Leptin Induces Odontogenic Differentiation and Angiogenesis in Human Dental Pulp Cells via Activation of the Mitogen-activated Protein Kinase Signaling Pathway

Viet Anh Ngo, DDS, MSD, Ji-Yeon Jung, MSD, PhD,† Jeong-Tae Koh, DDS, MSD, PhD,‡§ Won-Mann Oh, DDS, MSD, PhD,* Yun-Chan Hwang, DDS, MSD, PhD,*§ and Bin-Na Lee, DDS, MSD, PhD**

Abstract

Introduction: Up-regulation of odontogenic differentiation, dentin formation, and angiogenesis in dental pulp are key factors in vital pulp therapy. The aim of this study was to investigate whether leptin could promote odontogenic differentiation and angiogenesis in human dental pulp cells (hDPCs). In addition, the involvement of the intracellular signaling pathway in these effects was determined. **Methods:** The viability of hDPCs treated with leptin was examined using the water soluble tetrazolium salt-1 assay. Real-time polymerase chain reaction was performed to determine messenger RNA (mRNA) expression levels of odontogenic and angiogenic markers. Western blot analysis was used to measure odontogenic and angiogenic protein expression levels and assess mitogen-activated protein kinase (MAPK) pathway involvement. Alkaline phosphatase (ALP) and alizarin red staining were used to evaluate expression levels of ALP and calcified nodule formation after treatment with leptin and/or the presence of MAPK inhibitors. **Results:** All concentrations of leptin used in this study did not significantly affect the viability of hDPCs. However, mRNA and protein levels of odontogenic and angiogenic markers, ALP activity, and calcified nodule formation were significantly increased in the leptin-treated group compared with those in the control group. Leptin enhanced phosphorylation of extracellular signal-related kinases, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases within 5 minutes after treatment. However, leptin-induced dentin sialophosphoprotein and vascular endothelial growth factor protein expression and mineralization were appreciably blocked by the presence of MAPK inhibitors. **Conclusions:** Leptin can induce angiogenesis, odontogenic differentiation, and mineralization in hDPCs via activating the MAPK signaling pathway. *(J Endod 2017;■:1–7)*

Key Words

Angiogenesis, leptin, odontogenic differentiation, vital pulp therapy

Significance

Leptin has the ability to induce angiogenesis, odontogenic differentiation, and mineralization in hDPCs via activation of the MAPK signaling pathway. It will be great help in the clinical use of leptin for dentin-pulp regeneration, especially vital pulp therapy.

Vital pulp therapy such as direct pulp capping, indirect pulp capping, and pulpotomy can be used to preserve the health status of the tooth (1). Up-regulation of odontogenic differentiation, dentin formation, and angiogenesis of human dental pulp cells (hDPCs) are the key factors in vital pulp therapy.

Leptin, a 146 amino acid glycosylated hormone secreted mainly from adipose tissue, has various essential functions in the human body (2–5). It has been reported that leptin can induce endothelial cell proliferation and increase the secretion of vascular endothelial growth factor (VEGF) in human umbilical vein endothelial cells (6), enhance endothelial cell differentiation and angiogenesis in murine embryonic stem cells (7), and display a synergistic effect with basic fibroblast growth factor (FGF) 2 or VEGF in stimulating blood vessel growth (8).

In the field of dental research, it has been shown that leptin can enhance cemento/odontoblastic differentiation of periodontal ligament stem cells (9). Furthermore, the expression of leptin has a significant relationship with the expression of VEGF in ameloblasts and odontoblasts (10). Recently, it has been reported that leptin and its specific receptor are expressed in dental and periodontal tissues of primates (11). They are also up-regulated in inflamed human dental pulp (12, 13).

It has been shown that leptin can promote the expression of dentin sialophosphoprotein (DSPP) in human dental pulp (14), suggesting that leptin might affect odontogenic differentiation and angiogenesis in human dental pulp. However, the potential role of leptin as an agent for vital pulp therapy is currently unclear. Therefore, the aim of this study was to determine whether leptin could promote odontogenic differentiation and angiogenesis in human dental pulp. In addition, the involvement of the intracellular signaling pathway in these effects was determined.

From the Departments of *Conservative Dentistry, †Physiology, and ‡Pharmacology and Dental Therapeutics, School of Dentistry, Dental Science Research Institute and §Research Center for Biomineralization Disorders, Chonnam National University, Gwangju, Korea.

Address requests for reprints to Dr Bin-Na Lee or Dr Yun-Chan Hwang, Department of Conservative Dentistry, School of Dentistry, Chonnam National University, Young-bong ro 77, Bukgu, Gwangju, Korea. E-mail address: ychwang@chonnam.ac.kr or bnlee13@jnu.ac.kr

0099-2399/$ - see front matter

https://doi.org/10.1016/j.joen.2017.11.018

Materials and Methods

Cell Isolation and Culture of hDPCs

First, hDPCs were isolated from freshly extracted caries-free third molars of 10 healthy patients (19–25 years old). All procedures were performed after obtaining informed consent from each patient. The experimental protocol was approved by the Institutional Review Board of Chonnam National University Dental Hospital, Gwangju, Korea (CNUDH-2016-009). After tooth extraction, dental pulp tissues were instantly separated from the teeth under aseptic conditions and washed with Dulbecco phosphate-buffered saline solution (Welgene, Daegu, Korea), minced with fine scissors, and then transferred to 60-mm cell culture dishes. These cells were cultured in alpha minimum essential medium (Gibco Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco Invitrogen), 100 U/mL penicillin, and 100 mg/mL streptomycin. The medium was refreshed every 3 days until confluent cell monolayers were formed in a humidified atmosphere at 37°C with 5% CO_2. When cells reached confluence, subculture was performed. A pool of cells isolated from different patients was used in this study. Cells with passage numbers 3 to 8 were used for this study.

