingual glands of normal mice without cevimeline treatment and SS model mice with or without cevimeline treatment.

Parotid glands: H/E staining of acinar cells in the control mice revealed normal parotid glands with no lymphocyte infiltration around the vessels (Fig. 1A). AQP-5 immunohistochemical staining showed that AQP-5 was almost exclusively localized in the membranes of the apical region, except for the basolateral and basal side cell membranes (Fig. 1B and C).

H/E staining revealed that the acinar cells in the untreated SS model mice exhibited moderate lym-
phocyte infiltration around the blood vessels (Fig. 1D). AQP-5 expression was detected across the entire circumference of the acinar cells and within the cytoplasm (Fig. 1E and F).

H-E staining of the acinar cells of the cevimeline-treated SS model mice revealed lymphocyte infiltration around the blood vessels, comparable with the untreated SS model mice (Fig. 1G). However, the expression of AQP-5 was strongly localized to the membranes of the apical region cells, but not in the membranes of the basolateral and basal region of acinar cells (Fig. 1H and I).

**Submandibular glands:** H-E staining of the acinar cells of the untreated control mice revealed normal submandibular glands with no inflammatory changes, such as lymphocyte infiltration around the blood vessels (Fig. 2A). The expression of AQP-5 was predominantly localized in the membranes of the apical region cells, except for the basal and basolateral membrane (Fig. 2B and C).

H-E staining of the acinar cells of the untreated SS model mice revealed marked lymphocyte infiltration

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**Fig. 2.** H/E staining (A, C, D) and AQP-5 immunohistochemical staining (B, E, F, H, I) of the submandibular glands. A-C; Normal mice. AQP-5 existed on the apical cell membranes (black arrow) (C). D-F; Untreated SS model mice. Marked lymphocyte infiltration was observed around the blood vessels (white arrow) (D). AQP-5 expression was detected across the entire circumference of the acinar cells (black arrow and arrowheads) (E, F). G-I; Cevimeline-treated SS model mice. Lymphocyte infiltration was observed around the blood vessels (white arrow) (G). The AQP-5 expression was localized to the cell membranes in the apical region (black arrow) (I), thus presenting a similar distribution to that of untreated normal mice. (Scale bar = 100 μm)
around the blood vessels (Fig. 2D). Meanwhile, AQP-5 expression was detected across the entire circumference of the acinar cells and was present within the cytoplasm. The distribution of AQP-5 did not differ between the regions with or without lymphocyte infiltration (Fig. 2E and F).

H/E staining of the acinar cells of cevimeline-treated SS model mice revealed lymphocyte infiltration around the blood vessels, with no changes in inflammatory status versus the untreated SS model mice (Fig. 2G). Meanwhile, the expression of AQP-5 in the acinar cells was localized to the membranes of the cells in the apical region, including the lateral walls of the cells, thus presenting a similar distribution to that of untreated normal mice (Fig. 2H and I). The distribution of AQP-5 did not differ between the regions with versus without lymphocyte infiltration.

Sublingual glands: H/E staining revealed no inflammatory findings, such as lymphocyte infiltration around the vessels, in the sublingual glands in any group (Figs. 3A, C, E). We could not detect the expression of AQP-5 in the normal mice or in the untreated or cevimeline-treated SS model mice. (Figs. 3B, D, F).

Electron microscopic findings of submandibular acinar cells in normal mice

Untreated group: The submandibular acinar region consisted of an aggregate of a few cone-shaped acinar cells surrounding the lumen. Round-shaped nuclei tended to be localized to the basal region, away from the center of the cell bodies, and each nucleus possessed 1-2 nucleoli. Small-to-medium-sized secretory granules (maximum diameter: 1.7 μm) with low-to-moderate electron density were present between the upper regions of the nuclei and the apical regions. A large amount of laminated rough endoplasmic reticulum was observed between the periphery of the nuclei and the basal regions, and Golgi apparatuses were present in the upper regions of the nuclei (Fig. 4A).

Cevimeline-treated group: Medium-sized secretory granules with low-to-moderate electron density were present in the cytoplasm of the acinar cells near the luminal region, with fewer secretory granules compared with the untreated group. Although the location of the nuclei was similar to that of the untreated group, a larger amount of laminated rough endoplasmic reticulum was observed between the periphery of the nuclei and the basal region of the cell, as compared with the untreated group. Moreover, we also observed condensed vacuoles around the Golgi apparatuses, secretory granules bordering on the apical regions of the acinar cells, exocytosis and expansion of the intercel-

Fig. 3.  H/E staining (A, C, D) and AQP-5 immunohistochemical staining (B, D, F) of the sublingual glands. A, B; Normal mice. C, D; Untreated SS model mice. E, F; Cevimeline-treated SS model mice. No AQP-5 expression was detected on the cell membranes in all groups (B, D, F). (Scale bar = 100 μm)
lular spaces (Fig. 4C).

