Atrophy of myoepithelial cells in parotid glands of diabetic mice; detection using skeletal muscle actin, a novel marker

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ABSTRACT

In mouse parotid glands, we found expression of skeletal muscle actin (actin-α1) protein and mRNA. We isolated myoepithelial cells from the mouse parotid glands and investigated their actin-α1 expression because smooth muscle actin (actin-α2) has been used as a marker for myoepithelial cells. We used actin-α1 expression to identify pathological changes in diabetic non-obese diabetic (NOD; NOD/ShiJcl) mice—a mouse model for Sjögren's syndrome—and found myoepithelial cells to be decreased or atrophied in the diabetic state.

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1. Introduction

Myoepithelial cells have been found in association with intercalated ducts and with acini in all mammalian salivary glands [1]. Myoepithelial cells have been thought to be contractile because their fine structures are similar to those of smooth muscle [2]. Smooth muscle-specific actin (actin-α2), cytokeratin 14 and S100 protein have been used as markers of myoepithelial cells in histogenetic studies of salivary gland tumors [1,2], although these markers are not specific for salivary gland myoepithelial cells because of the number of capillary vessels in the glands.

Non-obese diabetic (NOD) mice develop salivary glands hypofunction and are commonly used to study autoimmune diabetes [3]. Salivary glands of NOD mice are histologically similar to salivary glands of patients with Sjögren’s syndrome, and are thus useful as a model for this disease [4,5]. We previously investigated differential gene expression in parotid glands of diabetic NOD mice, using a cDNA microarray assay, and found that the interspace between acinar cells was extended in diabetic mice [6]; we also found that some genes, including those for aquaporin 8 (Aqp8) and skeletal muscle actin-α (Acta1), were expressed less in diabetic NOD mice than in non-diabetic NOD mice and controls. As aquaporin 8 expression in parotid gland myoepithelial cells has been reported previously [7], we supposed that myoepithelial cells might decrease or shrink as diabetes develops, and myoepithelial cells might express skeletal muscle actin (actin-α1), although expression of skeletal muscle actin in the myoepithelial cells has not been reported.

Therefore, to clarify the pathogenesis of parotid glands in Sjögren’s syndrome using this mouse model, we investigated the decrease in myoepithelial cells in parotid glands of diabetic and non-diabetic NOD mice, and in control mice. We also studied expression of actin-α1 in parotid myoepithelial cells, and its potential as a novel marker for myoepithelial cells.

2. Materials and methods

2.1. Materials

Mouse monoclonal antibody specific for aquaporin 8 (M01) was purchased from Abnova (Taipei, Taiwan). Rabbit polyclonal antibody specific for aquaporin 5 was obtained from Millipore (Billerica, MA, USA). Rabbit polyclonal antibody specific for actin-α1 was obtained from Bioworld Technology (Minneapolis, MN, USA), and rabbit polyclonal antibody specific for actin-α2 was from Gene Tex Inc. (San Antonio, TX, USA). Alexa-Fluor 555 phallolidin was obtained from Molecular Probes (Eugene, OR, USA). DAPI (4′,6-diamidino-2-phenylindole) was obtained from Dojindo (Kumamoto, Japan).

2.2. Animals

All procedures were conducted to minimize pain and discomfort according to the Guidelines for the Care and Use of Laboratory Animals, The Nippon Dental University, School of Life Dentistry at Niigata. Female mice were bred and maintained under special pathogen-free
conditions in the mouse facility of our university. For the investigation of diabetic mice, female NOD/ShiJcl and C57BL/6Jcl mice (10 weeks old) were purchased from Clea Japan Inc. (Tokyo, Japan) and housed in an environmentally controlled room (12-h light–dark cycle, 23 ± 3°C and 50 ± 20% relative humidity). The animals had ad libitum access to certified rodent chow (Oriental Yeast, Tokyo, Japan) and water, and were acclimatized under these conditions. Mice were tested for blood glucose levels using OneTouch Ultra Test Strips (Life Scan Inc., Milpitas, CA, USA). Consecutive elevated fasting blood glucose levels >240 mg/dl were considered to represent the onset of diabetes. NOD mice between 25 and 28 weeks of age were separated into diabetic and non-diabetic groups (12 animals each), and were used for experiments, with C57BL/6Jcl mice (12 animals) at 25 weeks as the control group. For the investigation of actin-α1 in myoepithelial cells, Slc:ICR mice (Japan SLC, Inc., Hamamatsu, Japan) at 10 weeks were used.

