Original

A Study on the Effect of Human Dental Pulp Stem Cell Conditioned Medium on Human Oral Squamous Cell Carcinoma Cell Lines

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Abstract: In recent years, the application of conditioned medium (CM) in regenerative medicine has received much attention. However, the incidence of cancer in Japan is continuing to rise, with one in two people having cancer, among those who may be candidates for CM treatment and may be affected by or have a risk of cancer. We investigated the effects of human dental pulp stem cell (DPSC)-derived CM on human oral squamous cell carcinoma (OSCC) cell lines. CM was extracted from DPSC isolated from human dental pulp, and cell proliferation rates upon contact with OSCC cell lines were compared. Furthermore, chemosensitivity was evaluated with the collagen gel droplet embedded culture drug sensitivity test and was compared using Dulbecco’s modified Eagle’s medium (DMEM). To test the effect of DPSC-CM on xenograft tumors, an in vivo comparative study was conducted to investigate the tumor proliferation rate upon DPSC-CM and DMEM administration in tumor-bearing nude mice. The tumor growth factor production in culture medium over time was measured with enzyme-linked immunosorbent assay (ELISA) and was compared using DMEM. Vascular endothelial growth factor (VEGF) level significantly increased in the DPSC-CM contact group with ELISA. Therefore, VEGF-A mRNA expression in the OSCC cell line was studied with real-time polymerase chain reaction for comparison. No significant differences were observed in the cell proliferation rate or drug sensitivity between different culture media in each cell line or in vivo tumor proliferation rate. However, VEGF level contained in the cultured medium was significantly higher than that in the DPSC-CM group. VEGF-A mRNA level in OSCC cell lines was significantly higher in the DPSC-CM group, which increased over time. These results suggested that exposure to DPSC-CM does not immediately affect tumor growth or drug resistance, but induces VEGF overexpression in tumor cells.

Key words: Human dental pulp stem cell, Human oral squamous cell carcinoma, Tumor growth factor, Collagen gel droplet-embedded culture drug sensitivity test

Introduction

Many recent studies have shown that proteins, various growth factors, and cytokines secreted by transplanted cells in stem cell transplantation play important roles in regenerating tissue or treating various disorders1-3). These growth factors are produced by stem cells during culture and are secreted into the culture supernatant. The collected supernatant is called a conditioned medium (CM). Application to various types of regenerative medicine, using growth factors and cytokine effects contained in CM, is currently attracting significant attention. Among the various reported sources of stem cell-CM, including the bone marrow, fat, placenta, and umbilical cord, dental pulp stem cell (DPSC) can be harvested relatively easily and is known to have high differentiation potential. Many studies on regenerative medicine, using DPSC-derived CM, have been reported in several fields, indicating potential clinical applications4-6). The greatest benefit of using CM is that it contains no cellular substances and has no risk of becoming cancerous, unlike other cell types, such as induced pluripotent stem cells, since oncopogenes are not introduced into CM10-12). However, it is highly probable that the target patients for CM treatment in local administration or systemic administration are affected by or have a risk of cancer. There are a few reports on the effect of CM administration to various cancer cells, but reports involving DPSC-CM are particularly rare13) We investigated DPSC-CM’s effect on human oral squamous cell carcinoma (OSCC) cell proliferation, chemosensitivity, and tumor growth factor production to assess DPSC-CM potential effect on existing cancer cells when DPSC-CM is used for regenerative medicine.

Materials and Methods

Collection of DPSCs and extraction of CM

Dental pulp tissue was collected from the healthy teeth of participants aged 22–25 years and cut into small pieces using a scalpel on a culture dish. Subsequently, the primary culture was prepared using 10% fetal bovine serum (FBS; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 0.1% nonessential amino acid solution (Life Technologies; Thermo Fisher Scientific, Inc.), 1% penicillin–streptomycin (Life Technologies; Thermo Fisher Scientific, Inc.), and 0.1% fungizone (Life Technologies; Thermo Fisher Scientific, Inc.) added to Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12: Nihon Pharma-
ceutical Co., Ltd., Tokyo, Japan) (DMEM). Cultivation was performed in a humidified incubator at 37°C and under 5% CO₂. Subsequently, subculture was performed until passage (PS) 3, then used as DPSC. DPSC collection was approved by the Institutional Review Board of The Nippon Dental University College at Niigata (approval no., EC-NG-H-121: Niigata, Japan).

