Leptin expression in healthy and inflamed human dental pulp

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Abstract

Aim To investigate the expression of leptin in healthy and inflamed human dental pulp.

Methodology Twenty-one pulp samples were obtained from freshly caries- and restoration-free extracted human third molars. In seven-third molars (inflamed pulp group), inflammation was induced prior to extraction. Pulp samples were processed, and leptin expression was determined by quantitative real-time PCR (qRT-PCR) and the amount of leptin by immunoblot.

Results All healthy and inflamed dental pulp samples expressed leptin. Western blot analysis revealed the presence of a protein with an apparent molecular weight of ~16 kDa in human dental pulp, which corresponds to the estimated molecular weight of leptin. The expression of leptin mRNA in dental pulp was confirmed by qRT-PCR analysis, and the size of the amplified fragments (296 bp for leptin and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis. The expression of leptin in the inflamed pulp group was significantly greater ($P < 0.05$) than in healthy teeth. The relative amount of leptin in inflamed pulps was almost twice than in healthy pulps.

Conclusions For the first time, the presence of leptin in human dental pulp tissues has been demonstrated. The upregulation of leptin expression in inflamed pulp samples suggests that leptin can play a role in pulpal inflammatory and immune responses.

Keywords: dental pulp stem cells, leptin, pulpal immune response, pulpal inflammatory response, pulpitis.

Received 1 June 2012; accepted 4 September 2012

Introduction
The adipocyte-derived hormone leptin has been shown to regulate the immune response, both innate and adaptive responses, not only in normal but also in pathological conditions (Fernández-Riejos et al. 2010). Leptin is a 16-kDa-glycosylated protein product of the Ob gene (Zhang et al. 1994). Leptin is mainly synthesized and secreted from adipose tissue (Ahima & Flier 2000) and was originally described as an adipocyte-derived hormone to regulate weight control in a central manner, via its cognate receptor in the hypothalamus (Flier 1995). However, leptin has been classified as a cytokine because its primary amino acid sequence shows structural similarities to the long-chain helical cytokine family (Zhang et al. 1994, Sánchez-Margalet et al. 2003a, b). Moreover, the leptin receptor (Ob-R) is expressed not only in the central nervous system, but also in peripheral tissues, such as hematopoietic and immune systems (Sánchez-Margalet et al. 2003a,b). Therefore, a role for leptin in hematopoiesis and the immune
system has been proposed (Cioffi et al. 1996, Sánchez-Margalet et al. 2003a, b). It has been suggested that leptin orchestrates the immune host response by enhancing cytokine production and phagocytosis by macrophages (Fantuzzi & Faggioni 2000, Sánchez-Margalet et al. 2003a, b, Fernández- Riejos et al. 2010).

The presence of leptin has been reported both in healthy and inflamed gingival tissues (Johnson & Serio 2001), in gingival crevicular fluid (Bozkurt et al. 2006a, Karthikeyan & Pradeep 2007a, b, Dilsiza et al. 2010) and in human chronic periapical lesions (Haghighi et al. 2010). Elevated serum leptin concentration has been associated with increased chronic periodontitis (Gundala et al. 2011). Recently, the first evidence has emerged that leptin has effects on dental pulp stem cells, acting as an important modulator of pulp mesenchymal stem cell differentiation (Um et al. 2011), being expressed in ameloblasts, odontoblasts, dental papilla cells and stratum intermedium cells in rat and human tooth germs at the late bell stage (Ide et al. 2011).

It can be hypothesized that leptin is expressed by human dental pulp and that it plays a role in the modulation of pulpal inflammatory and immune responses. However, even though it has been previously shown that leptin is synthesized and secreted in vitro by pulp fibroblasts derived from extracted healthy molar teeth (El Karim et al. 2009), to date, no study has investigated its presence in human dental pulp. The aim of this study was to investigate the expression of leptin in healthy and inflamed human dental pulp.

**Material and methods**

The study was carried out with the understanding and written consent of each subject and according to the principles of the Declaration of Helsinki. The protocol was approved by the Ethical Board of the University of Sevilla, Spain.

**Dental pulp samples**

Human dental pulp was obtained from 21 freshly extracted third molars from nineteen healthy, non-smoking, human donors (22- to 35-year 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. Fourteen third molars were simply extracted and processed (healthy pulp group). However, in seven-third molars (inflamed pulp group), inflammation was induced prior to extraction using the method described previously by Caviedes-Buchelli et al. (2005). Briefly, the inflammatory process was generated by mechanical exposure of the pulp chamber using a no.1 round carbide bur in a high-speed handpiece without irrigation. After a period of 10 min, the teeth were extracted and processed.

The extracted teeth were washed with 5.25% sodium hypochlorite after extraction to eliminate remnants of periodontal ligament that could contaminate the pulp sample, and they were kept at −80 °C until use. The teeth were then sectioned by using a Zekrya bur (Dentsply Tulsa Dental, Tulsa, OK, USA) in a high-speed handpiece irrigated with saline solution. The pulp tissue was obtained by using a sterile endodontic excavator, washed 2–3 times in sterile PBS to remove excess blood and placed in an Eppendorf tube. Each sample was divided into two parts, one for the Western blotting analysis and the other for RNA extraction and quantitative real-time PCR (qRT-PCR) assay.

