Leptin Promotes Dentin Sialophosphoprotein Expression in Human Dental Pulp

Jénifer Martín-González, DDS, PhD, Antonio Pérez-Pérez, MSc, PhD,† Flora Sánchez-Jiménez, MSc, PhD,† Eduardo Manuel Díaz-Parrado, MSc, PhD,‡ Manuel de Miguel, MD, PhD,‡ Víctor Sánchez-Margalet, MD, PhD,† and Juan José Segura-Egea, MD, PhD, DDS**

Abstract

Introduction: Leptin, an inflammation-related adipokine, and its receptor (LEPR) are expressed in human dental pulp. Dentin sialophosphoprotein (DSPP) is involved in dentinogenesis and the dental pulp reparative response. The cell type expressing LEPR in dental human pulp and the resultant effect of the binding of leptin to LEPR remain unknown. This study describes the immmunohistochemical localization of LEPR and the effect of leptin on DSPP expression in human dental pulp. **Methods:** Twenty-five dental pulp specimens were obtained from freshly extracted caries-free and restoration-free human third molars. LEPR localization was examined by immunohistochemistry using the anti-human LEPR monoclonal antibody. The effect of leptin on DSPP expression was determined by immunoblot analysis and quantitative real-time polymerase chain reaction. **Results:** Immunoreactivity for LEPR concentrated in the odontoblast layer but was not evident in the central zone of the dental pulp. Leptin dose dependently stimulated DSPP expression. Western blot analysis revealed the presence of a protein with an apparent molecular weight of ~00 kDa, the estimated molecular weight of DSPP. The expression of DSPP messenger RNA was confirmed by quantitative real-time polymerase chain reaction, and the size of the amplified fragments (298 bp) was confirmed by agarose gel electrophoresis. **Conclusions:** The present study shows that human dental pulp is immunoreactive for LEPR, with the immunoreactivity concentrated in the odontoblast layer, and that leptin stimulates, in a dose-dependent manner, DSPP protein and messenger RNA (mRNA) expression in human dental pulp. These findings further support the functional role of leptin in the dentin mineralization process and/or in dental pulp reparative and immune responses. *(J Endod 2015;41:487–492)*

Key Words

Cytokine, dentinogenesis, immunity, inflammation, leptin receptor, odontoblast, pulp biology, receptors

Leptin, a nonglycosylated hormone of 146 amino acids released by adipocytes, regulates energy intake and expenditure, appetite and hunger, metabolism, and behavior (1). Recent evidence shows that leptin is an inflammation-related adipokine acting as a proinflammatory adipokine (1, 2). Consequently, a role for leptin regulating immunity, inflammation, and hematopoiesis has been accepted (3–5). In addition, it has been shown that leptin regulates both innate and adaptive immune responses in normal and pathological conditions (6). Leptin levels increase during acute infection, inflammation, and sepsis, particularly favored by lipopolysaccharide (LPS) and cytokines such as tumor necrosis factor α, interleukin (IL)-6, and IL-1b (7).

Compelling evidence has implicated leptin in dental pulp biology. Leptin acts as a modulator of pulp mesenchymal stem cell differentiation (8). Leptin is synthesized and secreted *in vitro* by human pulp fibroblasts derived from extracted healthy molar teeth (9) and is expressed in ameloblasts, odontoblasts, dental papilla cells, and stratum intermedium cells in tooth germs of human mandibular third molars at the late bell stage (10) and in rat dental pulp (10). Recently, the up-regulation of leptin and the expression of its receptor (LEPR) in inflamed human dental pulp have been reported (11, 12).

On the other hand, dentin sialophosphoprotein (DSPP), which is secreted by odontoblasts, is the most abundant noncollagenous protein in predentin (13). DSPP plays a main role in the biomineralization process of predentin (14, 15) and is expressed by odontoblastlike cells underlying reparative dentin (16, 17). Although there are no specific odontoblastic markers, DSPP has been used as an indicator of odontoblastic differentiation (18, 19).

There is evidence supporting the concept that the dental pulp inflammatory response to caries alters DSPP expression. Thus, DSPP gene expression is down-regulated by lipoteichoic acid (20), and tumor necrosis factor α, an inflammatory cytokine, increases the expression of DSPP in dental pulp cells (21).

The cell type expressing LEPR in dental pulp and the resultant effect of leptin binding to LEPR remain unknown. The aim of the present study was to describe the immunohistochemical localization of LEPR and to study the effect of leptin on DSPP expression in human dental pulp.

From the Departments of *Stomatology (Endodontics Section) and ‡Normal and Pathologic Cytology and Histology, University of Sevilla, Sevilla, Spain; and †Department of Medical Biochemistry and Molecular Biology, Virgen Macarena University Hospital, Sevilla, Spain.

Address requests for reprints to Dr Juan José Segura-Egea, Facultad de Odontología, University of Sevilla, C/ Avicena s/n, 41009-Sevilla, Spain or Dr Victor Sánchez-Margalet, Dpto de Bioquímica Médica y Biología Molecular, Hospital Universitario Virgen Macarena, University of Sevilla, Avda Dr Fedriani 3, 41071-Sevilla, Spain. E-mail address: margalet@us.es or segurajj@us.es

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Materials and Methods

The study was performed with the understanding and written consent of each subject and according to the principles of the World Medical Association Declaration of Helsinki. The protocol was approved by the Ethical Committee.

Reagents

Human recombinant leptin was provided from R&D Systems (Minneapolis, MN). Monoclonal mouse anti-DSPP (LFMb-21, 1:1500) and goat antihuman LEPR antibody C-20, a goat polyclonal immunoglobulin G specific against the long isoform of LEPR (c-terminal) of human origin (sc-1832), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal mouse anti–β-tubulin (1:5000) was provided from Sigma-Aldrich (St Louis, MO). Horseradish peroxidase–linked antimouse/antirabbit (1:10,000) immunoglobulins were purchased from Amersham Pharmacia Biotech (Barcelona, Spain).

