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Research paper

Maintenance of claudin-3 expression and the barrier functions of intercellular junctions in parotid acinar cells via the inhibition of Src signaling

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ABSTRACT

Objectives: Salivary acinar and duct cells show different expression patterns of claudins, which may reflect their different functions. To study the role of claudins in saliva secretion, we examined alterations in the expression patterns of cell adhesion molecules in parotid glands of γ-irradiated rats and analyzed the influence of those changes on intercellular barrier function using primary cultures of parotid acinar cells.

Design: Rats were γ-irradiated with doses of 5, 15 or 20 Gy, and expression levels of cell adhesion molecules were examined by immunoblotting analysis. Acinar cells were isolated from parotid glands and were cultured in the absence or presence of the Src kinase inhibitor PP1. Changes in protein and mRNA expression patterns were determined by immunoblotting and by RT-PCR analyses, respectively. Intercellular barrier function was examined by measuring transepithelial electrical resistance and the paracellular flux of FITC-dextran.

Results: In irradiated parotid glands, the expression of claudin-4 was enhanced at 15 Gy or higher, levels that induce the hyposecretion of saliva, although that increase was transient. At 30 days after irradiation, expression levels of cell adhesion molecules were decreased. In primary cultures, the expression of claudin-4 was also increased transiently but the expression of claudin-3 and E-cadherin was decreased. The barrier function of tight junctions was disrupted although the localization of occludin was maintained. The Src kinase inhibitor PP1 suppressed those changes in gene expression and retained the intercellular barrier function.

Conclusions: These results suggest that the inhibition of Src signaling maintains the barrier functions of intercellular junctions in salivary glands, which can be lost due to tissue injury.

1. Introduction

Cell-cell attachments, such as tight junctions (TJs), adherens junctions and desmosomes, play essential roles in maintaining the cell polarity of epithelial tissues. Claudins are TJ components that may contribute to the degree of paracellular permeability (Furuse & Tsukita, 2006; Heiskala, Peterson, & Yang, 2001; Van Itallie & Anderson, 2004). In rodent salivary glands, claudin-3 expression was detected both in acinar and in duct cells, but claudin-4 was expressed only in duct cells (Peppi & Ghabriel, 2004), which may affect the barrier properties of their TJs. We have previously reported that the expression of claudin-4 increased transepithelial electrical resistance (TER) and decreased the paracellular permeability of submandibular cells (Michikawa, Fujita-Yoshigaki, & Sugiya, 2008). The difference in claudin expression between acinar and duct cells is likely to be correlated with differences in their functions, the production of primary saliva and the modification of ion composition, respectively.

Decreased levels of saliva are a serious problem in clinical dentistry since saliva maintains a healthy environment of the oral cavity. The hyposecretion of saliva and consequent dry mouth leads to severe dental caries, periodontal disease and mucosal infections (Baum, 2000; Sreebny, 1996). Radiotherapy to treat head and neck cancers and autoimmune diseases such as Sjögren syndrome result in the hyposecretion of saliva due to the atrophy of acinar cells because primary saliva is produced by acinar cells. Tissue injuries caused by irradiation and inflammation may lead to the apoptosis and/or dysfunction of acinar cells, but the mechanism involved is unclear. In order to study the mechanism of dysfunction of salivary glands, we established a

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system to culture parotid acinar cells and focused on analyzing changes in their functions of generating and exocytosing secretory granules and in their morphology (Fujita-Yoshigaki, Tagashira, Yoshigaki, Furuyama, & Sugiya, 2005). During that analysis, we found that the expression patterns of claudins were remarkably changed (Qi et al., 2007). Those changes can be considered a process of dedifferentiation because the expression levels of other differentiation markers were also altered. Levels of immature duct markers, such as claudin-4, claudin-6 and cytokeratin-14, were increased but levels of acinar markers, such as claudin-10, aquaporin-5 and amylase, were decreased. Inhibitors of Src and p38 MAP kinases suppressed those changes and increased the expression of acinar marker proteins (Fujita-Yoshigaki, Matsuki-Fukushima, & Sugiya, 2008). The activation of p38 MAP kinase occurred during cell isolation from parotid glands, which could be suppressed by the Src kinase inhibitor PP1. The enzymatic digestion of parotid glands with collagenase and hyaluronidase used for cell isolation may have triggered the stress signal to activate p38 MAP kinase via Src kinase activation (Fujita-Yoshigaki et al., 2008; Moriyama, Yokoyama, & Katsumata-Kato, 2015). Cellular stresses induced during cell isolation are considered to cause dedifferentiation and the transition to duct-like cells, including the change in claudin expression through the Src-p38 MAP kinase signaling pathway. However, the analysis of gene expression patterns in the primary cultures in those previous studies was limited to a short period and the relevance of the changes in claudins to the production of fluid components of saliva has not been investigated.