**Antibodies and reagents**

The monoclonal mouse antitubulin (1 : 1000) was provided from Santa Cruz Biotechnology (Heidelberg, Germany), and the polyclonal rabbit anti-human leptin Y20 (1 : 1000) antibodies were from Sigma (Sigma Diagnostics, St Louis, MO, USA). Horseradish peroxidase-linked anti-mouse/anti-rabbit (1 : 10 000) immunoglobulins were purchased from Amersham Pharmacia (Amer sham Pharmacia Biotech, Barcelona, Spain).

**Western blotting analysis**

The pulp tissue samples were incubated in 75 µL of lysis buffer containing 20 mmol L⁻¹ Tris, pH 8, 1% Nonidet P-40, 137 mmol L⁻¹ NaCl, 1 mmol L⁻¹ MgCl₂, 1 mmol L⁻¹ CaCl₂, 1 mmol L⁻¹ dithiothreitol (DTT), 10% glycerol, 1 mmol L⁻¹ phenylmethylsulfonyl fluoride and 0.4 mmol L⁻¹ sodium orthovanadate, at 4 °C for 30 min on an orbital shaker and then centrifuged at 12 000 g for 15 min. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as standard. We added SDS stop buffer containing 100 mmol L⁻¹ of DTT to the pulp tissue samples followed by boiling for 5 min. The samples were then resolved by 15% SDS–PAGE and electrophoretically transferred onto nitrocellulose mem-branes (Martin-Romero et al. 2000). The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 3% albumin for 1 h at 23 °C.
The blots were then incubated with primary antibody for 1 h, washed in PBST and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/anti-rabbit immunoglobulins (Santa Cruz). Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce) (Sánchez-Margalet et al. 2003a,b). The bands obtained in the blots were scanned and analysed by the PCBASE 2.0 program. 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 quantitative real-time PCR (qRT-PCR) assay and agarose gel electrophoresis

Abundance of leptin mRNA was determined by quantitative real-time PCR (qRT-PCR). Total RNA was extracted from human dental pulp tissue samples using TRIzol reagent (Chomczynski 1993). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 μg of total RNA was reverse transcribed at 55 °C during 1 h using the Transcriptor first Strand cDNA synthesis kit (Roche, Mannheim, Germany). Quantitative real-time PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database: Leptin: forward, 5′-GAACCTCTGCGGATCT-3′; reverse, 5′-CTGCCGTTGGATAATTG-3′; cyclophilin: forward, 5′-CTTCCTGATACCT-3′; reverse, 5′-CCTTTGTGCTACCT-3′. Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (Bio-Rad, Hercules, CA, USA). A typical reaction contained 10 μmol L⁻¹ of forward and reverse primer, 3 μL of cDNA and the final reaction volume was 25 μL. The reaction was initiated by pre-heating at 50 °C for 2 min, followed by heating at 95 °C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 s at 95 °C and 1 min annealing and extension at 58 °C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the ΔΔCT method. For the treated samples, evaluation of 1/ΔΔCT indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin).

The retro-transcribed DNA samples were then resolved by 1% agarose gel. After the run of the electrophoresis, gels were visualized directly upon illumination with UV light (Voytas 2001).

Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as means ± SD in arbitrary units (AU). 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, USA). Statistically significant differences between healthy and inflamed dental pulp samples were tested using the Mann–Whitney rank sum test. Significance levels were set at P < 0.05.

Results

All healthy human dental pulp samples expressed leptin at both protein and mRNA levels. Western blot revealed the expression in dental pulp of an immunoreactive protein that migrated with recombinant leptin protein at an apparent molecular weight of ~16 kDa, which corresponds to the estimated molecular weight of leptin (Fig. 1).

To confirm the leptin expression in dental pulp, quantitative real-time PCR (qRT-PCR) assay was carried out to identify the leptin mRNA (Fig. 2, upper panel). The size of the amplified fragments (296 bp for leptin and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis (Fig. 2, lower panel).

All inflamed dental pulp samples expressed the protein leptin. The expression of leptin in the dental pulp of teeth with inflamed pulps was significantly greater than in healthy teeth (P < 0.05) (Fig. 3). The relative amount of leptin in inflamed pulps was almost twice than in healthy pulps.

Discussion

The present study is the first to demonstrate the expression of leptin in human healthy and inflamed dental pulp tissues. A recently study has also demonstrated the expression of leptin in rat dental pulp by Western blot analysis (Ide et al. 2011). In addition, this study is the first to demonstrate that leptin is upregulated in the inflamed dental pulp.

