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O.V. Horst^{1, 2, 3}, K.A. Tompkins⁴,
S.R. Coats⁴, P.H. Braham⁴,
R.P. Darveau^{1, 4}, and B.A. Dale^{1, 5, 6 *}

Departments of ¹Oral Biology, Box 357132, ²Endodontics, ³Dental Public Health Sciences, ⁴Periodontics, ⁵Biochemistry, and ⁶Medicine/Dermatology, University of Washington, Seattle, WA 98195-7132; *corresponding author, bdale@u.washington.edu

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ABSTRACT

TGF- β 1 exerts diverse functions in tooth development and tissue repair, but its role in microbial defenses of the tooth is not well-understood. Odontoblasts extending their cellular processes into the dentin are the first cells to recognize signals from TGF- β 1 and bacteria in carious dentin. This study aimed to determine the role of TGF- β 1 in modulating odontoblast responses to oral bacteria. We show that these responses depend upon the expression levels of microbial recognition receptors TLR2 and TLR4 on the cell surface. *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* activated both TLRs, but TLR4 played a greater role. Lack of cell-surface TLR2 was associated with poor response to *Streptococcus mutans*, *Enterococcus faecalis*, and *Lactobacillus casei*. TGF- β 1 inhibited TLR2 and TLR4 expression and attenuated odontoblast responses. Our findings suggest that the balance between TLR-mediated inflammation and TGF- β 1 anti-inflammatory activity plays an important role in pulpal inflammation.

KEY WORDS: Toll-like receptors, transforming growth factor beta 1, odontoblasts, chemokines, pro-inflammatory cytokines.

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TGF- β 1 Inhibits TLR-mediated Odontoblast Responses to Oral Bacteria

INTRODUCTION

Dental caries is the most common chronic infection in humans and can result in access for bacteria to the pulp tissue. Human dentin contains transforming growth factor- β 1 (TGF- β 1) (Finkelman *et al.*, 1990; Zhao *et al.*, 2000), which plays multiple roles in the formation and repair of the dentin-pulp complex (Smith, 2003). This cytokine also acts as a potent regulator for chemotaxis, activation, and survival of immunocompetent cells such as lymphocytes, macrophages, and granulocytes, to control the initiation and resolution of inflammatory responses (Li *et al.*, 2006). Odontoblasts establish the peripheral cellular layer of the dental pulp and extend their cellular processes into the tubular structure of dentin, such that they are the first cells able to detect signals from bacteria in the caries lesion and to respond to TGF- β 1 in the dentin matrix. Odontoblasts express receptors for TGF- β 1 (Smith *et al.*, 1998; Sloan *et al.*, 1999) and for microbes, Toll-like receptors (Veerayutthwilai *et al.*, 2007).

Toll-like receptors (TLRs) serve a major role in microbial detection. The TLR2 homodimer or the heterodimer of TLR2 with either TLR1 or TLR6 recognizes Gram-positive bacteria containing lipoteichoic acid (LTA) and lipopeptide, and also Gram-negative bacterial lipopeptide. TLR4, together with other accessory proteins such as MD2 and CD14, detects lipopolysaccharide (LPS) from Gram-negative bacteria. The regulation of various genes encoding inflammatory cytokines such as interleukin-8 (IL-8), tumor necrosis factor- α (TNF- α), IL-6, IL-1 β , and IL-12 is signaled via the TLRs on macrophages and other immunocompetent cells (Kawai and Akira, 2007). The role of several TLRs in human odontoblasts is emerging (Botero *et al.*, 2006; Durand *et al.*, 2006; Veerayutthwilai *et al.*, 2007); however, the functional significance of each TLR as well as the effect of variation in TLR level on odontoblast immune function have yet to be determined.

Very little is known about how TGF- β 1 and TLR signals may interact and contribute to odontoblast immune responses during infection. In this study, we first examined the contributions of TLR2 and TLR4 and the effect of TLR variation on odontoblast responses to oral bacteria by using 2 odontoblast-like cell clones that express different levels of TLR2 and TLR4 and an RNA silencing technique. We tested 3 Gram-positive bacteria (*i.e.*, *Streptococcus mutans*, *Enterococcus faecalis*, and *Lactobacillus casei*) and 3 Gram-negative bacteria (*i.e.*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum*), commonly involved in dental caries and pulpal infection. We then determined the effect of TGF- β 1 on expression levels of TLR2 and TLR4 and subsequent cellular responses to bacteria to examine the interrelationship of these factors in odontoblasts.

