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Cytokine production by human odontoblast-like cells upon Toll-like receptor-2 engagement

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ABSTRACT

Recent studies have suggested that odontoblasts are involved in the dental pulp immune response to oral pathogens that invade human dentin during the caries process. How odontoblasts regulate the early inflammatory and immune pulp response to Gram-positive bacteria, which predominate in shallow and moderate dentin caries, is still poorly understood. In this study, we investigated the production of pro- and anti-inflammatory cytokines by odontoblast-like cells upon engagement of Toll-like receptor (TLR) 2, a pattern recognition molecule activated by Gram-positive bacteria components. We used a highly sensitive Milliplex® kit for detecting cytokines released by cells stimulated with lipoteichoic acid (LTA), a cell wall component of Gram-positive bacteria, or with the potent TLR2 synthetic agonist Pam2CSK4. We found that odontoblasts produce the pro-inflammatory cytokines interleukin (IL)-6 and CXCL8, as well as the immunosuppressive cytokine IL-10 in response to TLR2 agonists. GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-7, IL-12(p70), IL-13 and TNF-α were not detected. These data indicate that TLR2 activation in human odontoblasts selectively induces production of mediators known to influence positively or negatively inflammatory and immune responses in pathogen-challenged tissues. We suggest that these molecules might be important in regulating the fine tuning of the pulp response to Gram-positive bacteria which enter dentin during the caries process.

Introduction

Human odontoblasts are neural crest-derived, dentin-producing mesenchymal cells aligned at the periphery of the dental pulp present in the center of the tooth. They become exposed to bacterial products as cariogenic oral bacteria progressively demineralise enamel and dentin to gain access to the pulp. Due to their location at the dentin–pulp interface, odontoblasts are the first cells encountered by invading pathogens and/or their released components, and represent, in the tooth, the first line of defence for the host. Our previous studies have shown that odontoblasts are able to sense pathogens and elicit innate immunity (Durand et al. 2006; Staquet et al. 2008). They express several pathogen recognition molecules of the Toll-like receptor (TLR) and NOD-like receptor (NLR) families, which allow them to recognize specific bacterial and viral components. Most studies aiming at elucidating the role of odontoblasts in the early innate response in the dental pulp have focused on Gram-positive bacteria, as these largely dominate the caries microflora in initial and moderate dentin caries lesions (Love and Jenkinson 2002; Farges et al. 2009). *In vitro*, odontoblasts were found to be sensitive to lipoteichoic acid (LTA), a Gram-positive bacteria-derived component recognized at the cell surface through TLR2. Engagement of odontoblast TLR2 by LTA triggers TLR2 and NOD2 up-regulation, NF-κB nuclear translocation, and production of various pro-inflammatory chemokines including CCL2, CXCL1, CXCL2, CXCL8 and CXCL10, while promoting immature dendritic cell recruitment (Durand et al. 2006; Staquet et al. 2008; Farges et al. 2009; Keller et al. 2009, 2010). Conversely, LTA down-regulates major dentin matrix components, including collagen type I and dentin sialophosphoprotein, and TGF-β1, a known inducer of dentin formation. These data indicate that odontoblasts constitute potential targets for preventing the development of immune events which lead to excessive inflammation and necrosis in the dental pulp challenged with cariogenic bacteria. In this context,

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Table 1
Primers used for PCR analysis.

Gene	Forward primer	Reverse primer	Annealing temperature	Amplicon size
IL6	GCCAGAGCTGTGCAGATGAGTAC	TCAGGGGTGGTTATTGCATCTAG	65 °C	86 bp
CXCL8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT	65 °C	69 bp
IL10	GAGACATCAGGGTGGCGACTCTA	CAATAAGGTTTCTCAAGGGGCTG	65 °C	109 bp
PPIA	GGATTGCTTGAGCCTAGAGTGA	CCTCTGCCTACCTTTGAGACAC	65 °C	87 bp

inhibition of Gram-positive bacteria recognition by specific pattern recognition receptors or modulation of pathogen-activated downstream signalling cascades appear to be valuable strategies to dampen pulp inflammation (Farges et al. 2009). Another strategy might be the neutralization of pro-inflammatory cytokines or, conversely, the induction of anti-inflammatory cytokines synthesized by odontoblasts in response to Gram-positive bacteria (Nishimoto 2010; Dinarello 2010). So far these cytokines have not been identified.