2.3. Preparation of homogenates from parotid glands

To obtain three individual samples from each group, minced parotid glands from a mouse were homogenized with ice-cold buffer containing 5 mM HEPES–NaOH (pH 7.5), 50 mM mannitol, 0.25 mM MgCl2, 25 mM β-mercaptoethanol, 0.1 mM ethyleneglycol-bis-(2-aminooxyethyl ether) N,N,N′,N′-tetra acetic acid (EGTA), 2 mM leupeptin, 2.5 μg/ml trypsin inhibitor, 0.1 mM 4-amidinophenyl methane sulfonyl fluoride hydrochloride, 5 mM benzamidine and 2 mg/ml apro tinin, using a glass homogenizer and a Teflon pestle. The mixture was then centrifuged at 500g for 10 min at 4°C, and the supernatant was collected as a homogenate.

2.4. Isolation of acinar cells and myoepithelial cells from mouse parotid glands

Mice were anesthetized by intra-peritoneal injection of sodium pentobarbital (50 mg/kg) and sacrificed by cardiac puncture. Parotid glands were digested with collagenase (0.2 U/ml medium) for 20 min after digestion with trypsin as described previously [8]. The digest was centrifuged at 160g for 1 min; the precipitated layer contained acinar cells and myoepithelial cells. A mixture of 65% RediGrad in 2 mM EDTA in HBSS without Ca2+ or Mg2+ containing 0.1% bovine serum albumin (BSA) was pre-centrifuged at 48,000 × g for 1 min; the precipitated layer contained acinar cells, as identified by immunoblotting and immunohistochemistry [9]; the lower-density fraction came from the myoepithelial cells.

2.4. Immunoblotting

Samples were solubilized in Laemmli sample buffer, boiled and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using pre-made 5–20% polyacrylamide gel plates (e-PAGEl, Atto, Tokyo, Japan). The separated proteins were electrotransferred from the gel to an Immun-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blots were probed with primary antibodies; immune complexes were detected with HRP-conjugated secondary antibodies and ECL plus reagents (GE Healthcare, Little Chalfont, UK), and visualized using X-OMAT film (Kodak, Rochester, NY, USA). The intensity of the immunoreactive bands on the films was quantified using the Image Gauze software (LAS-1000 software package (Fuji Film, Tokyo, Japan).

2.5. Extraction of total RNA

Parotid glands or cell fractions from the parotid glands were washed with PBS, soaked in 500 μl RNA later (Life Technologies, Carlsbad, CA, USA), and stored at 4°C overnight. Total RNA was prepared using an RNeasy Plus Mini Kit (Qiagen, Chatsworth, CA, USA) with a DNA deletion column according to the manufacturer’s instructions.

2.6. RT-PCR analysis

First-strand complementary DNA was synthesized from 5 μg of total RNA template using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) with oligo dT primers according to the manufacturer’s instructions. Primer sets used were: Actb (actin-β) sense: 5′-AGGACATGAGACTGACCCATCC-3′, antisense: 5′-CTCTACGTTTGCTGGTAAA-3′; Aqp5 (aquaporin-5) sense: 5′-GGAAATTTGAGGACCTC-3′, antisense: 5′-TGTCCTGAATCTGCTGGAG-3′; Aqp8 (aquaporin-8) sense: 5′-GCGAGGCTGAGCTAAGAG-3′, antisense: 5′-GATTTCAACCTTCTCAGC-3′; Acta1 (actin-α1) sense: 5′-CGAGGTAATCCAGCTGGAAA-3′, antisense: 5′-GAGAAATGCGCCACCTCAG-3′; and Acta2 (actin-α2) sense: 5′-CTGACAGGGACACTGAA-3′, antisense: 5′-CATCTCTGAGCTGACCA-3′. PCR reactions were performed using 1 unit of KOD-Plus DNA polymerase (Toyobo, Tokyo, Japan). Amplification was performed in an Applied Biosystems 2720 Thermal Cycler (Life Technologies) with 35 cycles of the following thermal profile: 94°C for 20 s, 60°C for 30 s, 68°C for 1 min. Amplified fragments were separated on a 1.8% agarose gel and detected with SYBR Green I (Lonza, Rockland, ME, USA).