**Physiological saline-treated group:** Cells from the physiological saline-treated group exhibited a morphology that was broadly comparable with that of the untreated group. The secretory granules were predominantly localized in the area between the upper regions of the nuclei and the apical region of the cells. The nuclei tended to be closer to the basal region of the cells, and they were surrounded by a large amount of rough endoplasmic reticulum. Furthermore, some Golgi apparatuses had condensed vacuoles (Fig. 4E).

**Postprandial group:** In contrast to the untreated group, there were fewer secretory granules with low-to-moderate electron density near the luminal region of the cell in the postprandial group. The nuclei maintained their roundness and possessed nucleoli, and tended to be localized closer to the basal region of the cells, away from the center of the cells. The rough endoplasmic reticulum in the periphery of the nuclei covered a greater area, and the mitochondria were of normal shape throughout the cytoplasm (Figure not shown).

**Electron microscopic findings of submandibular acinar cells in SS model mice**

**Untreated group:** Small-sized secretory granules with low-to-moderate electron density completely filled the cytoplasm of the acinar cells, reaching the basal region of the cells. In addition, concrescence of the secretory granules (maximum diameter: 3.1 μm) was observed. The numerous secretory granules translocated

**Fig. 4.** Electron micrographs of the submandibular acinar cells of normal mice (A, C, E) and SS model mice (B, D, F). A, B: Untreated group. C, D: Cevimeline-treated group. E, F: Physiological saline-treated group. Abundant secretory granules completely filled the cytoplasm of the acinar cells of SS model mice, reaching the basal region of the cells (B). The nucleus was translocated to the basal edge of the cells (B). In both cevimeline-treated groups, apparent decrease of the secretory granules and synthesis of some condense vacuoles (arrowheads) were observed (C, D). Especially, in the cevimeline-treated mice, the number of granules was markedly less than other groups (D). In physiological saline-treated SS model mice, abundant secretory granules filled the cytoplasm of the acinar cells to the basal region, as in the untreated group (F). (Scale bar = 2 μm)
the nuclei to the basal region of the cells. There was relatively little rough endoplasmic reticulum, and few Golgi apparatuses or mitochondria. In contrast, at the luminal border of the cells we observed a reduction in the intercellular space between the individual acinar cells (Fig. 4B).

**Cevimeline-treated group:** Medium-sized secretory granules with lower electron density than that in the untreated group were observed in the cytoplasm of the acinar cells, but the number of granules was markedly less than that of the untreated group and the granules were predominantly localized to the luminal border of the cells. The location of the nuclei was closer to the basal region of the cells, away from the center of the cells. Although the rough endoplasmic reticulum and mitochondria were not in disorderly alignment or displaced, the cells from the cevimeline-treated group contained a larger number of these organelles compared with the untreated group. Moreover, condensed vacuoles were observed in the Golgi apparatuses. In contrast, the luminal region of the cells exhibited an exocytotic profile, manifested by an expansion of the intercellular spaces (Fig. 4D).

**Physiological saline-treated group:** Small-sized secretory granules of low-to-moderate electron density filled the cytoplasm of the acinar cells to the basal region, as in the untreated group. As a result, the nuclei were translocated to the basal edge of the cells. However, the amount of rough endoplasmic reticulum and mitochondria was smaller than in the untreated group (Fig. 4F).

**Postprandial group:** Small-sized secretory granules with low-to-moderate electron density filled the entire cytoplasm of the acinar cells, albeit the number of granules was smaller than in the untreated group. The amounts of rough endoplasmic reticulum and mitochondria were also smaller than in the untreated group. Although an exocytotic profile was observed at the luminal border of the cells, as in the untreated group, there was no increase in the intercellular spaces in the postprandial group (Figure not shown).

**DISCUSSION**

SS is an autoimmune disease whose main symptoms include xerostomia and dry eyes, which are caused by dysfunctions of exocrine glands such as the salivary glands and lacrimal glands. Histologically, SS is characterized by extensive lymphocyte infiltration around blood vessels [21]. In the present study, we used MRL/l mice, which were originally obtained by hybridization of AKR/J, C57BL/6J, C3H/Di and LG/J mice, as SS model animals. MRL/l mice exhibit spontaneous autoimmune abnormalities, and commonly exhibit symptoms such as sialadenitis, lymphoma, vasculitis and polyarthritis [4-7,22]. Accordingly, MRL/l mice are widely used as a model for SS, and the histological features of these mice resemble SS disorders with lymphocyte infiltration around blood vessels. The severe lymphocyte infiltration around the vessels of the parotid glands and the submandibular glands of the SS model mice used in the present study indicate that the salivary glands are affected by SS.