Figure 1. Characteristics of 2 human odontoblast-like cell clones (*i.e.*, hOD1, hOD2). **(A-D)** Flow cytometric analyses showed TLR2 and TLR4 expression on the cell surfaces of intact hODs (black line, isotype control IgG; green line, primary antibodies to TLR2/TLR4) and in the whole cell (cell surface + cytoplasmic) of fixed and permeabilized hODs (grey line, isotype control IgG; orange line, primary antibodies to TLR2/TLR4). Cell-surface expression of TLR2 and TLR4 was much higher in hOD1. hOD2 lacked TLR2 on the cell surface and displayed lower cell-surface expression of TLR4. After the hOD2 cells were fixed and permeabilized, intracellular TLR2 was detected. In contrast, fixation and permeabilization did not significantly change the detection of TLR2 in hOD1 or TLR4 in both hODs. The data shown here are representative of 3 independent experiments. **(E,F)** DSPP and DMP1 mRNA expression in human gingival fibroblasts (hGF, negative control), native human odontoblasts isolated from the tooth crowns (TcOD, positive control), and 2 clones of human odontoblast-like cells (hOD1, and hOD2) by quantitative real-time PCR. Values are reported as logarithm of relative fold change in DSPP/DMP1 mRNA transcription in odontoblast samples vs. the negative control, which was set at 0. The data represent means and standard errors from triplicate wells of one experiment and are representative of 3 independent experiments. **(G,H)** Immunofluorescent labeling of DSP protein (Green) in both hODs (scale bar = 10 μm). Note the polarized cells and DSP-positive protein matrix secretion (arrows).

MATERIALS & METHODS

An *in vitro* Model of Human Odontoblasts

Freshly extracted intact human third molars were collected with consent following a protocol approved by the University of Washington Human Subjects Review Board. A library of human odontoblast-like cell clones was established. The protocol for cell culture and an *in vitro* OD differentiation, modified from a previous study (Couble *et al.*, 2000), can be found in the Appendices. Two representative clones (*i.e.*, hOD1, hOD2) with different levels of TLR2 and TLR4 were selected and used for: (1) cell transplantation in immunocompromised/SCID mice and (2) bacterial stimulation with and without TGF-β1 pre-treatment (0–20 ng/mL for 1–7 days, R&D Systems, Minneapolis, MN, USA; Catalog #240B). The detailed protocol for cell transplantation, bacterial culture, and immunohistochemistry can be found in the Appendices. The protocol for animal use was approved by the University of Washington Institutional Animal Care and Use Committee.

Dual Luciferase Assay for the Activation of TLR2 or TLR4

Cells from the immortalized human embryonic kidney cell line 293 (HEK293) were transfected with human TLR (TLR2, TLR2

plus TLR1, or TLR4 plus MD2), membrane CD14, NF-κB reporter (ELAM-1-firefly luciferase), and the transfection control (β-actin-*Renilla* luciferase) constructs, as previously described (Darveau *et al.*, 2004). Then they were stimulated with each of the 6 heat-killed bacteria for 4 hrs, subjected to lysis, and assayed for NF-κB-luciferase reporter activity.

Silencing of TLR4 Gene Expression

RNA silencing was performed with 2 sets of small interfering RNA (siRNA) targeting human TLR4 mRNA: (1) plasmid-based short hairpin small interfering RNA (InvivoGen, San Diego, CA, USA) and (2) non-plasmid siRNA (Qiagen, Valencia, CA, USA). The detailed protocol for cell transfections and silencing controls can be found in the Appendices.

RNA Isolation and Quantitative PCR Amplification

Total RNA isolation and quantitative PCR analyses were performed as previously described (Veerayutthilalai *et al.*, 2007). The detailed protocol

and specific primer pairs used for PCR amplification can be found in the Appendices.

Flow Cytometry (FACS) Analysis

Cells were detached with EDTA and washed with FACS buffer. For cell-surface staining, 5×10^5 live cells in suspension were stained with each fluorescent-dye-conjugated primary antibody or isotype control IgG for 30 min at 4°C. For whole-cell staining (cell surface and cytoplasmic), cells were fixed with 2% paraformaldehyde for 10 min at room temperature before being stained with primary antibody or isotype control IgG containing 0.5% saponin, with a similar protocol. Flow cytometric analyses were performed with a BD LSR II flowcytometer and FlowJo software. The detailed protocol and antibodies can be found in the Appendices.

Protein Isolation and Western Blot Analysis

We subjected cells to lysis in RIPA buffer to obtain total protein extracts. A 10-μg quantity of protein was separated by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, which was then probed with primary antibody and horseradish-peroxidase-linked secondary antibody, followed by Pierce Supersignal reagent. The detailed protocol and antibodies can be found in the Appendices.

Enzyme-linked Immunosorbent Assay (ELISA)

The presence of IL-8 in the cell culture supernatant was determined by use of the DuoSet ELISA Development kit for human CXCL8/IL-8 (catalog# DY208, R&D Systems), according to the manufacturer's instructions (Appendices).

