Effects of Human Dental Pulp Stem Cell-Derived Conditioned Medium on Atrophied Submandibular Gland after the Release from Ligation of the Main Excretory Duct in Mice

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Abstract: The salivary gland duct ligation model for the treatment of obstruction caused by salivary stones causes hypofunction of the salivary gland. However, sufficient results have not been obtained regarding the regeneration of acinar cells and the restoration of function, and the development of treatment methods is still ongoing. Here, we administered dental pulp stem cell-derived conditioned medium (DPSC-CM) containing various growth factors to mice and examined its effects. The main excretory duct of the submandibular gland was ligated; after 3 weeks, the ligation was released, and Dulbecco’s modified Eagle’s medium (DMEM) or DPSC-CM was administered biweekly via the right jugular vein. The mice were euthanized at 2, 4, and 8 weeks after the release of the ligation, and the submandibular glands were collected. After the release, the acinar cells had a time-dependent recovery. The DPSC-CM group exhibited a significant increase in the expression of progenitor cell marker cytokeratin 5 (CK5) at 4 weeks after the release of the ligation and acinar cell marker aquaporin 5 (AQP5) at 8 weeks compared with the control and DMEM groups by real-time RT-PCR. The administration of DPSC-CM in mice after the release of the submandibular main duct ligation promotes acinar cell regeneration.

Key words: Acinar cell marker aquaporin 5, Duct ligation, Regeneration, Dental pulp stem cells-conditioned medium, Salivary gland

Introduction

Salivary gland hypofunction is caused by changes in the salivary gland tissues associated with aging, salivary gland diseases1, autoimmune diseases2, and radiation exposure3. Salivary glands play various roles in the oral cavity, such as digestion, mucosal protection, self-cleaning, pH buffering, and remineralization. Salivary gland hypofunction causes dry mouth, dental caries, periodontal diseases, and ingestion/swallowing disorders and has a negative impact on the maintenance of the oral environment and daily life4. Salivary gland hypofunction treatments include the protection of oral mucosa with moisturizers, gargles, and artificial saliva; oral saliva stimulants and herbal medicines; and salivary gland massage. However, no fundamental treatment methods have been established.

There have been numerous studies on the salivary gland tissue after ligation and ligation release as a model of duct obstruction by salivary gland stones5-8. The atrophied salivary glands after ligation release express stem cell and progenitor cell markers and exhibit the remaining self-renewal capacity9-16. Therefore, adaptation of the salivary gland tissue to regenerative medicine has been considered. In recent years, regenerative medicine using stem cell culture supernatant conditioned medium (CM) derived from various sites of the body, such as dental pulp, bone marrow, adipose tissue, and skin, has been reported17-19, and its clinical application is expected. In this study, we administered dental pulp stem cell-derived CM (DPSC-CM) containing various growth factors to mice after releasing submandibular main duct ligation and examined the effects.

Materials and Methods

Animals

In this study, 60 10-week-old male ICR mice (CLEA Japan, Tokyo, Japan) were used, which were maintained in an animal room on a 12-h light/dark cycle with free access to solid food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. This study was conducted with the Animal Experiment Ethics Committee of the Nippon Dental University School of Life Dentistry at Niigata (approval reference number 139).

Sampling of DPSC-CM

Dental pulp tissues were collected from healthy teeth extracted from individuals aged 22–25 years and were cut into small pieces using a scalpel on a culture dish. Next, primary culture was established in Dulbecco’s modified Eagle’s medium/Ham’s Nutrient Mixture F-12 (DMEM) (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (Life Technologies, CA, USA), 0.1% non-essential amino acid solution (Life Technologies), 1% penicillin–streptomycin (Life Technologies), and 0.1% fungizone (Life Technologies). Cells were cultured in a humidified incubator set at 37°C and 5% CO2. Thereafter, the pulp cells were serially subcultured to passage 3 (P3). The media was switched to serum-free DMEM/F12 at 70%–80% confluence at P3, and the cells were incubated for an additional 48 h. The culture supernatant was collected, centrifuged at 1,500 ×g, filtered...
through a 0.45-μm pore filter, and used as DPSC-CM. The collection of DPSC was performed with the approval of the Ethical Review Board at the School of Life Dentistry at Niigata, Nippon Dental University (approval number ECNG-H-121).