To extract DPSC-CM, DPSC and PS 3 cultures obtained from the above method were used. At 70%–80% confluence, DMEM without 10% FBS replaced the medium, and serum-free culture was maintained for 48 h. Subsequently, only the supernatant was collected, centrifuged at 1,500 × g, and filtered through a 0.45-μm filter, then used as DPSC-CM. DPSC-CM was stored at −20°C and thawed before use.

**Cell lines and culture conditions**

The OSCC cell lines used were HSC-2, HSC-3, HSC-4, OSC-19, and SAS. Culture was performed with DMEM containing 10% FBS (Life Technologies; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C and under 5% CO₂. All OSCC cell lines used in the study were purchased from the Japanese Collection of Research Bioresources Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan).

**Evaluation of cell proliferation rate and susceptibility to anticancer agent with collagen gel droplet-embedded culture drug sensitivity test (CD-DST)**

In accordance with Kobayasi et al.’s method, each cell line was measured six times using the Primaster® human cancer cell primary culture system kit (Kurabo Industries, Ltd., Osaka, Japan) to investigate the culture medium’s influence on cell proliferation and susceptibility to anticancer agents (Fig. 1). For the experiment, solution A (Cellmatrix® Type CD), solution B (10-fold concentration F-12 medium), and solution C (reconstitution buffer) were mixed at a ratio of 8:1:1 in the culture kit. This was added to cancer cells for a concentration of 1 × 10⁵ cells/ml and mixed. The mixture was pipetted into a six-well plate, with three drops per well, for a total volume of 90 μl added in drops. Gelling was performed in an incubator at 37°C for 1 h, and the cells were cultured in DPSC-CM and DMEM for 24 h. Afterward, the cells were exposed to drugs commonly administered as multidrug therapy for oral cancer. Taxotere® cisplatin and 5-fluorouracil (TPF), standard cisplatin and 5-fluorouracil (PF), and cisplatin (CDDP) monotherapy were used for the experiment ((CDDP: Randa® Injection 50 mg/100 ml: Nippon Kayaku, Co., Ltd., Tokyo, Japan), 5-fluorouracil (5-FU injection 250 Kyowa®: Kyowa Hakko, Co., Ltd., Tokyo, Japan), Docetaxel (Taxotere® injection 20 mg, Sanofi, Inc., Tokyo, Japan)). Hereinafter, these abbreviations are used as a designation for an experimental group. The drug concentrations were formulated according to the method described by Sakuma et al. and Takamura et al. The cells were incubated with the anticancer drugs for 24 h, then washed several times with PBS (Takara Bio, Inc., Otsu, Japan) to completely remove the drugs. The medium was subsequently replaced with the serum-free medium, included with the kit, and cultured for 144 h. After 144 h, neutral red solution (Kurabo Industries, Ltd.) was added to each well, staining was performed for 2 h, and the solution was fixed with 10% neutral formalin solution for 40 min. A control group was cultured for 144 hours without contact with anticancer drugs. After fixation, the plate was washed with water and dried to prepare the samples. The prepared samples were then evaluated using image analysis (Solution Systems Inc., Chiba, Japan).

The cells were evaluated using an image analyzer (Primage®, Guangzhou Darui Biotechnology Co., Ltd., China) in accordance with Koezuka et al.’s method. Grayscale images were obtained using the image analyzer. Non-cancerous cells were identified, based on the difference in the degree of staining, and removed. Cancer cell proliferation and the media’s antitumor effect were determined by measuring the volume of colonies from the cell images. The cells’ proliferation rates after 24 and 144 h were compared (growth rate: 144 h/24 h). In addition, the ratio of the volume of the cells in the treatment group (Vₜ) to the volume in the control group (Vₐ) was used to calculate the tumor growth.
Determination of tumor growth-related factors by ELISA

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Table 1. PCR primers

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>VEGF-A</td>
<td>TCTGTCGATGGTATGGTG</td>
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Inhibition (T/C) value. A T/C value ≤50% was defined as high sensitivity and a value ≥50% was defined as low sensitivity. The evaluation criteria made it possible to evaluate tumor cells with a growth rate ≥0.8 times the baseline value established at the beginning of the study. Each cell line was measured six times, and a two-way analysis of variance (Bell Curve for Excel ver.2.14) was used for statistical analysis.