Statistical Analyses

The data were analyzed by analysis of variance followed by the Tukey test for multiple comparisons. Results were considered statistically significant when the P-value was less than 0.05.

RESULTS

Characterization of Human Odontoblast-like Cells

Two representative clones of human odontoblast-like cells (*i.e.*, hOD1, hOD2), derived from the dental pulp, expressed different level of TLRs; TLR2 and TLR4 cell-surface expression was significantly higher in hOD1 than in hOD2 (Figs. 1A-1D). However, both clones are functional cells possessing the odontoblastic phenotype (Figs. 1E-H) and a capacity to form extracellular matrix tissue that contained dentin matrix protein 2 *in vivo* (Appendix Figs. 1A-1F).

TLR2- or TLR4-mediated NF- κ B Activation in HEK 293 Cells by Heat-killed Gram-positive or Gram-negative Bacteria

Gram-negative bacteria (*i.e.*, *P. gingivalis*, *P. intermedia*, and *F. nucleatum*) activated TLR2, TLR2 plus TLR1, and TLR4, consistent with their LPS and lipopeptide components. In contrast, Gram-positive bacteria (*i.e.*, *S. mutans*, *E. faecalis*, and *L. casei*) activated only TLR2, and TLR2 plus TLR1, consistent with their LTA and lipopeptide (Appendix Figs. 2A, 2B). Similar results were obtained from TLR2 and TLR2 plus TLR1-expressing HEK cells, although the induction level was greater in the presence of TLR1 for all bacterial preparations.

Figure 2. TLR-mediated inflammatory responses to oral bacteria. (A-D) Regulation of IL-8 and TNF- α gene expression in hOD1 and hOD2 in response to oral bacteria. Two representative clones (hOD1, A&C; hOD2, B&D) were stimulated at bacteria-to-human-cell ratios of 100:1 (100) and 300:1 (300) for 24 hrs and processed for mRNA detection. (E-H) The role of TLR4 in odontoblast bacterial responses was validated by small interfering RNA targeting TLR4 (SiRNA-TLR4). TLR4 mRNA was knocked down by transfection of a siRNA-TLR4 into hOD1 cells (red bar, siRNA-TLR4 in E), but not by control non-silencing siRNA (ConSi, E) or without siRNA (NT, E). Cells were transfected for 48 hrs, then stimulated with each of the 6 heat-killed bacteria for 24 hrs, harvested, and assayed for mRNA expression. Values are reported as relative fold change/logarithm of relative fold change in IL-8/TNF- α mRNA transcription in bacterially stimulated vs. unstimulated samples. The data represent means and standard errors from triplicate wells of 1 experiment and are representative of 3 independent experiments. Asterisks indicate statistically significant changes, with $P < 0.05$.

Cell-surface Expression Levels of TLR2 and TLR4 are Associated with Odontoblast Bacterial Responses

We examined the bacterial responses of 2 hODs through the regulation of inflammatory cytokines, IL-8, and TNF- α gene expression. In both clones, IL-8 and TNF- α mRNA levels were greatly increased in response to *P. intermedia* and *F. nucleatum* (Figs. 2A-2D). hOD1, but not hOD2, showed enhanced cytokine gene expression in response to *S. mutans*, *L. casei*, and *E. faecalis*, consistent with the importance of TLR2 cell-surface expression for Gram-positive bacterial responses.

Figure 3. Effects of TGF-β1 on expression of TLR2, TLR4, DSPP, and 2 inflammatory markers, IL-8 and TNF-α. **(A-C)** hOD1 cells expressing TLR2 and TLR4 on the cell surface were treated with TGF-β1 at a range of doses and treatment (Tx) periods. Cells were then harvested and assayed for mRNA level by quantitative real-time PCR **(A-B)** and for protein expression by Western blot analysis **(C)**. Significant inhibitory effects of TGF-β1 on TLR2 and TLR4 expression were observed at 20 ng/mL for all tested time periods in both mRNA and protein levels. **(D-G)** The hOD cells were treated with 20 ng/mL of TGF-β1 for 1 wk, then stimulated with each of the 6 heat-killed bacteria for 24 hrs. The TGF-β1-treated hOD1 failed to up-regulate TLR2, TLR4, IL-8, and TNF-α gene expressions in response to all bacteria (data not shown for Gram-positive bacteria). Greater effects were noticed with Gram-negative bacteria (*P. gingivalis*, *P. intermedia*, and *F. nucleatum*). Note: Data for TLR2/4 are shown only for *P. intermedia*, but they represent the data for *P. gingivalis* and *F. nucleatum* **(D)**. Values are reported as relative fold change/logarithm of relative fold change in mRNA transcription in stimulated samples vs. unstimulated samples. The data represent means and standard errors from triplicate wells of 1 experiment and are representative of at least 3 independent experiments. Asterisks indicate statistically significant changes, with $P < 0.05$.