2.7. Real-time quantitative PCR

The quantitative PCR assay was performed using TaqMan probes selected from the Universal ProbeLibrary (Roche Diagnostics) and the LightCycler (Roche Diagnostics), according to the manufacturer’s instructions. Selection of TaqMan probes and primer design were performed at the Universal ProbeLibrary Assay Design Center Website. The primer sets used were: Acta1 (NM_009606.2) sense: 5′-AATGAGCCCTTTGCTTGC-3′, antisense: 5′-ATCCCGGAGACCTCATAC-3′; Acta2 (NM_007392.2) sense: 5′-CTCTTCTGACCCATCTTCCAT-3′, antisense: 5′-TATAGTTGCTTTGCCATGC-3′; and Actb (NM_007393) sense: 5′-CTAAGGCCAACCCTGGAAAAG-3′, antisense: 5′-ACGACAGCCATACAGGGACA-3′. Transcript copy numbers of target genes were calculated using amplicons: Acta1 804–920 fragment, Acta2 816–980 fragment, and Actb 371–534 fragment. Copy numbers were corrected using Actb as an internal standard.

2.8. Immunocytochemistry

Parotid glands and parotid acinar cells were fixed with 4% paraformaldehyde. Fixed specimens were then rapidly frozen at −35°C, and cut into 6-μm slices. For anti-aquaporin 8, parotid glands were rapidly frozen in precooled isopentane, cut into 8-μm-thick slices on a cryostat, mounted on slides and imersed in 4% paraformaldehyde. Individual semi-thin sections were incubated overnight with anti-aquaporin-8 (dilution 1:500), anti-aquaporin-5 (1:500), anti-actin-α1 (1:500), or anti-actin-α2 (1:500) antibody. After incubation and washing steps, these sections were exposed to fluorescent dye-conjugated secondary antibodies as described above. Alexa Fluor 555 phallolidin and DAPI were used to stain actin filaments and nuclei, respectively. Stained sections or cells were examined and photographed under a confocal laser scanning microscope (LSM 710; Zeiss, Oberkochen, Germany).
2.9. Statistical analysis

Data were analyzed using unpaired Student’s t-tests to assess differences. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of myoepithelial cells in mouse parotid glands

Myoepithelial cells are located on the basal surface of acini and ductal cells as a thin layer, and have star-shaped morphology. Immunocytochemistry was performed on mouse parotid glands with antibodies against actin-α2 (smooth muscle protein which has been used as a marker for myoepithelial cells), aquaporin 8 (expression of which in myoepithelial cells has been reported previously [7]), and actin-α1 which has not been examined. Myoepithelial cells in parotid glands were immunoreactive to anti-actin-α1 antibody, as well as the other antibodies (Fig. 1). To certify the results, we separated myoepithelial cells from the parotid glands; parotid glands were dispersed with collagenase after trypsin treatment; the precipitate obtained by centrifugation at 160g from the enzyme digests included acinar cells bound to myoepithelial cells (Fig. 2A). To separate myoepithelial cells from acinar cells, the fraction was treated with 2 mM EDTA following density gradient centrifugation. The high density fraction contained the acinar cells, as determined by RT-PCR (Fig. 2D) and the micrograph (data not shown). Immunocytochemistry of the low-density fraction showed that cells included in the fraction were immunoreactive both to anti-aquaporin 8 and anti-actin-α1 antibodies (Fig. 2B). As these cells tended to be round, unlike the typical shape of myoepithelial cells, we found their original shapes by the gradient-density centrifugation after sonicating the prefixed myoepithelial cell-bound acinar cells. The cells were then confirmed as myoepithelial cells by their shape (Fig. 2C). RT-PCR of these fractions showed expression of Aqp8, Acta2 and Acta1 in the MEC fraction, but little expression of Aqp5—a marker for acinar cells (Fig. 2D). These indicated that this skeletal muscle component was specifically expressed in myoepithelial cells in parotid glands, and actin-α1 could be a marker for myoepithelial cells.