According to the findings of prior studies in rats and the results of the present study, AQP-5 localization in the acinar cells of the salivary glands straddles the apical regions and the lateral walls of the cells [15,18,23]. Disordered AQP-5 localization has been reported in patients with SS and in SS model mice [20,24]. Furthermore, it has been reported that the administration of muscarinic agonists and adrenergic agonists induced the migration of the more than 90% of AQP-5 immunoreactivity to the cellular apical regions in rat submandibular acinar cells [18]. The present study revealed that the expression of AQP-5 in normal mice was predominantly localized to the apical and lateral walls of the submandibular acinar cells, but not the basal cell membrane. The localization of AQP-5 in the parotid and submandibular glands of the SS model mice was different from that of the normal mice, in that AQP-5 was expressed over a wider area including the basal membrane and the cytoplasm. However, administration of cevimeline to the SS model mice broadly normalized the localization of AQP-5 in the parotid and submandibular glands. These findings were consistent with the results of a stimulation experiment using muscarinic agonists and adrenergic agonists [18].

One study has reported the effect of cevimeline on AQP-5 expression in the interlobular ducts of rat parotid glands [19]. That study revealed that cevimeline activates M3 muscarinic receptors, which then form a complex with Gq/11 on the intracellular membrane. This complex activates phospholipase (PL) Cβ to stimulate the release of Ca^{2+} stored in the endoplasmic reticulum, to increase the intracellular Ca^{2+} concentration. The subsequent intracellular signal transduction pathway increases salivary secretion. It was proposed that the increase in intracellular Ca^{2+} concentration causes AQP-5 translocation from the cytoplasm to the apical cell membrane. Accordingly, the AQP-5 translocation in the cevimeline-treated SS model mice observed in the present study may have been caused by a similar mechanism.

In contrast, no lymphocyte infiltration was ob-
served in the sublingual acinar cells by H/E and immunohistochemical staining. No AQP-5 immunoreactivity was demonstrated in the sublingual acinar cells. A histological study of the salivary glands of cevimeline-treated MRL mice, in the present experiment, revealed little lymphocyte infiltration in the sublingual glands [25]. With respect to AQP-5 localization in the sublingual glands, it has been reported that the function of the mucous glandular cells was not affected in AQP-5 knockout mice; only the serous glands were affected by AQP-5 knockout [16]. These findings are consistent with the results of the present study. These results regarding mucous cells in the sublingual gland are very interesting, and we are planning to follow up with more investigations.

Histological observation of mouse submandibular glands revealed that they consist of terminal acinar parts and vessel parts, most of which are comprised of serous gland cells. The acinar parts consist of aggregates of 3-6 cone-shaped acinar cells, which surround the lumen [26]. Electron microscopy revealed that the nuclei tend to be closer to the basal region of the cell, away from the center of the cell bodies, and laminated rough endoplasmic reticulum extends from the perinuclear to the basal region of the cell. Moreover, secretory granules are present between the upper regions of the nuclei and the apical regions.

The results of the present study revealed that the number of secretory granules in the SS model mice was greater than that in the normal mice. Because the granules completely filled the cytoplasm, the nuclei had been translocated to the basal side of the cells in the SS model mice. This can be attributed to impaired raw saliva secretion in the SS model mice, thus resulting in accumulation of secretory granules. Furthermore, the administration of cevimeline to the SS model mice reduced the number of secretory granules and stimulated saliva secretion into the lumen. This was also observed in the cevimeline-treated normal mice, and condensed vacuoles were observed around the Golgi apparatuses in both groups of mice. Therefore, in the present experiment it seems that cevimeline treatment promoted the secretory function, and secondarily it also promoted the synthesis of secretory granules.

Because there were no changes in the number of secretory granules in either group of physiological saline-treated mice, the reduction in the number of secretory granules in the cevimeline-treated mice is more likely to be due to administration of the drug rather than from stimulation by oral reflection. In contrast to the untreated group, the normal mice in the postprandial group exhibited a translocation of secretory granules to the luminal region of the cell with a reduced number of granules. Although there was a small reduction in the number of secretory granules in the SS model mice, we did not observe any changes such as translocation of granules to the luminal region of the cell. In other words, the SS model mice secreted a reduced amount of saliva after eating as compared with normal mice, and a similar phenomenon occurs in patients with SS.

In the present study, we observed changes in AQP-5 localization in submandibular acinar cells in cevimeline-treated SS model mice. Moreover, the ultrastructural observation of secretory function in salivary acinar cells revealed that administering cevimeline to the SS model mice also enhanced saliva secretion in addition to normalizing the localization of AQP-5. We intend to investigate the detailed intracellular dynamics of AQP by extending our research to include other human exocrine glands. Once the mechanism of action of AQP has been clarified and the control of AQP expression becomes possible, we hope to be able to improve the quality of life of patients with SS and other diseases associated with AQP.

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