Effect of fibroblast growth factor-2 on dental pulp cells derived from human deciduous teeth *in vitro*

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Received January 25, 2010; Accepted March 2, 2010

DOI: 10.3892/etm_00000074

Abstract. Although dental pulp (DP) cells are indispensable for repair after injury, and blood supply is crucial for tissue healing and regeneration, the potential of DP cells derived from deciduous teeth to express endothelial cell (EC)-specific markers has yet to be determined. Therefore, this study investigated mRNA expression of the EC-specific markers vascular endothelial-cadherin (VE-cadherin), vascular endothelial growth factor receptor 2 (VEGFR2) and CD31 in DP cells derived from human deciduous teeth and treated with 10 ng/ml fibroblast growth factor (FGF)-2 *in vitro*. Quantitative PCR was used to determine the mRNA expression levels of the EC-specific markers, and showed that the FGF-2-cultured DP cells exhibited mRNA expression of VE-cadherin and VEGFR2, and marked CD31 mRNA expression. Western blot analysis showed that CD31 protein was induced in the DP cells following 3 weeks of treatment with FGF-2. DP cells derived from deciduous teeth inducibly expressed EC-specific markers, and thus have the potential to differentiate into cells of the vascular lineage.

Introduction

Endothelial cells (ECs) derived from vascular progenitor cells are responsible for angiogenesis and the events of wound healing (1). They are characterized by the expression of vascular endothelial-cadherin (VE-cadherin, CDH5) (2-4), vascular endothelial growth factor receptor 2 (VEGFR2, KDR) (5,6) and CD31 (also called platelet endothelial cell adhesion molecule-1, PECAM-1) (7). As blood supply is crucial for wound healing and tissue regeneration, it is important to determine how the progenitors of ECs differentiate into vascular cells in order to establish a practical strategy for regenerative therapy (1).

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Key words: endothelial cell markers, dental pulp, deciduous teeth

For regenerative therapy, biologically active soluble factors such as cytokines and growth factors are being evaluated for clinical use in the regeneration of periodontal tissue damaged or lost as a result of periodontitis. Among these factors, fibroblast growth factor (FGF)-2 is a multifunctional growth factor that exerts a variety of effects, including the induction of proliferation and differentiation in a wide range of mesodermal and neuro-ectodermal cells. Moreover, FGF-2 is one of the most potent angiogenesis inducers (8). Therefore, we investigated whether FGF-2 induces EC-specific markers in cultured dental pulp (DP) cells *in vitro*.

Dental pulp (DP) tissue is a non-hematopoietic connective tissue that is almost completely surrounded by hard tissue (9). After tooth maturation, DP tissues act only in a reparative capacity in response to general mechanical erosion or disruption and dentinal degradation caused by bacteria. Recently, dental pulp progenitor/stem cells (DPSCs) have been shown to be capable of differentiating into osteoblasts, adipocytes and neural cells *in vivo* (10). However, despite extensive investigation of DPSCs, the characteristics and properties of postnatal stem cells derived from DP cells from deciduous teeth have not yet been sufficiently studied in terms of the expression of the phenotype of endothelial cells and the regulation of differentiation in DP cells.

In the present study, we demonstrated that VE-cadherin, VEGFR2 and CD31 mRNA are expressed in cultured DP cells upon treatment with FGF-2. We also demonstrated CD31 protein expression in DP cell cultures using Western blot analysis. This is the first report regarding the inducible expression of the endothelial cell phenotype by DP cells derived from human deciduous teeth.

Materials and methods

Reagents. FGF-2 was obtained from R&D Systems (Minneapolis, MN, USA). Anti-CD31 monoclonal antibody for the Western blot analysis was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The dental pulp (DP) tissues were obtained from the crown and root of healthy human deciduous teeth from three donors, aged 7-8 years. Informed consent was obtained from

the donors' parents prior to tooth extraction, which was carried out in our hospital during the course of orthodontic treatment. The study protocol was approved by the Ethics Committee of Iwate Medical University, School of Dentistry (no. 01101).