Leptin Treatment

Recombinant human leptin (Sigma-Aldrich, St Louis, MO) was directly added to the growth media at concentrations of 0.01, 0.1, 1, and 10 μg/mL to determine its effect on hDPCs.

Cytotoxicity Test

The effect of leptin on the viability of hDPCs was examined using an Ez-Cytox Enhanced Cell Viability Assay Kit (Daeil Lab Service Co, Seoul, Korea) according to the manufacturer's instructions. Briefly, hDPCs were seeded into 96-well cell culture plates at a density of 1×10^4 cells per well and incubated for 24 hours. They were then treated with leptin at different concentrations (0.01, 0.1, 1, and 10 μg/mL) for 24 hours. Ex-Cytox reagent was then added to each well and incubated for 4 hours in an incubator. The absorbance of each well was measured at a wavelength of 420 nm with 650 nm for background subtraction using a spectrophotometer (VERSAmax Multiplate Reader; Molecular Devices, Sunnyvale, CA).

RNA Extraction and Quantitative Real-time Polymerase Chain Reaction Assay

After seeding cells into 6-well cell culture plates at a density of 2×10^5 cells per well, cells were stimulated with leptin at different concentrations (0.1, 1, and 10 μg/mL) for 1 and 3 days. Total RNA extraction and complementary DNA synthesis were performed using Trizol reagent (Invitrogen, Carlsbad, CA) and the AccessQuick RT-PCR System (Promega, Madison, WI), respectively. Quantitative real-time polymerase chain reaction was performed in triplicates for each sample using the Quanti-Tect SYBR Green PCR Kit (Qiagen, Valencia, CA). All primers were synthesized by Bioneer (Daejeon, Korea). Relative gene expression was quantified after normalizing against the expression level of glyceraldehyde 3-phosphate dehydrogenase as an endogenous control. The gene-specific primer sequences used in this study are shown in Table 1. The data of gene expression were analyzed using the $\Delta\Delta$Ct method (15).

Western Blot Analysis

To measure odontogenic and angiogenic protein expression levels and assess the mitogen-activated protein kinase (MAPK) pathway involvement, Western blot analysis was performed under 3 different conditions:

1. hDPCs were treated with or without leptin at concentrations of 0.1, 1, and 10 μg/mL for 3 days.
2. hDPCs were incubated with leptin at a concentration of 1 μg/mL for 0, 5, 10, 30, and 60 minutes.
3. hDPCs were pretreated with MAPK inhibitors (U0126, extracellular signal-related kinases (ERK) inhibitor, 10 μmol/L; SP600125, c-Jun N-terminal kinase (JNK) inhibitor, 10 μmol/L; and SB202190, p38 mitogen-activated protein kinases (p38) inhibitor, 10 μmol/L) for 1 hour followed by treatment with leptin at 1 μg/mL for 3 days.

After treatment, cells in different groups were washed twice with cold PBS and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA). After removing cell debris by centrifugation, protein concentrations of cell lysates (supernatants) were evaluated with Lowry protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western Blot analysis were performed using polyvinylidene difluoride membranes. After a blocking step with blocking solution (5% nonfat dried skimmed milk powder, 0.01 mol/L PBS, and 0.1% Tween 20 [Biosesang, Sungnam, Korea]) for 1 hour, membranes were incubated with antileptin receptor (Thermo Fisher Scientific Inc, Rockford, IL), anti-DSPP, anti-VEGF, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-p38, or anti-phospho-p38 (Cell Signaling Technology) at 4°C overnight. Afterward, they were incubated with a secondary antibody such as horseradish peroxidase–conjugated antimouse immunoglobulin G or antirabbit immunoglobulin G (Sigma-Aldrich) at room temperature for 1 hour. Finally, antibody-loaded membranes were visualized using chemiluminescent horseradish peroxidase (Millipore, Billerica, MA) with a chemiluminescence imaging system (Ez-capture; Atto, Tokyo, Japan).

Alkaline Phosphatase Staining and Alizarin Red Staining Assays

For alkaline phosphatase (ALP) staining and alizarin red staining assays, hDPCs were seeded onto 24-well culture plates at a density of 2×10^4 cells per well with or without pretreatment with MAPK

TABLE 1. List of Primers Used for Real-time Polymerase Chain Reaction

Gene symbol	Full name	Primer sequences
DSPP	Dentin sialophosphoprotein	Forward: GGG AAT ATT GAG GGC TFF AA Reverse: TCA TTG TGA CCT GCA TCG CC
DMP-1	Dentin matrix protein-1	Forward: TGG TCC CAG CAG TGA GTC CA Reverse: TGT GTG CGA GCT GTC CTC CT
FGF	Fibroblast growth factor	Forward: GAC GAC TCT ATG CTT CGG AGC Reverse: AGG CGT ACT AGA CAC CGT CC
VEGF	Vascular endothelial growth factor	Forward: GAG GAG CAG TTA CGG TCT GTG Reverse: TCC TTT CCT TAG CTG ACA CTT GT
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Forward: CAT CAC CAT CTT CCA GCA G Reverse: AGG CTG TTG TCA TAC TTC TC