**Experimental group**

To confirm the condition of the salivary glands after 3 weeks of ligation and the non-ligated salivary glands, tissue samples were collected from the right submandibular glands after 3 weeks of main duct ligation (3W lig group, n = 6) and the left submandibular gland that did not undergo ligation (Non-lig group, n = 6). Mice in which the ligation was released 3 weeks later were divided into three groups: the DMEM biweekly administered group (DMEM group, Number of mice = 18), to which 0.4 ml DMEM was administered via the right jugular vein biweekly under general anesthesia; the DPSC-CM biweekly administered group (CM group, Number of mice = 18), to which 0.4 ml DPSC-CM was administered using a similar method; and the control group (Number of mice = 18), in which mice underwent only the release of the ligation. Six mice each from the DMEM, CM, and control groups were sacrificed at 2, 4, and 8 weeks after the release of the ligation to collect samples.

**Ligation of the main excretory duct of the right submandibular salivary gland, jugular vein administration, and tissue collection**

General anesthesia was performed on mice by intraperitoneal administration of a combination of three anesthetics: medetomidine hydrochloride (0.3 mg/kg; Kyoritsu, Tokyo, Japan), midazolam (5 mg/kg; Astellas Pharma, Tokyo, Japan), and butorphanol tartrate (5 mg/kg; FALMA, Tokyo, Japan). The mice were restrained in dorsal recumbency and a midline skin incision was made on the neck; the right submandibular gland main excretory duct was exposed under a dissecting microscope through the midline skin incision on the neck (Fig. 2A). The duct was ligated approximately 2 mm superior to the main body of the right submandibular gland with a Sugita titanium aneurysm clip (Mizuho Ika

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**Table 1. List of primary antibodies**

| Antibody           | Source (catalog number)       | Dilution |
|--------------------|--------------------------------|----------|
| Anti-c-kit         | Abcam (ab5506)                | 1:500    |
| Anti-cytokeratin 5 | Covance (PRB-160P)            | 1:3,000  |
| Anti-aquaporin 5   | Abcam (ab104751)              | 1:2,000  |
| Anti-α-amylase     | Sigma-Aldrich (AR273)         | 1:1,000  |

Sources: Abcam (Tokyo, Japan), Covance (Princeton, NJ, US), Sigma-Aldrich (St. Louis, USA).
Kogyo Co., Tokyo, Japan), and the wound was closed (Fig. 2B). Three weeks later, the ligation was released via a similar approach (Fig. 2C). Either DMEM or DPSC-CM was administered biweekly through the right jugular vein to the mice in the experimental group in which the ligation was released after 3 weeks.

After collecting submandibular gland tissue for gene expression analysis, the thorax was opened, and the submandibular gland was perfused first with phosphate-buffered saline (PBS, 9.57 mM, pH 7.35–7.65; Takara Bio, Inc., Shiga, Japan) to remove the blood from the capillaries followed by 4% paraformaldehyde (PFA; WAKO Pure Chemical Industries, Osaka, Japan) for perfusion fixation to collect tissue samples for histological analysis.

**Submandibular gland measurement**

The dimensions of the major and minor axes of the submandibular glands were measured with calipers before collecting tissues for gene expression analysis. A two-way analysis of variance (ANOVA) and Tukey’s multiple comparison test were performed for statistical analyses (BellCurve for Excel: Social Survey Research Information, Co., Ltd., Tokyo, Japan). A p value of <0.05 was considered to be statistically significant.

**Tissue preparation and general staining**

Samples for histological analysis underwent immersion fixation with 4% paraformaldehyde (PFA, Wako Pure Chemical Industries, Osaka, Japan) and were dehydrated using an ascending ethanol series before being embedded in paraffin. A rotating microtome was used to create 5-μm sections, which were then subjected to hematoxylin–eosin (HE) staining. Histological observations were conducted under an optical microscope (Olympus BX51, Tokyo, Japan).