DP tissues were cut into pieces using a surgical blade and digested with collagenase (2 mg/ml) at 37˚C for 30 min. The tissues were then washed with Dulbecco's phosphate-buffered saline (PBS), placed on culture dishes and maintained in α-modified minimum essential medium (α-MEM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL). Fibroblastic cells that outgrew from the DP tissues were used as DP cells. When the cells reached confluence, they were detached with 0.2% trypsin and 0.02% EDTA (4 Na) in PBS and subcultured at a 1:4 split ratio. Experiments were performed using 4th passage cells cultured in α-MEM supplemented with 10% FBS in the absence or presence of 10 ng/ml FGF-2 for 2 days. The cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Isolation of total RNA. Total RNA was extracted from the cultured DP cells using Isogen (Nippon Gene, Tokyo, Japan) as previously described (11,12). The pellet of total RNA was washed briefly with 75% ethanol, resuspended in 30 μl of diethylpyrocarbonate (DEPC)-treated water, and stored at -80˚C. The concentration of total RNA was determined spectrophotometrically by measuring the optical density at 260 nm.

Quantitative real-time reverse transcription-polymerase chain reaction. Using a PrimeScript RT reagent kit (Takara Shuzo, Kyoto, Japan), 1 μg of RNA sample was reverse-transcribed to first-strand cDNA according to the manufacturer's protocol. A Thermal Cycler Dice Real-Time system (Takara Shuzo) was used for the two-step reverse transcription-polymerase chain reaction. cDNA was amplified with SYBR Premix ExTaq and specific oligonucleotide primers for target sequences encoding parts of VE-cadherin, VEGFR2 and CD31. The primers (listed in Table 1) were designed based on the cDNA sequences of human mRNA for VE-cadherin, VEGFR2, CD31 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Amplification conditions consisted of 10 sec at 95˚C followed by 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec, with a final 15 sec at 95˚C and 30 sec at 60˚C in the Thermal Cycler Dice Real-Time system.

Western blot analysis of cell surface CD31 expression in DP cells. After treatment with FGF-2 for 21 days, DP cells were washed twice with PBS and then treated with lysis buffer [10 mM HEPES-KOH (pH 7.5), 100 mM KCL and 0.1% NP-40]. Protein concentration in the cell lysate was measured using a BioRad Protein Assay kit (BioRad, Hercules, CA). Each sample containing equal amounts of protein was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After being blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membrane was incubated with mouse anti-human CD31 antibodies and subsequently with anti-mouse secondary antibodies (Zymed Laboratories Inc., San Francisco, CA). Specific protein bands on the membrane were detected using an enhanced AP conjugate substrate kit (BioRad Laboratories, CA) as previously described (11,12).

Table I. Primers used in the quantitative real-time reverse transcription-polymerase chain reaction.

Gene name	Primer	Oligonucleotide sequence (5'-3')
VE-cadherin	Forward	GAGACCTCATCAGCCTTGGGATAG
	Reverse	CTGGATTTGCCAGCATTTGAGA
VEGFR2	Forward	CCAGGCAACGTAAGTGTTCGAG
	Reverse	GGGACCCACGTCCTAAACAAAG
CD31	Forward	GACGTGCAGTACACGGAAGTTCA
	Reverse	GTGCATCTGGCCTTGCTGTC
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA

VE-cadherin, vascular endothelial-cadherin; VEGFR2, vascular endothelial growth factor receptor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis. Results are expressed as the means ± SEM. Statistical significance was determined by one-way analysis of variance and Bonferroni comparisons between pairs of groups. Data with P-values <0.01 were considered statistically significant.

Results

Varying cell morphology of DP cells derived from deciduous teeth in primary culture. Using phase-contrast microscopy, DP cells were observed to differentiate into various types of cells 10 days after isolation from DP tissues (Fig. 1). Most of the DP cells derived from deciduous teeth exhibited a fibroblastic cell morphology (Fig. 1A). Some of the cells showed polygonal-like epithelial cell and mature osteoblast morphologies (Fig. 1A, arrow). A few cells showed a senescent fibroblastic cell-like morphology (Fig. 1A, arrowhead). After reaching confluence and undergoing subculture, it was no longer possible to distinguish between these cell morphologies (Fig. 1B).

