Effects of [6]-Gingerol on Dedifferentiation of Salivary Acinar Cells

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Abstract

Salivary gland atrophy is an important problem in clinical dentistry since salivary glands produce and secrete saliva that creates and regulates the environment of the oral cavity. To clarify mechanisms of salivary gland dysfunction, we have established a system for primary culture of parotid acinar cells. We have found that the process of cell isolation induced the stress signal mediated by Src and p38 MAP kinases, which causes the alteration in expression patterns of various differentiation markers. Claudin-4 began to be expressed after the cell isolation and increased during the culture while the original acinar cells do not express claudin-4. In addition, the expression of salivary acinar markers such as amylase and aquaporin-5 were rapidly decreased during the culture, which implies the dedifferentiation of acinar cells. In this study, we examined the effects of [6]-gingerol, which is an ingredient of ginger, on the dedifferentiation of parotid acinar cells. We found that the increase of expression level of claudin-4 mRNA during the culture was suppressed by addition of [6]-gingerol. The effect was confirmed by immunoblot analysis. In addition, the decrease of amylase and aquaporin-5 during the culture was also suppressed by [6]-gingerol. These results suggest that [6]-gingerol has a protective effect against the cellular stresses that induce dysfunction of salivary glands.

Introduction

Salivary gland dysfunction is an important problem in clinical dentistry since salivary glands produce and secrete saliva that maintains the health environment of the oral cavity. Decrease of saliva causes severe dental caries and periodontal disease(1, 2). To clarify mechanisms of salivary gland dysfunction, we have established a system for primary culture of parotid acinar cells(3, 4). Using the primary culture system, we have found that the process of cell isolation using digestive enzymes induced the stress signal mediated by Src and p38 MAP kinases, which results in the dedifferentiation of parotid acinar cells(5). For example, the expression pattern of tight junction (TJ) proteins was changed(4).

TJs function as selective barriers that allow the passage of some ions and solutes through the paracellular pathway, which is an essential function for epithelial tissues. The claudin family consists of more than 24 members and the combinations of different claudins expressed determine the overall barrier properties of the TJs(6, 7). Which claudin members are expressed is important for functions of epithelial tissues. In salivary glands, claudin-4 was expressed only in ducts but not in acinar cells while claudin-3 is expressed in both ducts and acinar cells(8). It is proposed that TJs of acini have high permeability to produce primary saliva(9). Since expression of claudin-4 decreased the permeability of TJ in salivary cells, the absence of claudin-4

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expression in acinar cells may be important for production of saliva. We found that the expression of claudin-4 increased with time in culture, which may correlate with loss of function as acinar cells(10).

To develop the therapeutic approach for dry mouth, we focused the effect of ginger. Ginger (*Zingiber officinale*) is used as an oriental medicine. [6]-Gingerol is a major active component of ginger and exhibits various pharmacological effects including antioxidant and anti-inflammatory activities. [6]-Gingerol has been reported to inhibit the signal pathways mediated by Erk and p38 MAP kinases, which induce inflammation reactions(11). In this study, we examined the effect of [6]-gingerol on the alteration in expression of claudin-4 and differentiation markers in the primary culture of parotid acinar cells.

**Materials and Methods**

**Preparation and culture of isolated acinar cells**

Parotid glands were taken from male Sprague–Dawley rats (150–200g each) anesthetized with sodium pentobarbital (Dainippon Pharmaceutical, Osaka, Japan). The experiment conforms with institutional guidelines for the use of experimental animals and was approved by the Experimental Animal Ethical Committee of Nihon University School of Dentistry at Matsudo. Acinar cells were isolated by digestion with collagenase A and hyaluronidase in isolating buffer (Hanks’ balanced salt solution containing 20mM Hepes/NaOH, pH 7.4) as described previously (5). 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, 1μM hydrocortisone, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10 nM cystatin in the absence or presence of 50μM [6]–gingerol, and were cultured at 37℃ in 5% CO₂. [6]–gingerol was purchased from Sigma-Aldrich and claudin-4 were designed according to the previous studies (4, 12). PCR products were evaluated by melting curve analysis according to the manufacturer’s instructions and by examining the size of the PCR products separated on 2.0% agarose gels. Relative RNA equivalents for each sample were obtained by normalizing to GAPDH levels. Each sample was run in duplicate to determine sample reproducibility, and the average relative RNA equivalents per sample pair were used for further analysis.

