Leptin Promotes Dentin Sialophosphoprotein Expression in Human Dental Pulp

Jénifer Martín-González, DDS, PbD, * Antonio Pérez-Pérez, MSc, PbD, † Flora Sánchez-Jiménez, MSc, PbD, ‡ Eduardo Manuel Díaz-Parrado, MSc, PbD, §§ Manuel de Miguel, MD, PbD, ‡ Victor Sánchez-Margalet, MD, PbD, §§ and Juan José Segura-Egea, MD, PbD, 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 immunohistochemical 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 ~10 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 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-1β (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.
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 antiamouse/antibrituminoglobulins (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 PBCAS 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.

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 evacuator 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 pulp was randomly distributed in tubes containing 1 mL Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-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% CO₂ 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, Hohenbrunn, Germany) for 1 hour at 23°C. After this, membranes were immunoblotting overnight with monoclonal mouse antialkylphosphoprotein (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 antiamouse/antibrituminoglobulins (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 PBCAS 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’-AGAAGGCTGCTGAAAAAT-3’; reverse, 5’-CTCTCTC GGTACTGTGTTT-3’ and cyclophilin: forward, 5’-CTCCGGGATA CTCTA-3’; reverse, 5’-CTTGTGCTACCTT-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-ΔΔCT method. For the treated samples, evaluation of 2-ΔΔCT 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...
citrate buffer (pH = 6.0) for 40 minutes at 96°C to unmask antigens. The sections were cooled and washed in PBS (pH = 7.2). Specimens were incubated in a blocking agent (5% albumin bovine serum in PBS) for 1 hour. Endogenous peroxidase activity was quenched by incubating the sections in a solution of 3% H2O2 in 10% methanol and PBS for 15 minutes. Sections were incubated with goat antihuman LEPR (C-20) polyclonal primary antibodies at 1:100 dilution overnight at 4°C. In the negative control sections, PBS with no primary antibody was used. The sections were then washed in PBS (3×, 5 minutes each) and incubated with goat secondary antibodies. The manufacturer’s instructions were followed for the sequential incubation and durations for the exposure to the secondary antibodies. A diaminobenzidine (DAB) substrate kit (code K3467; Dako, Carpinteria, CA), a high-sensitivity DAB system suitable for use in peroxidase-based immunohistochemical analysis, was used following the manufacturer’s instructions at a final concentration of 20 μL DAB/mL of hydrogen peroxide. After washing with PBS, the sections were incubated with DAB for 5 minutes, which resulted in a brown-colored precipitate at the antigen-antibody binding sites, and the reaction was stopped in distilled water. After removing the slides from water, all the sections were dehydrated, 1 drop of aqueous mounting medium (Faramount, Dako) was applied, and the sections were coverslipped.

Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analyzed by 2 different operators under magnifications up to 100×. A cell was considered positive when it demonstrated distinct brown surface staining. Representative sites in each sample were photographed with 4, 10, 40, 60, and 100× objectives and a 10× ocular lens and captured with a software system (CS3, Adobe Photoshop version 10.0.1; Adobe Systems Inc, San Jose, CA).

Data Analysis

Experiments were repeated separately at least 3 times to ensure reproducible results. Results are expressed as means ± standard deviation in arbitrary units. Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the GraphPad Prism computer program (GraphPad Software, San Diego, CA). Significance levels were set at P < .05.

Results

Microscopic analyses of all human dental pulp samples (n = 10) showed that dental pulp specimens were reactive to anti-LEPR antibody. Immunohistochemical analysis showed the presence and localization of LEPR in human dental pulp (Figs. 1A–D and 2A–D). Immunoreactivity for LEPR concentrated in the odontoblast layer (Figs. 1 and 2) but was not evident in the central zone of the dental pulp. However, some endothelial cells showing immunostaining for LEPR were found (Fig. 1C, red arrowheads).

To check whether leptin may stimulate DSPP expression in human dental pulp, dose-response experiments stimulating the pulp samples with human leptin were performed, analyzing the amount of DSPP protein and mRNA levels.

The amount of DSPP proteins in every sample was controlled by antitubulin immunoblotting. As shown in Figure 3, the effect of leptin on DSPP protein synthesis was dose dependent. A concentration of 0.1 nmol/L leptin was sufficient to increase the expression of DSPP, and 1 nmol/L leptin significantly increases DSPP expression. The maximal expression of DSPP was observed at 10 nmol/L leptin, which increased the basal protein synthesis rate 10-fold (P < .05).