FGF-2-induced morphological changes in DP cells after 2 days of treatment. DP cells were cultured in control media (Fig. 2A) or in the presence of FGF-2 (Fig. 2B), and reached subconfluence after 2 days of culture. FGF-2-treated DP cells reached confluence and exhibited an altered morphology of long and thin spindle-shaped fibroblasts (Fig. 2B).

FGF-2-induced EC-specific markers in DP cells after 2 days of treatment. In DP cells cultured in the presence of FGF-2, VE-cadherin and VEGFR2 mRNA expression was increased, though not significantly (Fig. 3A and B). However, CD31 expression was significantly induced by treatment with FGF-2 (Fig. 3C).

FGF-2-induced morphological changes in DP cells after 3 weeks of treatment. After three weeks of culture with FGF-2, DP cells reached confluence and exhibited a long and thin spindle-shaped fibroblastic morphology (Fig. 4B) compared to the cells cultured in control media (Fig. 4A).

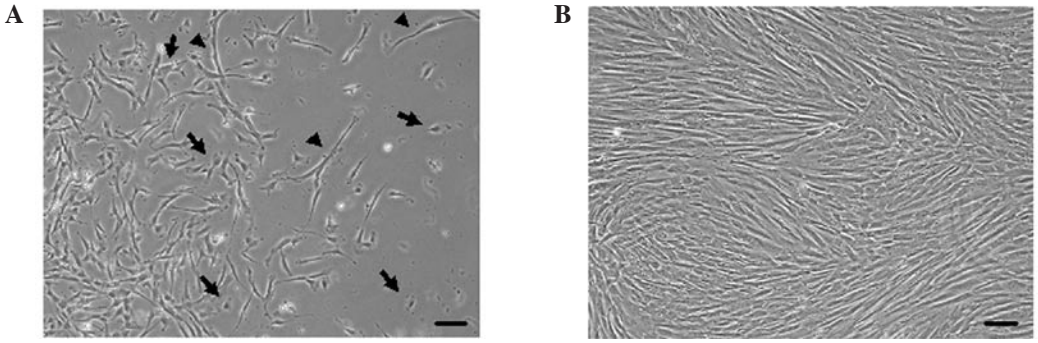

Figure 1. Morphologies of DP cells derived from deciduous teeth in primary culture observed using phase-contrast microscopy. (A) Most of the DP cells outgrown from DP tissues showed fibroblastic cell morphology (arrowheads). Some of the cells showed polygonal-like epithelial cell and mature osteoblast morphologies (arrows) 10 days after isolation. A few cells exhibited a senescent fibroblast-like cell morphology. Bar, 100 μm. (B) After reaching confluence and undergoing subculture, it was not possible to distinguish between these cell morphologies.

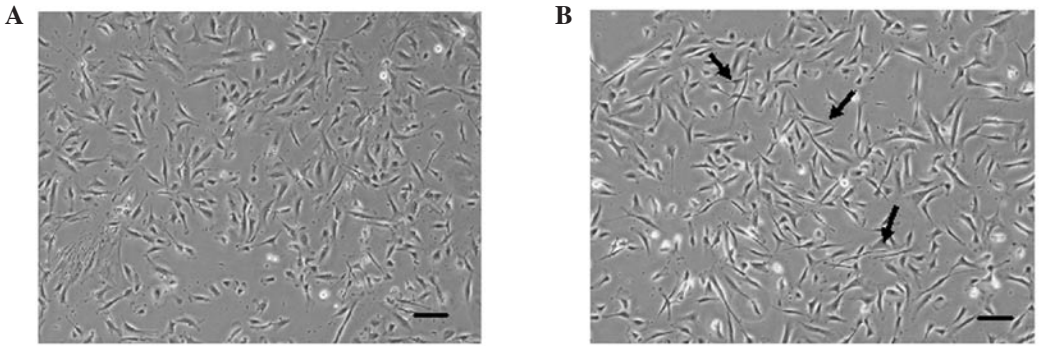

Figure 2. Effect of FGF-2 on the morphology of DP cells derived from deciduous teeth after 2 days of culture. (A) DP cells cultured in the absence of FGF-2 for 2 days. (B) DP cells cultured in the presence of FGF-2 for 2 days. The cells exhibited a long and thin spindle-shaped fibroblastic morphology. Bar, 100 μm.