Evaluation of tumor growth rate in nude mice

To investigate the effect of DPSC-CM on xenografted tumors, six-week-old female BALB/c nu/nu nude mice (Clea Japan, Inc., Tokyo, Japan) were raised under pathogen-free conditions and evaluated in vivo. In accordance with Geran et al.'s method, one of five cell lines (HSC-2, HSC-3, HSC-4, OSC-19, and SAS) was subcutaneously transplanted into the dorsal region of a nude mouse. When the tumor volume (TV) (1/2 × long diameter × short diameter) reached 100–300 mm³, 0.2 ml of DPSC-CM or DMEM was administered intraperitoneally, and measurement was initiated. TV was measured every three days. The mice were sacrificed on day 21, and the growth rates of the transplanted tumors were compared. Six animals were used from each cell line, and the Mann–Whitney U test (Bell Curve for Excel ver.2.14) was used for statistical analysis.

Figure 2. The results showed no significant difference in cell proliferation rate between the DPSC-CM and control groups in any of the cell lines (a) (control: DMEM). The results showed no significant difference in sensitivity to anticancer drugs between the DPSC-CM and control groups in any of the cell lines (b) (c) (d) (control: DMEM).

Comparison of OSCC cell line VEGF-A-mRNA levels

The ELISA results revealed that the VEGF level significantly increased in the DPSC-CM contact group. Therefore, real-time reverse transcription (RT)-polymerase chain reaction (PCR) was performed on the five cell lines to measure the VEGF-A level, which is best known within the VEGF family and is said to have the highest efficiency at inducing tumor angiogenesis. First, to determine the appropriate primers for experimental primers, VEGF-A mRNA expression in each cell line was confirmed with RT-PCR (Table 1). Each cell line was seeded in a 35-mm petri dish and cultured in a serum culture medium. Total RNA was extracted from the five cell lines according to the standard protocol using ISOGEN II (Nippon Gene, Co., Ltd., Tokyo, Japan). cDNA was synthesized with a high-capacity cDNA reverse transcription kit (Life Technologies; Thermo Fisher Scientific, Inc) using 1 µg of extracted total RNA. PCR amplification was performed using Platinum PCR Super Mix (Life Technologies; Thermo Fisher Scientific, Inc) and VEGF-A for the PCR cycle: after initial heating at 95°C for 20 s, denaturation 94°C, 30 s; annealing, 55°C, 30 s; extension 72°C, 60 s; 35 cycles. PCR amplification was performed using glyceraldehyde-3-phosphate dehydrogenase as an internal standard under the same conditions. Amplified PCR products were electrophoresed on 2% agarose gel (Nippon Gene, Co., Ltd., Tokyo, Japan) and visualized with ethidium bromide to confirm the bands. Because VEGF-A mRNA expression was confirmed in the five cell lines with RT-PCR, the levels in the medium were further compared using real-time RT-PCR. Each cell line was seeded in a 35-mm petri dish and cultured in the appropriate culture medium. When
Figure 3. There was no significant difference in the xenograft tumors’ growth rate between the DPSC-CM and control groups in any of the cell lines (a) (b) (c) (d) (e) (control: DMEM). (TV: tumor volume)

Figure 4. When compared the levels between 24 h and 96 h time points, VEGF levels were significantly higher in DPSC-CM than control in HSC-2, OSC-19, HSC-4, and HSC-3. This tendency was also observed in SAS, although not significant (a). The dominance of DPSC-CM over control was only observed in VEGF, while this consistent pattern was not observed in the result of EGF, IL-8 and FGF-2 (b) (c) (d). Multi-way analysis of variance using the data from all cell lines showed a significant induction only in VEGF result (a).
cell confluence reached 70%-80%, the culture medium was changed to DPSC-CM or DMEM (control). Cells were collected 24 h and 96 h after changing the medium. cDNA was synthesized using the extracted total RNA 1 μg, PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real-Time) (TaKaRa Clontech, Inc., Kusatsu, Japan). Subsequently, the reaction solution was prepared (Fast SYBR Green Master Mix, Applied Biosystems, Inc., Foster City, CA, USA), and real-time RT-PCR was performed (StepOnePlus, Applied Biosystems). After initial heating at 95°C for 20 s, PCR conditions were set to 40 cycles each at 95°C for 3 s and at 60°C for 30 s, respectively. The RNA expression level in the 2 M Riga group was regarded as the baseline and analyzed using the ΔΔCmethod. Each cell line was cultured six times, and statistical analysis was performed using multi-way analysis of variance (Bell Curve for Excel ver.2.14).

