Lipopolysaccharide-binding Protein Inhibits Toll-like Receptor 2 Activation by Lipoteichoic Acid in Human Odontoblast-like Cells

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Abstract

Introduction: Previous studies have suggested that odontoblasts sense gram-positive bacteria components through Toll-like receptor 2 (TLR2) and trigger dental pulp immunity by producing proinflammatory cytokines. Currently, the factors that modulate odontoblast TLR2 activation are unknown. Our aim was to investigate lipopolysaccharide-binding protein (LBP) effects on the TLR2-mediated odontoblast response. **Methods:** Human odontoblast-like cells were stimulated with lipoteichoic acid (LTA) (a TLR2 ligand), LBP, CD14 (a TLR2 cofactor), or various combinations of LTA/LBP, LTA/CD14, or LTA/CD14/LBP. *CXCL8, IL6,* and *TLR2* gene expression was assessed by real-time polymerase chain reaction. CXCL8 and interleukin (IL)-6 production was determined by enzyme-linked immunosorbent assay in culture supernatants of cells stimulated with LTA, LTA/CD14, or LTA/CD14/LBP. LBP effects on nuclear factor kappa B (NF-κB), p38, JNK, ERK, STAT3, and p70S6 signaling pathways were determined in LTA-stimulated odontoblast-like cells with a multiplex biometric immunoassay. LBP effects were compared with specific inhibitors of these signaling pathways. LBP transcript and protein were investigated *in vivo* in healthy and inflamed dental pulps by real-time polymerase chain reaction and immunohistochemistry. **Results:** Activation of *CXCL8, IL6,* and *TLR2* gene expression and CXCL8 and IL-6 secretion in LTA- and LTA/CD14-stimulated odontoblast-like cells was significantly decreased by LBP. LBP inhibited NF-κB and p38 signaling pathways in LTA-stimulated cells in a similar way to NF-κB and p38 inhibitors. LBP transcript and protein were detected *in vivo* in inflamed dental pulps but not in healthy ones. **Conclusions:** These results demonstrate that LBP reduces TLR2-dependent production of inflammatory cytokines by odontoblast-like cells. We suggest that in this way it could modulate host defense in human dental pulp. *(J Endod 2013;39:1008–1014)*

Key Words

CD14, CXCL8, human dental pulp, IL-6, innate immunity, LBP, NF-κB, pathogen-associated molecular pattern, p38, Toll-like receptor

Odontoblasts are neural crest–derived, dentin-producing mesenchymal cells organized as a densely packed layer at the periphery of the dental pulp. They become exposed to bacterial by-products as cariogenic oral microorganisms demineralize enamel and then dentin to gain access to the pulp (1). Because of their specific location at the pulp-dentin interface and entrapment of a long cell process in dentin, odontoblasts are the first cells encountered by tooth-invading pathogens and their released components, and they have been suggested to initiate pulp immune and inflammatory responses to these pathogens (2, 3). How this initiation is modulated is unknown.

Studies that aimed at elucidating the triggering of dental pulp immunity by odontoblasts have mainly focused on gram-positive bacteria, because these largely dominate the microflora in initial and moderate dentin caries lesions (1). In particular, we found that odontoblasts were responsive to lipoteichoic acid (LTA), a gram-positive bacteria component recognized at cell surface through the pattern recognition receptor Toll-like receptor-2 (TLR2). Activation of odontoblast TLR2 by LTA triggered (1) up-regulation of TLR2 itself and the cytosolic pattern recognition receptor nucleotide-binding oligomerization domain 2, (2) production of the proinflammatory chemokines and cytokines CCL2, CXCL1, CXCL2, CXCL8, CXCL10, and interleukin (IL)-6, and (3) recruitment of immature dendritic cells (2, 4–8). Conversely, LTA down-regulated 2 major dentin matrix components, type I collagen and dentin sialophosphoprotein, as well as the dentinogenesis inducer transforming growth factor-β1 (2). Collectively, these results suggest that on TLR2 activation, odontoblasts undergo a functional switch to decrease their specialized function of dentin matrix synthesis, while orienting their metabolic activity toward the production of molecules responsible for the triggering

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of pulp immune and inflammatory responses. How this production is controlled remains unknown.

Sensing by TLR2 is modulated by co-receptors such as TLR1 and TLR6, which confer ligand specificity to TLR2, and accessory molecules, including CD14, CD36, and lipopolysaccharide-binding protein (LBP), whose main function is to amplify or attenuate ligand recognition by TLR2 and subsequent cell response intensity (9–12). Although it is generally admitted that CD14 and CD36 bind LTA to improve its delivery to TLR2, thus increasing cell sensitivity and innate response to LTA, the role of LBP in LTA-triggered TLR2 engagement appears to be more complex. LBP is an acute phase protein mainly synthesized by hepatocytes and secreted in serum. It is also produced in healthy and/or pathologic conditions by epithelial cells from various organs and tissues including lung, intestine, and gingiva (13–15). First regarded to specifically bind lipopolysaccharide (LPS) from gram-negative bacteria, LBP is now known to interact with LTA as well. LBP was shown to reduce or increase LTA effects in macrophages and monocytes, respectively (16, 17), possibly reflecting a dual role in innate immune response control (18). Nothing is known about LBP effect on LTA-stimulated odontoblasts. Therefore, the aim of this study was to analyze LBP influence on the LTA-triggered odontoblast immune response *in vitro* by using the increase of *CXCL8*, *IL6,* and *TLR2* gene expression as readout of this response (6, 7). *In vivo* relevance of these findings was examined by comparing the levels of LBP transcript and protein in healthy and bacteria-challenged inflamed dental pulps.