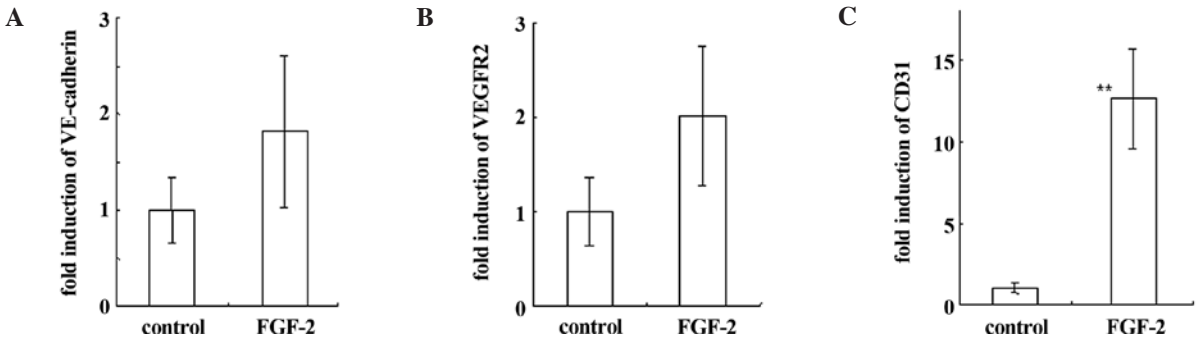

Figure 3. Effect of FGF-2 on endothelial cell (EC) marker mRNA expression of DP cells. (A) VE-cadherin, (B) VEGFR2 and (C) CD31 mRNA expression in the cultured DP cells. VE-cadherin and VEGFR2 mRNA expression was increased upon treatment with FGF-2, though not significantly, while expression of CD31 was significantly induced only in the presence of FGF-2 ($P<0.01$).

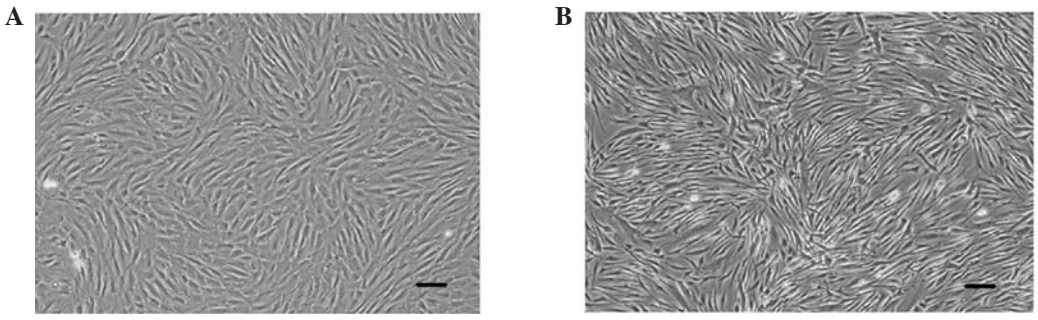

Figure 4. Effect of FGF-2 on the morphology of DP cells derived from deciduous teeth after 3 weeks of culture. (A) DP cells cultured in the absence of FGF-2 for 3 weeks. (B) DP cells cultured in the presence of FGF-2 for 3 weeks. Th cells exhibited a long and thin spindle-shaped fibroblastic morphology. Bar, 100 μm.