Results
Comparison of the proliferation rate and susceptibility to anticancer drugs in OSCC cell lines

The cell proliferation rate and susceptibility to anticancer drugs were compared between DPSC-CM and DMEM group (control) using the collagen gel droplet-embedded culture drug sensitivity test (CD-DST). The results showed no significant difference in cell proliferation rate between the DPSC-CM and control groups (Fig. 2a) in any of the cell lines.

Similarly, anticancer drug sensitivity was evaluated using three drugs: TPF, PF, and CDDP. The results showed no significant difference between the DPSC-CM and control groups in any of the cell lines (Fig. 2b, c, d).

Comparison of OSCC xenograft tumor growth rates in mice

To examine DPSC-CM’s effects on xenograft tumors, either DPSC-CM or DMEM was administered to nude mice bearing the OSCC cell line-derived xenografts, and the change in TV was measured. There was no significant difference in the xenograft tumors’ growth rate between the DPSC-CM and control groups in any of the cell lines (Fig. 3).

Confirmation of mRNA expression of VEGF-A and comparison of VEGF level

We then compared VEGF, EGF, IL-8, and FGF-2 levels in each cell line using ELISA. When compared the levels between 24h and 96h time points, VEGF levels were significantly higher in DPSC-CM than control in HSC-2, OSC-19, HSC-4, and HSC-3. This tendency was also observed in SAS, although not significant (Fig. 4a). The dominance of DPSC-CM over control was only observed in VEGF, while this consistent pattern was not observed in the result of EGF, IL-8 and FGF-2 (Fig. 4b, c, d). Multi-way analysis of variance using the data from all cell lines
showed a significant induction only in VEGF result.

VEGF-A gene expression was then tested with RT-PCR, which showed that VEGF-A mRNA was expressed in all five cell lines (Fig. 5a). Real-time RT-PCR showed that VEGF-A mRNA levels were significantly higher in the DPSC-CM group than control at each time point of 24 and 96 h (p < 0.05) (Fig. 5b), confirming the results of ELISA. Comparing VEGF-A levels between exposure time of 24 and 96 h using Multi-way analysis of variance also showed the VEGF-A levels were significantly higher in the DPSC-CM group (Fig. 5b).

**Discussion**

CM contains various components produced by cultured cells, and the application of stem cell-CM in regenerative medicine has been anticipated in recent years. DPSC is currently attracting attention in various fields due to several advantages, including ease of collection with low risk during dental treatments and a superior differentiation ability compared to bone marrow-derived stem cells. Specifically, it has been demonstrated that DPSC-CM reduces cardiac damage after ischemia–reperfusion and improves cognitive function in a mouse model of Alzheimer’s disease. Therefore, potential applications for DPSC-CM in regenerative medicine are greatly anticipated in many fields.

However, the potential negative effects of CM are also anticipated as various growth factors, cytokines, and proteins secreted by stem cells not only promote normal cell growth but may also have negative effects on cancer cells. Among the factors secreted by stem cells, angiogenic factors are thought to strongly promote tumor growth and metastasis. Angiogenic factors are considered tumor growth factors and, to date, many studies on their interaction with cancer cells have been reported. Ji et al. reported that adipose-derived stem cell-CM promoted proliferation and infiltration of pancreatic cancer cells, and Zhang et al. further reported that human omental adipose-derived mesenchymal stem cell-CM altered the proteomic profile of ovarian cancer cell lines to be malignant. However, there have been few reports on DPSC-CM’s effect on cancer cells. Therefore, in order to investigate the potential impact on oral cancer patients for whom there has been no previous research report before its clinical application in the stomatognathic field, the cell proliferation rates of OSCC cell lines, their sensitivity to anticancer agents, and the tumor growth factors produced by the cell lines were comparatively assessed by culturing OSCC cell lines in either DPSC-CM or DMEM.

The cell proliferation rate and sensitivity to anticancer agents of OSCC cell lines were evaluated using the CD-DST method. The CD-DST method has the advantage of evaluating cancer cells’ physiological metabolism in vivo because micro three-dimensional culture with an extracellular matrix type I collagen gel permits infiltration and excretion of various growth factors and cytokines into the collagen gel. Another advantage of the CD-DST method is that it can be used to test the antitumor effect of an anticancer agent at a physiological drug concentration, and the antitumor effect can be quantified for evaluation using an image analyzer.