**Immunohistochemistry (DAB method)**

Immunohistochemical staining for c-kit, cytokeratin (CK5), aquaporin 5 (AQP5), and α-amylase was performed using the 3,3’-diaminobenzidine (DAB) detection method. The primary antibodies used are listed in Table 1. Paraffin-embedded tissue sections were deparaffinized and rehydrated in xylene and a series of ethanol gradients, respectively, followed by endogenous peroxidase blocking (Dako REALTM peroxidase blocking solution, Agilent Technologies, CA, USA) and antigen retrieval (HistoVT One, Nacalai Tesque, Kyoto, Japan). Sections were subsequently incubated with each primary antibody and then a secondary antibody (EnVisionTM + Dual link System-HRP, Agilent Technologies, CA, USA); detection and visualization of antibody binding were performed using a DAB substrate (ImmPACTTM DAB, Vector Laboratories, CA, USA). After nuclear staining with hematoxylin, sections were cleared and mounted.

**RNA preparation and real-time RT-PCR**

After collection, tissue samples were immediately immersed in RNAlater RNA Stabilization reagent (QIAGEN, Holden, Germany) and frozen until use. Total RNA was extracted using ISOGEN II (Wako Pure Chemical Industries, Osaka, Japan) and BioMasher III (Nippi, Co., Ltd., Tokyo, Japan) according to the manufacturers’ protocols. After purification, complementary DNA (cDNA) was synthesized with a high-capacity cDNA reverse transcription kit (Life Technologies, CA, USA). Gene expressions were confirmed using the reverse transcription-polymerase chain reaction (RT-PCR) and quantified using real-time RT-PCR. A reaction solution was prepared using Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) with the primers used for RT-PCR. Real-time RT-PCR was performed using StepOnePlusTM (Applied Biosystems, Foster City, CA, USA). After initial heating at 95°C for 20 s, PCR conditions were set at 95°C for 3 s and 60°C for 30 s for 40 cycles each. After normalization of the samples with GAPDH, the expression levels of each RNA were analyzed using the ΔΔCT method, with those in the 3W lig group considered as the baseline. Primers used are listed in Table 2. Two-way ANOVA and Bonferroni multiple comparisons were performed for statistical analyses (BellCurve for Excel: Social Survey Research Information, Co., Ltd., Tokyo, Japan). A p value of <0.05 was considered to be statistically significant.

**Results**

**SMG measurement**

The major and minor axes of the submandibular gland were significantly smaller after 3 weeks of ligation. After the ligation release, the size of the gland body showed an increasing, time-dependent trend. A significant increase in size was observed at 2, 4, and 8 weeks after the ligation release (p < 0.01). However, during the period of observation following the release of the ligation, there were no significant changes in the size of the glands among the DMEM, CM, and control groups.

**Histological analysis**

The HE-stained sections of submandibular gland tissues taken from the Non-lig group showed abundant acinar cells and ducts, which are seen in normal submandibular tissues (Fig. 3A). The sections from the 3W lig group showed atrophy and disappearance of acinar cells, with signs of atrophied salivary gland tissue that included the dilation of the interlobular ducts and acini.
ductal lumen and the appearance of duct-like structures (Fig. 3B). At 2 weeks following the release of the ligation, no evident changes from the time of the ligation release were observed in any of the groups (Fig. 3C, F, I). At 4 weeks following the release, signs of atrophy showed a slight improvement; however, no apparent changes were noted between the groups (Fig. 3D, G, J). At 8 weeks after the release, signs of regenerated acinar cells were observed in each group (Fig. 3E, H, K). Notably, these signs were exhibited more by the CM group than by the DMEM and control groups (Fig. 3K). The regenerated acinar cells tended to be smaller in the 3W lig group than in the Non-lig group.
Immunohistochemistry analysis

In the Non-lig group, some cells in the ducts were positive for c-kit, which was used as a stem cell marker (Fig. 4A). In the 3W lig group, areas in and around some ducts and duct-like structures were positive for c-kit and the number of c-kit positive cells had increased by the 3-week ligation stage (Fig. 4B). At 2 weeks after the release of ligation, the number of positive cells showed a slightly decreasing tendency (Fig. 4C, F, I). At 4 and 8 weeks after the release of ligation, the number of c-kit positive cells further decreased; however, there was no apparent difference between the groups (Fig. 4D, E, G, H, J, K).