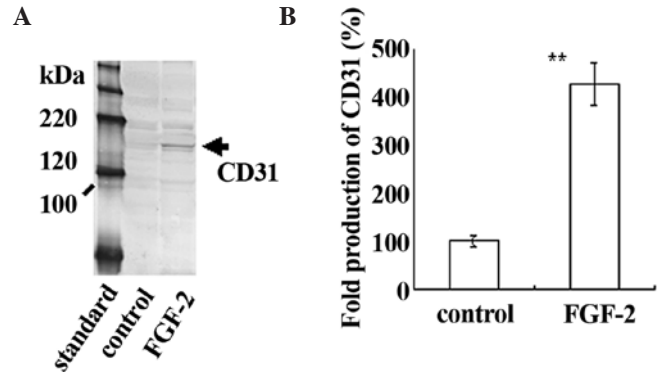

Figure 5. Effect of FGF-2 on CD31 expression in DP cells after 3 weeks of culture (A) In DP cells cultured in control media, CD31 expression was weakly detected. Upon treatment with FGF-2, CD31 expression was increased. (B) CD31 expression in FGF-2-treated DP cells compared to cells cultured in control media (control values normalized to 100%). Data are expressed as the mean ± SEM. CD31 expression was stimulated by treatment with FGF-2.

FGF-2-induced CD31 expression in DP cells. To determine whether CD31 protein was induced in DP cells cultured with FGF-2 (Fig. 5), Western blot analysis was conducted. CD31 expression was not detected after 2 days of treatment with FGF-2 (data not shown), nor after 2 weeks of culture. After treatment with FGF-2 for 3 weeks, CD31 expression was detected by Western blot analysis as compared with the control (Fig. 5A). Therefore, the production of CD31 was induced in DP cells by FGF-2 treatment (Fig. 5B).

Discussion

Tissue regeneration and homeostasis in response to pathological and environmental changes such as dental injury (e.g., preparation of a deep cavity) are thought to depend in large part upon angiogenesis in DP tissue. DP tissues exist in the pulp chamber surrounding dentin, and thus are likely to play an key role in protection against dental caries (9). DP cells were shown to have biological characteristics in common with bone marrow mesenchymal cells, suggesting that multipotent stem cells are present in DP tissue (10). However, it remains unclear whether DP cells derived from human deciduous teeth give rise to the endothelial cell (EC) lineage *in vitro.* To investigate DP tissue regeneration and homeostasis, it is crucial to determine whether DP cells have the ability to differentiate into ECs.

In the present study, we used DP cells derived from human deciduous teeth to investigate the effects of FGF-2 on the expression of markers specific for mature ECs: VE-cadherin (2-4), VEGFR2 (5,6) and CD31 (7). Previous findings have demonstrated that DP cells unstimulated by FGF-2 basally express EC markers such as CD31, though very weakly (13,14). In agreement with these findings, using real-time PCR we observed that DP cells without FGF-2 treatment expressed VE-cadherin, VEGFR2 or CD31. Unexpectedly, the expression of VE-cadherin and VEGFR2 was increased in DP cells cultured in the presence of FGF-2. The expression of CD31 was also significantly increased in DP cells cultured with FGF-2.

Compared with mRNA expression, CD31 protein production showed relatively small changes in the DP cells. However, treatment with FGF-2 was sufficient to induce CD31 protein expression. As shown in Fig. 5, in DP cells treated with FGF-2, CD31 production was increased by approximately 4-fold compared with the control group. The amount of protein, which is determined not only at the mRNA level but also by multiple processes of protein synthesis and degradation, might be a critical factor.

Here, we demonstrated for the first time that DP cells derived from human deciduous teeth inducibly express the EC-specific markers VE-cadherin, VEGFR2 and CD31 upon treatment with FGF-2 *in vitro*. These findings may aid in understanding the regeneration of DP tissue through the induction of angiogenesis.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research (no. 18592026 to A.I., no. 19791370 to N.C. and no. 18592239 to T.H.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Open Research Project and High-Tech Research Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Akiyama Foundation (to T.H., 2005), and by a grant from the Keiryokai Research Foundation (no. 100 to N.C., 2008; no. 106 to T.H., 2009).

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