**Immunoblot analysis**

Mouse monoclonal anti–claudin–4 was purchased from Invitrogen. Rabbit polyclonal anti–aquaporin–5 (AQP5) antibody was purchased from Millipore (Billerica, MA). Rabbit polyclonal anti–GAPDH antibody was purchased from Sigma–Aldrich. Cells were harvested and lysed in homogenizing buffer (150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM PMSF, 10mM NaF, 1mM Na₃VO₄, 50mM β–glycerophosphate, 20mM Hepes/NaOH, pH 7.4) containing 1 X Complete Protease Inhibitor Cocktail. Proteins were separated by SDS–PAGE, and were transferred to Hybond–LFP membranes (GE Healthcare, Buckinghamshire, UK). The same amounts of proteins were applied to each lane of SDS–PAGE. The membranes were blocked at room temperature for 50 minutes in Blocking Agent (GE Healthcare) and blotted with antibodies. Immunoreactivity was determined by using an ECF Western blotting kit (GE Healthcare) and the images were acquired by using Typhoon Trio (GE Healthcare) and the fluorescence intensities were measured with ImageQuant (GE Healthcare).

**Amylase assay**

The activity of amylase was measured by the method reported previously (13), modified for incubation at 30℃ for 5 minutes. In this method, one unit of amylase is defined as the quantity of enzyme that liberates 1mg of maltose per minute at 30℃.
Statistical analysis

Data are compared using Student’s t-test for unpaired groups. When \( p < 0.05 \), it was considered as statistically significant.

Results

Effect of [6]-gingerol on expression of claudin-4

Real time RT-PCR revealed that expression level of claudin-4 mRNA was transiently increased during the culture (Fig.1). Its expression level was the highest at 1 day after the cell isolation and gradually decreased. At 7 days, the expression level became similar to that immediately after cell isolation. The increase of claudin-4 was suppressed by addition of 50 \( \mu \)M [6]-gingerol. Although no significant difference was observed at 1 or 3 days, its expression levels in the presence of [6]-gingerol were lower than the control at all time points examined in this study. There is a significant difference between the expression levels in the absence and the presence of [6]-gingerol at 2 days. To confirm the suppression of claudin-4 expression by [6]-gingerol, immunoblot analysis using anti-claudin-4 antibody was performed. Because the amount of the claudin-4 protein is increasing for 3 days in the control culture (Fig.1), we compared the amounts of claudin-4 in the cell lysates harvested at 3 days. As shown in Fig.2, the protein level of the claudin-4 in the cells cultured for 3 days in the presence of [6]-gingerol was lower than the control. These results indicate that [6]-gingerol suppressed the increase of claudin-4 expression, which may rescue the decline in the permeability of TJs after tissue damages.

Effect of [6]-gingerol on maintenance of salivary acinar markers

Because the expression of claudin-4 is negatively correlated to the differentiation degree in the previous studies (4), we examined the expression of salivary acinar markers, amylase and aquaporin-5. We harvested the cells cultured in the absence or presence of [6]-gingerol for 3 days and 7 days, and examined the relative amylase activity in the cell lysates. Although relative amylase activity was declined during the culture as previously reported (5), the activity of the cells cultured in the presence of [6]-gingerol was significantly higher than the control (Fig.3). Amylase is stored in secretory granules of parotid acinar cells, and the abundance of secretory granules indicates the maintenance of functions as acinar cells. The increase of amylase activity implies the maintenance of differentiation degree. Next, we investigated the protein levels of aquaporin-5 in the culture. Aquaporin-5 is specifically expressed in acinar cells, but not in ducts in the original salivary glands (14). Whereas aquaporin-5 decreased during the culture, addition of [6]-gingerol maintained its expression (Fig.4). These results suggest that [6]-gingerol maintained the differentiation degree of salivary acinar cells.
Discussion

Water of primary saliva is derived from blood serum. There are two pathways that water passes through the epithelium of salivary glands: paracellular and transcellular pathways. TJ's regulate the paracellular pathway and channels and transporters control the transcellular pathway. Claudins determine the permeability of TJs. The expression of claudin-4 decreases permeability of TJ (10) and could play a major role in the rapid restoration of barrier function and protection of the organism following disruption of the epithelium (15). At the same time, the expression may decrease saliva secretion because TJs expressing claudin-4 have low permeability for Na+ and the paracellular transport of Na+ is essential for the generation of primary saliva (16). For transcellular pathway, aquaporin-5 is considered an essential component for water secretion. Aquaporin-5 is a major water channel in salivary glands and it was reported that knockout of aquaporin-5 results in remarkable decrease of saliva secretion (17). The high expression of claudin-4 and decrease of aquaporin-5 inhibit the water transport through both intercellular and intracellular pathways. To suppress the alterations in gene expression by [6]-gingerol may rescue the dysfunction of salivary glands induced by cellular stresses.

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