To further validate the effect of leptin on DSPP expression in a dose-dependent manner in human dental pulp tissue, the real-time

Figure 1. Immunohistochemical localization and expression of LEPR in human dental pulp. Representative sections from several specimens of human dental pulp. (A) Hematoxylin-eosin staining (original magnification 100×). (B) Negative control (lacking primary antibody); no staining in the odontoblast region is evident (original magnification 100×). (C and D) In situ LEPR expression by immunohistochemical analysis; immunostaining is evident in the odontoblastic layer and odontoblast processes (black arrowheads) as well as in 2 endothelial cells (red arrowheads) (original magnification |C| 40× and |D| 100×); odontoblasts’ nuclei are counterstained with hematoxylin.
polymerase chain reaction assay was performed to examine the mRNA levels of DSPP in these tissue samples. As shown in Figure 4 (upper panel), leptin enhanced mRNA DSPP levels in a dose-dependent manner. A concentration of 0.1 nM leptin increased DSPP expression. A concentration of 1 nM leptin caused a significant increase in DSPP expression. The maximal effect was achieved at 10 nmol/L leptin, the dose at which the expression level of DSPP increased almost 10-fold ($P < .05$). The size of the amplified fragments (298 bp for DSPP and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis (Fig. 4, lower panel).

**Discussion**

The present study shows that DSPP expression in human dental pulp, both protein and mRNA, increases with leptin stimulation. Moreover, immunohistochemical evidence indicating that the odontoblast layer expresses LEPR has been provided for the first time. Taking into account that DSPP is an indicator of odontoblastic differentiation (18, 19), these findings, taken together, provide evidence that LEPR is expressed by human odontoblasts.

Immunohistochemical results showed LEPR expression in the odontoblast layer where the brown positive immunostaining was evident. The staining method used is a high-sensitivity DAB system suitable for use in peroxidase-based immunohistochemical staining methods (24). In this system, upon oxidation, DAB forms a brown end product at the site of the target antigen or nucleic acid. In the present study, positive and negative controls have accompanied each staining run. Positive controls have shown that specimen processing and handling have been performed correctly, and negative controls have demonstrated the absence of nonspecific staining.

LEPR expression has been shown previously in periapical lesions in experimental rats (25), in normal and inflamed human dental pulp (12), and, recently, in human periapical granulomas (26). Moreover, up-regulation of LEPR in experimentally inflamed human dental pulps has been shown (12). Although human dental pulp contained several cell types expressing LEPR, such as dental pulp stem cells (DPSCs) (8), monocytes (27), natural killer cells (28), and CD4 T and CD8 T cells (6), the stimulation of DSPP mRNA and protein expression in
The link between an adipokine, such as leptin, whose first functional role is the control of appetite and hunger (1, 2), and the expression of DSPP, a dentin protein implicated in dental pulp defensive and reparative responses, could also be interpreted as new evidence of the relationship between obesity, inflammation, and oral infections. In this respect, several possible pathways involving the host response and bacterial challenge have been proposed to explain the association between obesity and inflammation (34). Obesity could alter the host immune responses to oral infections, increasing individuals’ susceptibility (35). Several possible pathways involving the host response and bacterial challenge have been proposed to explain the association between obesity and inflammation (34). Obesity could alter the host immune responses to oral infections, increasing individuals’ susceptibility (35). Among these potential pathways, leptin and vascular endothelial growth factor (VEGF) have been identified as key regulators of immune responses and angiogenesis in gingival tissue. Leptin can stimulate angiogenesis through direct effects on endothelial cells and indirect effects on immune cells. VEGF is a key mediator of angiogenesis and immune responses in the oral cavity, and its expression is up-regulated in inflamed human dental pulp (4, 5). Therefore, the expression of leptin and VEGF in inflamed human dental pulp suggests that these factors play a role in the regulation of immune responses and angiogenesis in this tissue.

**References**


**Figure 4.** The stimulatory effect of leptin on DSPP mRNA expression in human dental pulp samples. Human dental pulp were incubated for 12 hours in the presence of different concentrations of leptin (0.1, 1, and 10 nmol/L), washed, and lysed. Total RNA was extracted from each sample. DSPP mRNA was quantified using the qRT-PCR assay and visualized by agarose gel electrophoresis. Cyclophilin was used as the internal standard (right band). Markers bands (300 and 200 bp) are on the left. Results from a representative experiment performed in triplicate are shown. Data represent the fold change from the mean values of cyclophilin and are expressed as means ± standard deviation. *P < 0.05 versus control (0 nmol/L leptin).