Expression of the Stem Cell Marker Nestin in Response to Tissue Injuries of Parotid Acinar Cells

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Abstract
Since saliva is essential for maintenance of a healthy oral environment, the secretory function of the salivary gland is important in clinical dentistry. Although tissue injuries result in a decrease in acinar cells and consequent dysfunction of saliva secretion, the number of acinar cells recovers if the damage is not too severe. The origin of the regenerated acinar cells is unclear. One hypothesis is that acinar cells that are atrophied in response to tissue injuries can re-differentiate. We have previously established a primary culture of parotid acinar cells to study the mechanism of dysfunction and regeneration of salivary glands. During the culture, the expression levels of acinar markers decreased, whereas immature ductal markers increased, suggesting that the cells changed to immature duct-like cells. In this study, to clarify the properties of the cultured cells, the expression of nestin, a stem cell marker of the pancreas, was examined. It was found that nestin began to be expressed and increased during the culture. On immunofluorescence microscopy, nestin-expressing cells had secretory granules, indicating that the cells were derived from acinar cells. Src family kinase inhibitor PP1 suppressed the expression of nestin. It is possible that nestin expression is a programmed response to survive cellular stresses and to acquire re-differentiation potential.

Introduction
Irradiation treatment for head and neck cancer frequently causes dysfunction of salivary glands and consequent reduction of quality of life. With decreased saliva, patients are much more likely to suffer serious dental caries, periodontitis, and mucus infections, as well as difficulties in mastication and swallowing (1). Thus, it is important in clinical dentistry to clarify the mechanism for protecting and recovering salivary gland function in the face of tissue injuries such as γ-irradiation.

In irradiated salivary glands, the number of acinar cells decreases and ducts become prominent, which leads to hypossecretion of saliva. If the damage is not too severe, acinar cells increase again, and their function to generate saliva is recovered after the irradiation (2). The origin of regenerated acinar cells is still under debate. The presence of adult stem cells that reside in intercalated ducts has been proposed (3). The stem cells are activated and increase in response to tissue injuries and differentiate into both acinar and duct cells. In contrast, acinar cells have been reported to have the capacity of self-duplication (4). Tissue injuries change the morphology and gene expression patterns of acinar cells into duct-like cells, and the cells re-differentiate to acinar cells after the injuries (5, 6).

We have established a system of primary culture of parotid acinar cells to study the mechanism of salivary gland dysfunction induced by tissue injuries (7). Although the cultured acinar cells have maintained their capacities of granule generation and stimulus-dependent exocytosis for at least three days, the functions decline gradually. The expressions of acinar cells, such as aquaporin-5 and amylase, decreased, and ductal markers such as claudin-4 began to be expressed (8). These changes were induced by the process of cell isolation, which triggers cellular stress signals. During the cell isolation process, activation of Src family kinases...
(SFKs) and p38 MAP kinase (MAPK) occurred, which is essential for the changes in gene expression patterns (9, 10). Therefore, these changes are considered a programmed response to tissue injuries. The cultured acinar cells also expressed Claudin-6, which is transiently expressed during salivary gland development, and Cytokeratin-14, which is expressed in the basal cells of excretory ducts, suggesting that the character of the cultured cells is similar to that of undifferentiated duct cells (9). There is a possibility that the changes of acinar cells induced by tissue injuries imply not just dysfunction, but the acquisition of re-differentiation potential.

As a candidate stem cell marker of parotid acinar cells, we focused on nestin. Nestin was first identified as a stem cell marker of neurons (11) and was also reported to be expressed in other developing organs (12, 13). Pancreatic exocrine cells, which have a similarity to parotid acinar cells, are differentiated from nestin-positive precursor cells (14). To investigate the character of the cultured cells, the expression of nestin was examined.

Materials and Methods

Primary culture of rat parotid acinar cells

Parotid glands were taken from male Sprague-Dawley rats (150–200 g each) under anesthesia. Acinar cells were isolated and cultured as described previously (7). Isolated acinar cells were cultured in the absence and presence of the SFK inhibitor PP1 (Calbiochem, La Jolla, CA, USA; final concentration of 10 μM). Media were changed at 1 and 4 days after cell isolation, and PP1 was added to the fresh medium.

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

Total RNA was isolated from the parotid acinar cells immediately after isolation or culture for 1–7 days, using the TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini kits (Qiagen, Hilden, Germany). Amounts of RNA were quantified by measuring the absorbance at 260 nm. The expression level of nestin mRNA was determined with the QuantiTect® SYBR® RT-PCR kit (Qiagen) using Thermal Cycler Dice (Takara Bio, Kusatsu, Japan). Amplification of rat GAPDH was performed according to a previous study (8). The primer pair for the amplification of rat nestin was as follows: forward 5'-GCT ACA TAC AGG ACT CTG CTG-3' and reverse 5'-GAG CAC AGA TTC CAG GTA CT-3', with a predicted size of 147 bp. PCR products were evaluated by melting curve analysis and by examining the sizes of the PCR products separated on 2.0% agarose gels. Relative RNA expression levels for each sample were obtained by normalizing to GAPDH.