Materials and Methods

Reagents

Purified *Staphylococcus aureus* LTA, Celastrol (nuclear factor kappa B [NF-κB] inhibitor), SB203580 (p38 inhibitor), SP600125 (Jun N-terminal [JNK] inhibitor), and PD98059 (extracellular signal-regulated kinase [ERK] inhibitor) were from InvivoGen (San Diego, CA). Stattic (STAT3 inhibitor) and anti-LBP rabbit polyclonal antibody were from Sigma-Aldrich (St Louis, MO). Recombinant human LBP and CD14 were from R&D Systems Europe (Lille, France).

Cell Culture and Treatments

Clinically healthy, nonerupted human third molars were obtained with informed consent of the patients, in accordance with the World Medical Association's Declaration of Helsinki and following a protocol approved by the local ethics committee. Odontoblast-like cells were differentiated from dental pulp explants as previously described (19) and then used for stimulation experiments. *CXCL8, IL6,* and *TLR2* expression was analyzed after cell challenge for 4 hours with 1 or 10 μg/mL LTA, 0.2 or 2 μg/mL LBP, 1 or 10 μg/mL CD14, or various combinations of LTA and LBP, LTA and CD14, or LTA and CD14 and LBP, as indicated in Figures 1 through 4. To assess LBP effect on LTA-modulated signaling pathways, IκBα, p38, JNK, ERK, STAT3, and p70S6 phosphorylated and total proteins were measured after cell treatment for 15, 30, or 60 minutes with 10 μg/mL LTA or 10 μg/mL LTA and 2 μg/mL LBP. IκBα was chosen because its phosphorylation is necessary for NF-κB nuclear translocation, and it thus directly reflects NF-κB signaling activity. LBP effect was compared with specific inhibitors of these signaling pathways by incubating odontoblast-like cells with 2 μg/mL LBP or inhibitors to NF-κB (Celastrol, 5 μmol/L), p38 (SB203580, 20 μmol/L), JNK (SP600125, 50 μmol/L), ERK (PD98059, 50 μmol/L), or STAT3 (Stattic, 10 μmol/L) for 30 minutes before addition of 10 μg/mL LTA for 4 hours.

Real-time Polymerase Chain Reaction

RNA extraction and reverse transcription were performed from odontoblast-like cell cultures as previously described (6). Real-time polymerase chain reaction (PCR) was performed in a CFX96 Real-Time PCR Detection System with the iQ SYBR Green Supermix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's specifications. The cyclophilin A housekeeping gene (*PPIA*) was used for sample normalization. Gene-specific primer sequences for *CXCL8*, *IL6*, *TLR2,* and *PPIA* were reported in our previous articles (2, 6). For each target gene, relative expression was determined after normalization by using the Bio-Rad CFX Manager software. Results were expressed as fold-change values relative to unstimulated control odontoblast-like cell cultures.

Enzyme-linked Immunosorbent Assay

To confirm the effects of LTA, CD14, and LBP at protein level, CXCL8 and IL-6 concentration was measured in supernatants of odontoblast-like cell cultures stimulated with 10 μg/mL LTA, 10 μg/mL LTA and 10 μg/mL CD14, or 10 μg/mL LTA and 10 μg/mL CD14 and 2 μg/mL LBP for 4 hours by using specific Instant ELISA kits (Bender MedSystems, Vienna, Austria).

Milliplex Assay

A multiplex biometric immunoassay containing fluorescent dyed microbeads (Milliplex MAP Cell Signaling kit; Millipore Corp, St Charles, MO) was used for measuring phosphorylated and total IκBα, p38, ERK, JNK, STAT3, and p70S6 protein levels. Mean fluorescence intensity (MFI) was calculated by Luminex technology (Bio-Plex Workstation; Bio-Rad). Data were analyzed by using the Bio-Plex Manager 5.0 software (Bio-Rad).

Conventional PCR

RNA extraction and reverse transcription were performed from pulp samples obtained from healthy and decayed, irreversibly inflamed human molars as described previously (6). Liver RNA was from Clontech (Palo Alto, CA). Total cDNA was amplified by conventional PCR with the GoTaq Green Master Mix (Promega, Witchburg, WI). LBP primers were the following: forward: 5′AGGGCCTGAGTCTCAGCATCT3′, reverse: 5′CAGGCTGGCCGTGTTGAAGAC3′. Amplicon size was 578 base pairs, annealing temperature was 55°C, and amplification cycle number was 35. PCR products were analyzed with 2% agarose gel electrophoresis.

Immunohistochemistry

Healthy and decayed human molars were collected with informed consent of the patients, in accordance with the World Medical Association's Declaration of Helsinki and following a protocol approved by the local ethics committee. In brief, teeth were fixed in 4% paraformaldehyde–phosphate-buffered saline solution for 1 week, demineralized in 10% acetic acid for 4 months, and routinely treated for paraffin embedding (2). Eight-micrometer serial sections were then cut, deparaffinized, and rehydrated. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide for 15 minutes. Sections were incubated for antigen retrieval in 10 mmol/L citrate buffer (pH 6.0, 98°C) for 10 minutes and then blocked with normal goat serum for 30 minutes. They were then incubated with 1.5 μg/mL anti-LBP rabbit polyclonal antibody (Ref. HPA001508; Sigma-Aldrich) overnight at 4°C. Staining controls were performed by omitting the primary antibody or by using normal rabbit immunoglobulin G. Antibody detection was performed by using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Sections were slightly counterstained with hematoxylin.