3.2. Use of actin-α1 expression to detect pathogenesis of parotid glands of NOD mice

At 28 weeks of age, NOD mice were separated and housed into two groups—diabetic and non-diabetic; the latter was recognized as a pre-diabetic group, because both might have the same autoimmune characters as defined previously [3,5]. Comparative experiments used four mice in each group. First, we compared expression of aquaporins 5 and 8 in parotid glands among the mouse groups (Fig. 3A, upper). Aquaporin 5 expression was similar, but that of aquaporin 8 was less in the diabetic NOD mice. The extracellular area stained by phalloidin (actin filaments) was also smaller in diabetic NOD mice (Fig. 3A, lower). Expression of aquaporin 8, actin-α1 and actin-α2 was less in diabetic NOD mice, but that of aquaporin 5 was similar (Fig. 3B). Decreased mRNA expression of Aqp8, Aqp1 and Aqp2 was also seen (Fig. 3C). Quantitative real-time PCR was used to study reduced gene expression in the diabetic NOD mice (Fig. 4). Relative expression of both Acta1 and Acta2 to the internal standard Actb was lower in the diabetic NOD mice than in control mice.

4. Discussion

Although myoepithelial cells have been isolated from, and their function identified in, mammary glands [10,11], those in salivary glands have not been isolated. Myoepithelial cells surround ductal and acinar cells in salivary glands, and they reportedly support acinar and ductal cells [12], and support stimulation of saliva secretion by...
contraction [13], but these roles are not well studied. Myoepithelial cells in mammary glands and those in salivary glands may have different characters; CD10 (CALLA, common acute lymphoblastic leukemia antigen), a marker of mammary myoepithelial cells, is reportedly not expressed in salivary glands [14]. The characters of these myoepithelial cells should be further compared.

Myoepithelial cells contain components of smooth muscle, including actin-α2, which is used as a marker [15], but is not specific for these cells [12]. Here, we isolated myoepithelial cells from mouse parotid glands; this investigation was our first attempt at salivary gland research. The isolated myoepithelial cells of parotid glands expressed mRNA transcripts of the skeletal muscle-related genes, Acta1 (Fig. 2D), and were immunoreactive to actin-α1 antibody (Fig. 2B). The presence of skeletal muscle components in myoepithelial cells has not been reported. Epithelial cells are of mesodermal origin and contain smooth muscle, whereas, myoepithelial cells are of ectodermal origin, similar to that of sphincter pupillae muscle and iris dilator. Expression of the skeletal muscle components in myoepithelial cells may relate to their origin; by extension, further studies might find such skeletal muscle components in sphincter pupillae muscle and iris dilators. We would not expect to find other skeletal muscle components in the parotid glands, as shown in our experiments (Fig. 1C); therefore, actin-α1 and its mRNA could be used as markers for myoepithelial cells in the parotid glands.

In this study, we found that actin filaments outside acinar cells and expression of Aqp8, Acta2 and Acta1 genes decreased in parotid glands of diabetic NOD mice, indicating atrophy of myoepithelial cells in such mice. Quantitative real-time PCR confirmed that the expression of Acta1 and Acta2 in diabetic mice was also lower than that of controls. Our previous study of parotid glands from diabetic mice indicated increased interspaces between acinar cells [6]. Atrophy of myoepithelial cells may therefore increase the interspace in the parotid glands of diabetic NOD mice.

Loss of myoepithelial cells has been reported in sialadenosis patients [16]. Decrease of actin-α1, actin-α2 and aquaporin-8 may depend on the atrophy or destruction of myoepithelial cells caused by diabetes mellitus as a secondary phenomenon of sialadenosis. As the pathogenesis of xerostomia may include autoimmunity, diabetic NOD mice might develop pathologies in various tissues and salivary glands, similar to patients with Sjögren’s syndrome [17]. The possible relationship between the pathology of Sjögren’s syndrome and that of the model animals used here should be further investigated.

In conclusion, we have shown expression of Acta1, Acta2 and Aqp8, and the proteins encoded by their mRNAs, were significantly reduced in parotid glands of diabetic mice compared with control mice. As non-diabetic NOD mice (which may be in pre-immune states) did not show the critical reduction, the altered protein and gene expressions might result from various etiologies, including immune responses, inflammation, or atrophy, as the near-final stage of tissue destruction, rather than the onset of diabetes. Fewer or atrophied myoepithelial cells in diabetic NOD mice imply the importance of these cells in salivary glands. Further investigation into their role is needed to define the mechanisms of onset of Sjögren’s syndrome and its relationship to saliva secretion.

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