Leptin is one of the most important hormones secreted by adipose tissue, and its implication in energetic homeostasis at central level has been largely
Leptin is also secreted by other types of tissues, such as placenta (trophoblast), ovaries, skeletal muscle, stomach, mammary epithelial cells, bone marrow, pituitary and liver (Margetic et al. 2002). Although adipocytes are not a normal cellular component in dental pulp, human dental pulp stem cells (DPSCs) have been found to be capable of differentiating into oil red-O-positive lipid-containing adipocytes (Gronthos et al. 2002), expressing in vitro the early adipogenic master gene PPARγ2 (peroxisome proliferator-activated receptor gamma two) and the late marker lipoprotein lipase (LPL), two adipocyte-specific transcripts (Koyama et al. 2009). So, pulpal leptin could be secreted by DPSCs suffering adipogenic differentiation. Nevertheless, other dental pulp cells can also be the source of leptin found in human dental pulp tissue. Thus, it has been demonstrated that human pulp fibroblasts in culture are able to synthesize and secrete leptin (El Karim et al. 2009), and it has been found that leptin is expressed in odontoblasts in rat and human tooth germs (Ide et al. 2011). Therefore, these cell types could also be the origin of pulpal leptin found in the present study.

One of the physiological roles of leptin is the connection between nutritional status and immune competence (Sánchez-Margalet et al. 2003a,b, Fernández-Riejos et al. 2010). Leptin modulates the immune system at the development, proliferation, antiapoptotic, maturation and activation levels. In fact, leptin receptors have been found in neutrophils, monocytes and lymphocytes, and the leptin receptor belongs to the family of class I cytokine receptors. Moreover, leptin activates similar signalling pathways to those engaged by other members of the family (Sánchez-Margalet et al. 2003b, Fernández-Riejos et al. 2010). Leptin signalling deficiency impairs humoral and cellular immune responses (Bennett et al. 1996).

The overall leptin action in the immune system is a pro-inflammatory effect during adaptive immune response, activating pro-inflammatory cells, promoting T-helper 1 responses and mediating the production of the other pro-inflammatory cytokines, such as tumour necrosis factor-α, IL-2 or IL-6 (Fernández-Riejos et al. 2010). In processes involving innate immunity, leptin circulating levels increase acutely upon infectious and inflammatory stimuli such as Lipopolysaccharide (LPS), turpentine and cytokines (Sarrafi et al. 1997, Bernotiene et al. 2006).
The expression of leptin in human dental pulp revealed in the present study suggests that this protein could modulate pulpal immune and inflammatory responses. Moreover, the present investigation has demonstrated the upregulation of leptin in inflamed dental pulp. The significantly higher relative amount of leptin in inflamed dental pulps found in the present study support the concept that leptin plays a role in dental pulp inflammatory processes. Pulp inflammation is characterized by the infiltration of inflammatory cells such as lymphocytes, macrophages, dendritic cells and neutrophils, and consequently, pulpitis is formed (Hahn et al. 2000). It is well known that chemokines regulate the trafficking of lymphocytes, and CC-chemokine ligand 20 (CCL20) has been shown to play a crucial role in the recruitment of memory T cells (Liao et al. 1999) and immature dendritic cells into inflammatory lesions (Dieu et al. 1998). Taking into account that leptin is associated with an increased expression of CCL20 (Farquharson et al. 2012), the high relative amount of leptin in inflamed pulp suggests that leptin could regulate the trafficking of lymphocytes during pulpal inflammatory response. It has been demonstrated that CCL20 expression is induced by stimulation with caries-related bacteria invading deeply into the dental tubules as well as by proinflammatory cytokines in the inflamed pulpal lesions (Takahashi et al. 2008).

When the number of macrophages increases during the innate response of the dentin/pulp complex to caries (Kamal et al. 1997), leptin can regulate macrophage function as assessed by in vitro experiments measuring free radical production (Maingrette & Renier 2003, Sánchez-Pozo et al. 2003). Thus, leptin has been found to stimulate the oxidative burst in control monocytes (Sánchez-Pozo et al. 2003) and macrophages (Maingrette & Renier 2003), and binding of leptin at the macrophage cell surface increases lipoprotein lipase expression through oxidative stress- and PKC-dependent pathways (Maingrette & Renier 2003).

Leptin dose-dependently stimulates the production of pro-inflammatory cytokines by monocytes, such as TNF-α and IL-6, and enhances CC-chemokine ligand expression in cultured murine macrophage, through phosphorylation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) pathways (Kiguchi et al. 2009). Taking into account that JAK2–STAT3 signalling pathway has been identified in human dental pulp (Zhou et al. 2011), leptin could use the same signalling pathway in human dental pulp.

Conclusion

As a result of the nature of the present study, it was not possible to identify which type of human pulp cell expresses leptin. Further investigations must be carried out to elucidate which pulp cell type expresses leptin, as well as whether leptin receptors are expressed by human dental pulp cells. Nevertheless, the upregulation of leptin expression in inflamed pulp samples showed in this study suggests that leptin plays a role in pulpal inflammatory and immune responses.

Acknowledgements

The authors deny any conflict of interests. This work was supported by a Grant from the Instituto de Salud Carlos III (ISCIII PS09/00119). Flora Sánchez-Jiménez and Antonio Pérez-Pérez are research fellow supported by the Instituto de Salud Carlos III.
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