Human Dental Pulp

Human dental pulp was obtained from 25 freshly extracted third molars from 22 healthy, nonsmoking, human donors (20–54 years old) who gave their written informed consent to donate their pulp tissue. All teeth were caries and restoration free and without signs of periodontal disease. The extracted teeth were washed with 5.25% sodium hypochlorite after extraction to eliminate remains of the periodontal ligament, which could contaminate the pulp sample. The teeth were then sectioned by using a Zekrya bur (Dentsply Maillefer, Tulsa, OK) in a high-speed handpiece irrigated with saline solution. The pulp tissue was obtained by using a sterile endodontic excavator and washed 2 to 3 times in sterile ice-cold phosphate buffered saline (PBS) to remove excess blood. Fifteen samples were cut into 2 parts, one for the Western blot analysis and another one for RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) assay, and 10 samples were analyzed by immunohistochemistry.

Dental Pulp Samples

Human dental pulps were randomly distributed in tubes containing 1 mL Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEN-F12) medium (Gibco Invitrogen, Carlsbad, CA). Dental pulps were maintained in a shaking water bath at 37°C for 5 minutes to equilibrate temperature. Dental pulps were incubated at 37°C in 5% CO_2 (22) for 12 hours in the same medium supplemented or not with 0.1, 1, and 10 nmol/L human recombinant leptin (R&D Systems). Then, dental pulps were removed and resuspended in 75 μL lysis buffer (1× PBS, 1% Nonidet P-40 [Roche Diagnosis GmbH, Mannheim, Germany], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], and 10 mg/mL phenylmethanesulfonyl fluoride) for 30 minutes at 4°C on an orbital shaker and later centrifuged at 10,000*g* for 20 minutes. Supernatants were analyzed by Western blot.

Western Blot Analysis

Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL) using bovine serum albumin as the standard. SDS stop buffer containing 100 mmol/L dithiothreitol (DTT) was added to the pulp tissue samples followed by 5 minutes of boiling. In each lane, 50 μg protein was loaded. Then, samples were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto nitrocellulose membranes (Hybond, Amersham Pharmacia Biotech). The membranes were blocked with PBS with 0.05% Tween 20 containing 3% bovine serum albumin (Merck-Schuchardt, Hohanbrunn, Germany) for 1 hour at 23°C. After this, membranes were immunoblotted overnight with monoclonal mouse antisialophosphoprotein (LFMb-21, 1:1500, Santa Cruz Biotechnology). Loading controls were performed by immunoblotting the same membranes with monoclonal anti–β-tubulin (1:5000, Sigma-Aldrich). Then, the blots were washed in PBS with 0.05% Tween 20 and further incubated with secondary antibodies using horseradish peroxidase–linked antimouse/antirabbit immunoglobulins (1:10,000, Amersham Pharmacia). Bound horseradish peroxidase was visualized using a highly sensitive chemiluminescence system (Super Signal; Pierce Biotechnology, Rockford, IL). Quantification of protein bands was determined by densitometry using the PCBAS 2.0 densitometry software (Raytest, Straubenhardt, Germany). The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

RNA Extraction and qRT-PCR Assay and Agarose Gel Electrophoresis

The abundance of DSPP messenger RNA (mRNA) was determined by qRT-PCR. Total RNA was extracted from human dental pulp tissue samples using TRISURE reagent (Bioline, London, UK) (21). The concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For complementary DNA synthesis, 5 μg total RNA was reverse transcribed at 55°C for 1 hour using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). qRT-PCR was performed using the primers based on the following sequences of the National Center for Biotechnology Information GenBank database: DSPP: forward, 5′AGAAGGACCTGGCCAAAAAT-3′; reverse, 5′-TCTCCT CGGCTACTGCTGTT-3' and cyclophilin: forward, 5-CTTCCCCGATA CTTCA-3′; reverse, 5′-TCTTGGTGCTACCTC-3′. The qRT-PCR Master Mix Reagent kit was obtained from Roche (FastStart Universal SYBR Green), and polymerase chain reactions were performed on a MiniOpticon (Bio-Rad, Hercules, CA). A typical reaction contained 10 μmol/L forward and reverse primer and 3 μL complementary DNA, and the final reaction volume was 25 μL. The reaction was initiated by preheating at 50°C for 2 minutes followed by heating at 95°C for 10 minutes. Subsequently, 40 amplification cycles were performed as follows: denaturation for 15 seconds at 95°C and 1 minute of annealing and extension at 59°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program (Bio-Rad). Relative quantification was calculated using the $2-\Delta\Delta CT$ method. For the treated samples, evaluation of $2-\Delta\Delta CT$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin) and relative to the untreated control. The retrotranscribed DNA samples were then resolved by 1% agarose gel. After the run of the electrophoresis, gels were visualized directly upon illumination with ultraviolet light (23).

Immunohistochemistry

The dental pulp samples were fixed in 10% formalin for at least 24 hours and then processed in paraffin and processed routinely. A series of 4-μm sections from each tissue sample were cut. The first section of each series was stained with hematoxylin-eosin to study the histology. The following sections were used for immunohistochemical staining for the expression of LEPR and counterstained using hematoxylin to identify immunoreactive cells.

Pilot studies were undertaken to establish the optimum dilution of the antibody as well as confirming the concentration and application times for the chemicals used during the immunohistochemistry procedure. Sections were picked up on a slide, deparaffinized in xylene, rehydrated by sequential immersion in a graded series of alcohols, transferred into water for 10 minutes, and then heat treated in sodium