In the Non-lig group, cells in some ducts were found to be positive for CK5, which was used as a precursor marker for acinar cells (Fig. 5A). In the 3W lig group, CK5 positive cells were observed in some ducts and duct-like structures (Fig. 5B). At 2 weeks after the release of
ligation, no apparent changes in CK5 staining were observed (Fig. 5C, F, I). At 4 weeks after the release of ligation, the number of CK5 positive cells increased in the duct-like structures in each group (Fig. 5D, G, J). In particular, there were more CK5-positive cells in the CM group than in the DMEM and control groups (Fig. 5J). At 8 weeks after the release of ligation, the number of CK5-positive cells showed a decreasing tendency in the CM group (Fig. 5K). Regenerated acinar cells were negative for staining.

Acinar cells in the Non-lig group (Fig. 6A) were positive for AQP5, which is an acinar cell marker. In the 3W lig group, the number of AQP5-positive cells markedly decreased with atrophy of the acinar cells (Fig. 6B). At 2 weeks after the release of ligation, no apparent changes...
were observed in each group (Fig. 6C, F, I). At 4 and 8 weeks after the release, the number of AQP5-positive cells increased in a time-dependent manner. (Fig. 6D, E, G, H, J, K). In particular, at 8 weeks after the release, the number of AQP5-positive cells observed in the CM group was higher than in the DMEM and control groups (Fig. 6K).

Additionally, "α-amylase staining was positive in ducts in the Non-lig group (Fig. 7A), whereas in the 3W lig group, the "α-amylase-positive cells disappeared (Fig. 7B). At 2, 4, and 8 weeks after the release, no apparent changes in "α-amylase staining were observed (Fig. 7C-K).

**Gene expression analysis**

The c-kit expression level (Fig. 8A) was increased by ligation, which showed a decreasing tendency in a time-dependent manner after release, with a significant reduction at 2 and 4 weeks after the ligation release (p < 0.01). There was no significant difference in the c-kit expression levels between the groups.

The CK5 expression level (Fig. 8B) was increased by ligation and subsequently showed an increasing tendency in a time-dependent manner. At 4 weeks after release of the ligation, the CM group showed significantly higher CK5 expression levels compared with the DMEM and control groups (p < 0.01). At 8 weeks after release of the ligation, the CM group showed a decreasing trend in CK5 expression (p < 0.05).

The AQP5 expression level (Fig. 8C) was markedly decreased by ligation and significantly increased after the release of ligation in a time-dependent manner (p < 0.01). At 8 weeks after release of ligation, the expression level of AQP5 was significantly higher in the CM group than in the DMEM and control groups (p < 0.01).

The "α-amylase expression level (Fig. 8D) was markedly decreased by ligation and exhibited a slightly increasing trend over time after the release; however, the changes were not significant.

**Discussion**

Several studies on regenerative medicine for salivary glands have been conducted, and elucidation of the mechanisms involved in the regeneration and development of clinically applicable treatment methods are underway. To restore the salivary gland function, gene therapy has been conducted, and elucidation of the mechanisms involved in the regeneration and differentiation capacities of stem cells, such as growth factors, cytokines, and exosomes; these substances are expected to be therapeutic. The DPSCs used in the present study are relatively easy to obtain and have superior proliferation and differentiation capacities. Moreover, because the culture supernatant does not contain cell components, the risks of developing malignancy and immune rejection are low, and thus, it is expected to be safe. A supply system, called the dental pulp bank, has been established, and these samples can also be frozen to ensure a stable and steady supply. In this study, we administered DPSC-CM to mouse atrophied salivary glands, aiming to develop a novel treatment method for atrophied salivary glands.