In this study, we examined the effects of γ-irradiation on the expression patterns of cell adhesion molecules and compared it to the expression patterns in primary culture, since the dysfunction of salivary glands caused by γ-irradiation has been analyzed in detail. We found that irradiation at 15 Gy or higher, which has been reported to cause irreversible hyposecretion of saliva from parotid glands, induced a transient increase of claudin-4 and a subsequent decrease in claudin-3 and E-cadherin. We also found that similar changes occurred in primary cultures of parotid acinar cells. When the decrease in claudin-3 and E-cadherin was suppressed by the inhibition of Src family kinases (SFK), the barrier functions of TJs were maintained. The expression of occludin and zonula occluden-1 (ZO-1) did not affect the barrier functions of TJs.

2. Materials and methods

2.1. Materials

Mouse monoclonal anti-E-cadherin and anti-vimentin antibodies (RV202) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). For immunofluorescence microscopy, the mouse monoclonal anti-vimentin (V9) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the mouse monoclonal anti-occludin and anti-claudin-4 and rabbit polyclonal anti-claudin-3 and anti-ZO-1 antibodies were from Zymed (San Francisco, CA). The goat polyclonal anti-claudin-6 antibody was from Santa Cruz Biotechnology. The rabbit polyclonal anti-amylase antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Src kinase inhibitor PP1 was purchased from Calbiochem (San Diego, CA, USA).

2.2. Animals

Male young adult Sprague-Dawley rats weighing 150–200 g each were obtained from Sankyo Labo Service Corporation (Tokyo, Japan). The rats were kept under controlled conditions and were allowed free access to food and water. All animal experiments conformed with institutional guidelines for the use of experimental animals and were approved by the Experimental Animal Ethical Committee of Nihon University School of Dentistry at Matsudo.

2.3. Irradiation

Under anesthesia with sodium pentobarbital (30 mg/kg body weight), rats were wrapped in bubble cushion and were retained on polystyrene foam in order to maintain their body temperature. They were irradiated in a single exposure at a dose rate of 0.9 Gy min^{-1} using a ^{137}Cs γ-ray (662 keV) source (69.5 TBq) under anesthesia at the Research Center for Nuclear Science and Technology, University of Tokyo. A 20-mm lead shield with a portal 2.5 × 10 cm^2 was positioned over the body so that only the salivary gland region was exposed to γ-ray irradiation. The gland area was irradiated with a dose of 5, 15 or 20 Gy. Dose rates were measured in air with a dosimeter. The control rats were sham-irradiated. For measurement of saliva flow rates, rats were anesthetized with sodium pentobarbital (30 mg/kg body weight). Samples of saliva were collected with rolled cotton for 5 min, and amounts of saliva were determined by weight. Triplicate measurements for each rat were done to determine the saliva flow rates reproducibly. Glands were taken at 2, 3, 7 or 30 days after irradiation and were used for immunoblotting analysis and immunofluorescence microscopy.

2.4. Preparation and culture of isolated acinar cells

Parotid glands were taken from male Sprague-Dawley rats (150–200 g each) under anesthesia with 3% sevoflurane. Acinar cells were isolated by digestion with collagenase and hyaluronidase in isolating buffer (Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4) as described previously (Fujita-Yoshigaki et al., 2005). The cells were over 90% viable as determined by Trypan blue exclusion. Cells were diluted to 0.3 mg/ml with Waymouth's medium containing 10% rat serum, ITS-X supplement, 1 μM hydrocortisone, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 nM cystatin, and were cultured at 37 °C in 5% CO_2. The SFK inhibitor PP1 was dissolved in dimethylsulfoxide (DMSO) to a concentration of 20 mM as a stock solution and was diluted 1:2000 (final concentration of 10 μM) with the culture medium. The same volume of DMSO was added to the control medium (final concentration of 0.05%). We confirmed that 0.05% DMSO had no effect on the changes in gene expression reported in this study. Media were changed at 1 day and at 4 days after the cell isolation and PP1 was added to the fresh medium.

2.5. SDS-PAGE and immunoblotting analysis

Glands and cells were lysed with 20 mM HEPES (pH 7.4) containing 0.1% Triton X-100 and 1 × Complete Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland). Proteins were separated by SDS-PAGE and were transferred to Hybond-LFP membranes (GE Healthcare, Buckinghamshire, UK). To compare the expression levels of the proteins, we performed transfer and blotting of samples from the control and from the PP1-treated glands on the same membrane. We also used the same homogenates and cell lysates of 0 day when the expression levels were compared. Each membrane was blocked at room temperature for 1 h in ECL Prime Blocking Reagent (GE Healthcare), and was probed for 2 h with appropriate primary antibodies. Immunoreactivity was determined using ECL-Plex (GE Healthcare) and images were acquired using Typhoon Trio (GE Healthcare). Intensities of immunoreactivities were quantified using ImageQuantTL software (GE Healthcare).

2.6. RNA preparation and real time RT-PCR analysis of mRNA expression levels

Total RNA was isolated from parotid glands and from parotid acinar cells immediately after their isolation, or from cells after 2–168 h in culture, using the TRIzol reagent (Invitrogen, Carlsbad, CA). After treatment with DNase I, RNA was purified using an RNeasy Mini kit (Qiagen, Hilden, Germany). Amounts of RNA were quantified by