Testing the proliferation rate of the five cell lines using the CD-DST method revealed the proliferation rate was slightly higher in DPSC-CM in four cell lines other than HSC-4; however, the differences between the culture media were not significant. No significant difference in the sensitivity to anticancer agents was observed for the culture media when exposed to the CDPP single agent or a multidrug combination of PF and TPF. In addition, in vivo experiments with xenograft tumors showed no significant difference between DPSC-CM and DMEM in any of the cell lines used for xenograft tumors, indicating that DPSC-CM had no promoting effect on OSCC cell lines’ proliferation. However, DPSC-CM safety cannot be confirmed based only on cell proliferation or sensitivity to anticancer agents. For example, Isabele et al. reported that adipose-derived stem cell-CM did not affect cell proliferation but enhanced glioma cells’ migratory capacity. Li et al. also reported that mesenchymal stem cell-CM did not alter growth but enhanced hepatocarcinoma cells’ migratory capacities.

Therefore, time-dependent changes in the production of growth factors that promoted OSCC cell proliferation were measured by ELISA and comparatively analyzed. Referring to the reports by Folkman et al., VEGF, EGF, IL-8, and FGF-2 were involved in angiogenesis to supply oxygen and nutrients in the microenvironment of growing or metastasizing tumors. They were selected and measured using ELISA. EGF, IL-8, and FGF-2 showed no differences between the DPSC-CM and DMEM groups; however, the VEGF was significantly higher in the DPSC-CM group. VEGF is the most important tumor-derived angiogenic factor, as it is overexpressed in OSCC patients, promotes growth in OSCC cell lines, and is strongly related to poor prognosis. An association between VEGF expression and lymph node metastasis has also been reported. VEGF levels in OSCC cell lines cultured in DPSC-CM and DMEM were measured and compared to real-time RT-PCR. Since VEGF-A is considered to have the highest tumor vasculature induction efficiency in the VEGF family, its levels were also examined. The VEGF-A level was significantly higher in the DPSC-CM group. Furthermore, the increase VEGF-A level from the 24-h to the 96-h time point was about three times greater in the DPSC-CM group than in the DMEM group, suggesting that VEGF expression is enhanced as the exposure time to DPSC-CM increases. Uregulation of VEGF expression and subsequent increase in VEGF level by DPSC-CM are involved in promoting growth and promotion of metastatic ability of OSCC cell lines. Aggarwal et al. reported that VEGF-mRNA expression in tumor cells was seven times higher than that in normal cells, while Ko et al. reported that higher levels of VEGF-mRNA were observed in more advanced tumors. Ji et al. reported cell proliferation was upregulated in pancreatic cancer cells cultured in adipose-derived stem cell-CM from the start of culture to the 72-h time point. Altogether, these reports indicated that VEGF expression was more upregulated and cell proliferation was promoted with longer DPSC-CM exposure.

In CD-DST, the exposure time to DPSC-CM was 24 h. This could explain the lack of significant difference in the growth rates and sensitivity to anticancer agents, suggesting that a 24 h exposure is not sufficient in the CD-DST method. A study by Pegram et al., discussing VEGF expression and sensitivity to anticancer agents, reported that high VEGF level might increase chemotherapy resistance in advanced breast cancer. In our study, we saw a difference in sensitivity between the OSCC cell lines. Therefore, resistance to anticancer agents may occur with a longer exposure time.

There have been many reports on the application of stem cell-CM in regenerative medicine; however, many ambiguous points remain regarding its effect on malignant tumors, and its safety is yet to be established. Although the current study found no significant difference in growth rates or sensitivity to anticancer agents in the DPSC-CM group, upregulation of VEGF expression was observed. Therefore, DPSC-CM safety in cancer patients remains to be determined. However, the promising features of DPSC-CM in regenerative medicine are highly anticipated, and confirming DPSC-CM’s usefulness should be an urgent issue.

In the future, in addition to confirming VEGF changes, cellular proliferation rate, and sensitivity to anticancer agents over a long period of time, further investigation of changes in tumor growth factors is neces-
sary, including factors other than VEGF in the microenvironment of cancer in vivo and in other types of cancer.

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Conflict of Interest

The authors have declared that no COI exists.

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