The results of this study revealed that in the CM group, in which DPSC-CM was administered biweekly, significantly elevated expressions of CK5 and AQP5 were seen at 4 and 8 weeks after the release of ligation, respectively, suggesting that DPSC-CM may promote acinar cell regeneration. This could be due to the effects of growth factors contained in DPSC-CM, which are involved in the development and growth of salivary glands that are of epithelial origin. Studies focusing on the growth factors for salivary gland tissues reported that the promoting effects on salivary gland regeneration were obtained by addition of epidermal growth factor and basic fibroblast growth factor (bFGF) in vitro and by bFGF administration in vivo. Growth factors are also indispensable for induction and differentiation of salivary gland tissues during the developmental process. Many growth factors are present in the CM, and the mechanisms of action and factors have not been identified to date; these remain the scope of future studies. Substances secreted from stem cells include exosomes, which are cellular secretory vesicles involved in intercellular communication, tissue regeneration, and in the immune system. Exosomes secreted from stem cells contain various proteins, lipids, and mRNAs. Some studies have also reported the therapeutic effects of exosomes on myocardial injury, stroke, and kidney injury. It is necessary to analyze DPSC-CM-derived exosomes and investigate the details for clinical application.

The expression of c-kit, a salivary gland stem cell marker, did not exhibit a significant difference between the groups, whereas the expression of precursor cell marker CK5 was significantly high in the CM group at 4 weeks after the release. Furthermore, the expression of an acinar cell marker, AQP5, was significantly high in the CM group at 8 weeks after the release. These results indicate that DPSC-CM may promote the precursor cells and acinar cells differentiation in atrophic salivary glands. AQP5 is a water channel protein that is expressed in the salivary glands, lungs, and lacrimal glands. AQP5 is present in the acinar cell membrane and plays an important role in exocrine function. The expression of AQP5 reportedly correlates with the amount of salivary secretion, suggesting that the secretory function of the salivary glands in the CM group may be increased compared with that in the DMEM and control groups. If combined with salivary secretion stimulants that have already been used, further recovery of the salivary gland secretory function may be possible by stimulating acinar cells whose regeneration is promoted by CM. Gene expression analysis revealed that "α-amylase, a marker for the salivary gland function, exhibited a slightly increasing trend after the release of ligation; however, there was no significant difference during the observation period or between the groups. It was considered that at 8 weeks after the release, acinar cells in the CM group had a higher tendency for recovery compared with the DMEM and control groups; however, the recovery of the function was not sufficient enough to show an increase in the "α-amylase expression level.

Experiments using ligation of salivary glands have been reported to be of various lengths of duration, ranging from 1 week to as long as 3 months. In this study, the main duct of the right submandibular gland was ligated for 3 weeks. As presented in the 3W lig group, at the time of the release of ligation, sufficient atrophy of the salivary gland tissues was confirmed by HE and PAS staining, immunostaining for various proteins, and real-time PCR, which was consistent with the report by Akadomari et al. On the other hand, studies on observation after the release of ligation have reported to be of various durations after the release, ranging from 1 week to as long as 2 months. Moreover, the salivary gland tissue is thought to recover after the release of ligation if the ligation is within a certain period of time; however, the longer the ligation period, the more severe the irreversible degeneration and the more difficult the self-renewal process. By the 3-week ligation in this study, self-renewal of the salivary gland tissue proceeded slowly in the DMEM and control groups, which appeared to be an appropriate ligation period for comparison between the CM, control, and DMEM groups.

Regarding the method of administration, CM and DMEM were ad-
ministered via the right jugular vein in this study. In mouse experiments, administration through the jugular vein enables CM administration to the ligated submandibular gland at a high level, although it is retrograde, which is considered to be a factor to enhance the effects. However, jugular vein administration is relatively more invasive compared with other administration routes. Thus, it is necessary to examine other administration routes for clinical application.

For salivary gland diseases that lead to severe damage or salivary gland excision, treatment is difficult, except for tissue or cell transplantation. For salivary gland diseases that can be conservatively treated, such as salivary gland stones, salivary gland tissue regeneration may be promoted and recovered by DPSC-CM-containing growth factors. It is necessary to elucidate the factors and mechanisms of action and examine the efficient administration routes for future clinical application.

In conclusion, we administered DPSC-CM to mice after releasing the submandibular gland main duct ligation. Significant elevated expression levels of CK5 and AQP5 were observed at 4 and 8 weeks after the release, respectively. Growth factors contained in DPSC-CM were considered to be responsible for the effects. Altogether, the results of DPSC-CM administration to mice after releasing the submandibular gland main duct ligation suggested that DPSC-CM may promote acinar cell regeneration of atrophied salivary glands.

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Conflicts of Interest
The authors have declared that no COI exists.

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