The aim of this study was to identify cytokines produced by human odontoblasts upon TLR2 engagement by LTA or by Pam2CSK4, a highly potent synthetic TLR2 activator. We also demonstrated increase of gene expression for cytokines secreted by odontoblasts upon TLR2 engagement, and *in vivo* relevance of these findings was obtained by comparing the levels of cytokine gene expression in healthy and inflamed bacteria-challenged human dental pulps.

Material and methods

Cell culture and stimulation

Four clinically healthy, non-erupted human third molars were collected from four different donors with informed consent of the patients, in accordance with the French Public Health Code and following a protocol approved by the local ethics committee. Odontoblast-like cells were obtained by culturing dental pulp explants as detailed (Couble et al. 2000). After 4 weeks, cells were stimulated for 4, 8 or 16 h with 10 μg/mL purified *Staphylococcus aureus* LTA or with the same concentration of the TLR2 synthetic agonist Pam2CSK4 (both from Invivogen, San Diego, CA, USA). We previously showed that these conditions functionally activated TLR2 and led to chemokine production by human odontoblast-like cells and dental pulp fibroblasts (Durand et al. 2006; Staquet et al. 2008; Keller et al. 2009).

Milliplex® assay

For quantifying cytokine production by odontoblast-like cells, culture supernatants were analyzed with a Milliplex® assay (High Sensitivity Human Cytokine kit; Millipore Corp., St. Charles, MO, USA), which allows the simultaneous quantification of the 13 following human cytokines: GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13 and TNF-α.

Dental pulp samples

Healthy dental pulps were taken from five non-erupted human third molars. Inflamed pulps were taken from five decayed erupted molars with clinical features of irreversible acute pulpitis (deep dentin caries lesions, severe spontaneous dental pain for 12–24 h, no sensitivity to vertical or horizontal percussion, lack of periapical lesions) and in the absence of anti-inflammatory treatment.

Real-time PCR

Cell culture samples corresponding to supernatants used for cytokine detection were analyzed for gene expression. Ribonucleic acid extraction and reverse transcription were performed essentially as described (Keller et al. 2010). Total RNA was extracted with a Nucleospin RNA XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Ribonucleic acid samples (1 μg) were then converted to first-strand cDNAs using 500 ng oligo$(dT)_{15}$ primers (Roche Diagnostics, Meylan, France) and SuperScript III Reverse Transcriptase (Invitrogen Life Technologies, Grand Island, NY, USA). Real-time PCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the iQ SYBR Green Supermix kit (Bio-Rad) according to the manufacturer's specifications. All runs were performed in duplicate. Cyclophilin A housekeeping gene (*PPIA*) was used for sample normalization. Primer sets, annealing temperatures and amplicon sizes for *IL6*, *CXCL8*, *IL10* and *PPIA* genes are listed in Table 1. For each target gene, relative expression was determined after normalization using the Bio-Rad CFX Manager software. Results were expressed as fold change values relative to unstimulated control samples.

Conventional PCR

Ribonucleic acid extraction and reverse transcription were performed from pulp samples from five healthy and five decayed teeth as described above. Total cDNA was amplified by conventional PCR with the GoTaq Green Master Mix (Promega, Charbonnières-les-bains, France) according to the manufacturer's specifications. Primers were the same as those used for real-time PCR. Thirty cycles of PCR amplification were performed. PCR products were analyzed with 3% agarose gel electrophoresis.

Statistical analysis

Results were expressed as mean values ± standard deviation (SD) obtained from the four different donors. Statistical analysis was performed with unpaired Student's *t*-test. Results were considered statistically significant when $p < 0.05$.

Results

Odontoblast-like cells treated by 10 μg/mL LTA or Pam2CSK4 for 4, 8 or 16 h were assessed for the secretion of 13 cytokines using a High Sensitivity Human Cytokine detection kit and Milliplex® technology. Three cytokines were detected in supernatants from LTA- or Pam2CSK4-stimulated cells: IL-6, CXCL8 and IL-10 (Fig. 1). GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-7, IL-12(p70), IL-13 and TNF-α were not detected, or were detected at extremely low concentrations in a non-reproducible manner between cultures obtained from different patients (not shown). Concentrations of released IL-6 and CXCL8 were relatively similar and about four times higher than IL-10 concentrations. Pam2CSK4 was a more potent inducer than LTA for each of the three detected cytokines regardless of the stimulation time. Despite this strong effect, stim-