Immunoblotting analysis

Glands and cultured 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 transferred to Hybond-LFP membranes (GE Healthcare, Buckinghamshire, UK). Each membrane was blocked in ECL Prime Blocking Reagent (GE Healthcare) and was probed with mouse monoclonal anti-nestin antibody (Cell Signaling). Immunoreactivity was determined by ECL-Plex (GE Healthcare), and images were acquired using Typhoon Trio (GE Healthcare). Intensities of immunoreactivities were quantified using ImageQuantTL software (GE Healthcare).

Immunofluorescence microscopy of cultured acinar cells

Isolated acinar cells were cultured in collagen I-coated glass-base dishes (AGC Techno Glass, Haibara, Japan). Cells were fixed with 10% formalin in phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100/PBS. After blocking with 1% bovine serum albumin and 0.05% pre-immune goat IgG in PBS, the cells were labeled with mouse monoclonal anti-nestin and rabbit polyclonal anti-amylase (Sigma-Aldrich, St. Louis, MO, USA) antibodies, followed by Alexa Fluor 568-conjugated anti-mouse IgG and Alexa Fluor 647-conjugated anti-rabbit IgG antibodies. Images were acquired using an LSM-510 confocal microscope.

Statistical analysis

Statistical testing was performed using the paired t-test. Values are shown as means ± SEM.

Results

Expression of the stem cell marker nestin in primary culture of parotid acinar cells

Real time RT-PCR analysis showed that the expression level of nestin mRNA in parotid acinar cells increased during the culture and reached its peak at 3 days. After that, its expression decreased slightly (Fig. 1). The nestin protein
was faintly detected in the homogenates of parotid glands, but not in the isolated acinar cells. Nestin was detected in the cell lysates at 1 day, and its amount increased over the culture (Fig. 2).

Because the parotid gland consists of several types of cells, such as acinar, duct, myoepithelial, and mesenchymal cells, there is a possibility that nestin is expressed in other than acinar cells in the culture. Although we confirmed that more than 90% of cells were acinar cells after cell isolation, there was contamination by cells other than acinar cells in the culture (9). To identify the nestin-positive cells in the culture, immunofluorescence microscopy was performed using anti-nestin and anti-amylase antibodies. Amylase is a good marker of acinar cells, and its antibody labels secretory granules. At 3 days after cell isolation, secretory granules were observed in the cultured cells, indicating that the cells were derived from acinar cells. The same cells that had secretory granules were also labeled with anti-nestin antibody (Fig. 3). Nestin signals were observed as a filament meshwork. Nestin is an intermediate filament and forms network structures in the cytosol. These results showed that nestin is expressed in acinar cells and increased during the culture.

**SFK signaling is involved in nestin expression**

In the previous studies, we reported that tissue injuries triggered the SFK-p38 MAPK signal via generation of reactive oxygen species (10). Inhibition of SFK and p38 MAPK suppressed the changes in gene expression patterns of cultured acinar cells (9). To clarify whether the same signaling pathway is involved in nestin expression, the effect of PP1, an SFK inhibitor, was examined. Addition of PP1 suppressed the increase of nestin, which was shown by both real time RT-PCR and immunoblotting analysis. The expression level of nestin mRNA was significantly less in the cells cultured in the presence of PP1 than in its absence (**P < 0.01; ***P < 0.001, paired t-test; Fig. 1). The nestin protein was also decreased by addition of PP1 (Fig. 2). These results indicate that the expression of nestin is mediated by activation of SFK.

**Discussion**

In this study, it was found that nestin, which is considered a stem cell marker of the pancreas, began to be expressed in the cultured parotid acinar cells. Its expression may be a programmed response to tissue injuries, which is mediated by the SFK-p38 MAPK signaling pathway. It has been
reported that nestin-positive cells in the pancreas can differentiate into not only exocrine, but also endocrine cells, which indicates that nestin-positive cells are multipotential (15). It is likely that the changes in gene expression in the cultured parotid acinar cells mean not just dysfunction, but a process to revert to a primitive phenotype. There is a possibility that parotid acinar cells in the primary culture also maintain the potential for re-differentiation into acinar cells.

It has been reported that nestin increased transiently in response to EGF, which causes pancreatic acinar-ductal metaplasia (16). Since metaplastic ductal epithelium is considered a premalignant lesion in the pancreas, the expression of nestin is likely involved in tumorigenesis. Nestin was reported to be observed in human salivary gland tumors, whereas it was rarely expressed in non-neoplastic glands (17). The possibility is raised that the dedifferentiated acinar cells induced by tissue injuries are a source of salivary gland tumors. Further studies are required to uncover the involvement of oncogenic transformation and alteration of acinar cells in salivary glands.

**Conflicts of interest**

The authors have no potential conflicts of interest.

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