TLR4 Silencing Diminished Odontoblast Immune Responses to Gram-negative Bacteria

We utilized 2 systems for TLR4 RNA silencing: (1) a non-plasmid siRNA (Figs. 2E-2H), and (2) a plasmid-based siRNA (data not shown). TLR4 silencing with the non-plasmid siRNA resulted in over 80% reduction of TLR4 mRNA expression, compared with both negative controls: transfection reagent alone (NT) and non-silencing control (ConSi) (Fig. 2E). Although a slight inhibitory action on TLR2 mRNA was observed, this effect was not significant compared with the negative controls (data not shown). The up-regulation of IL-8 and TNF-α mRNA, induced by *P. gingivalis*, *P. intermedia*, and *F. nucleatum*, was abolished or significantly reduced in TLR4-knocked-down cells (hOD1 in Figs. 2F-2H, data not shown for hOD2), suggesting that TLR4 plays a major role in signaling the odontoblast responses to these bacteria. Similar results were obtained when TLR4 silencing was done with the plasmid-based siRNA.

TGF-β1 Decreased mRNA and Protein Expression of TLR2 and TLR4 in Human Odontoblast-like Cells and Suppressed Odontoblast Inflammatory Responses to Oral Bacteria

TLR2 and TLR4 expression significantly decreased in both hOD1 (Figs. 3A-3C) and hOD2 (data not shown), treated with 20 ng/mL of TGF-β1 at all treatment periods (1-7 days, $P < 0.05$, Figs. 3A, 3B). Although TGF-β1 treatment suppressed DSPP gene expression, in agreement with a previous report of TGF-β1 treatment at 5 ng/mL for 24 hrs (Unterbrink *et al.*, 2002), we found that this effect occurred regardless of TGF-β1 treatment (data shown for 0 and 20 ng/mL, not shown for 0.05, 0.5, 5, and 10 ng/mL) and noticed that the DSPP expression recovered after 1 wk (Appendix Fig. 3), suggesting that the odontoblast phenotype was maintained. We then treated hOD cells with 20 ng/mL of TGF-β1 for 1 wk before testing the effect of TGF-β1 on bacterial responses. Expression of TLR2 and TLR4 mRNA increased significantly in hOD1 and hOD2 with bacterial exposure in the absence, but not in the presence, of TGF-β1 treatment (Fig. 3D with *P. intermedia* stimulation, data not shown for stimulation

with *P. gingivalis* or *F. nucleatum*). The TGF-β1-treated hOD1 (Figs. 3E-3G) and hOD2 (data not shown) failed to up-regulate IL-8 and TNF-α mRNA transcription in response to Gram-negative bacteria, *P. gingivalis*, *P. intermedia*, and *F. nucleatum*. The inhibitory effect of TGF-β1 on IL-8 protein production in response to the 3 Gram-negative bacteria was confirmed in both hOD1 (data not shown) and hOD2 (Appendix Fig. 4). The contributions of TLR-driven inflammatory signals and TGF-β1 anti-inflammatory activities on the odontoblast bacterial responses are summarized in Fig. 4.

DISCUSSION

We previously demonstrated TLR function in human odontoblasts maintained in cultured teeth (Veerayuthwilai *et al.*, 2007). Our previous findings for TLR2- and TLR4-immunolocalization on human teeth suggested the heterogeneous TLR2 and TLR4 expression in odontoblasts. In this study, we induced the differentiation of pulp progenitor cells into

odontoblast-like cells and demonstrated heterogeneity of TLR2 and TLR4 cell-surface expression. We showed that these variations significantly influenced the odontoblast response to oral bacteria. Despite the different level of TLRs, both clones were highly responsive to *P. intermedia* and *F. nucleatum*, for which the activation signal occurred mainly through TLR4 in these cells. We also showed that TGF- β 1 inhibited both TLR2 and TLR4 expression in human odontoblast-like cells and resulted in attenuated bacterial responses. These findings suggest that odontoblast-bacterial interactions through TLRs are modulated by TGF- β 1 *in vivo*.

Teeth are constantly exposed to a multitude of Gram-positive and Gram-negative bacteria in the oral cavity (Love and Jenkinson, 2002). Odontoblasts, the first cells to be exposed to bacteria within the tooth, monitor the invasion of these microbes using TLRs to mount innate host defenses. Previous findings in cultured tooth crown odontoblasts (Levin *et al.*, 1999; Veerayutthwilai *et al.*, 2007) and odontoblast-like cells (Botero *et al.*, 2006; Durand *et al.*, 2006) support these results. In addition, our findings suggest that human odontoblasts are highly responsive to the Gram-negative bacteria, *P. intermedia* and *F. nucleatum*.

Although various components of Gram-negative bacteria can activate many immunomodulating receptors (Akira *et al.*, 2006), analysis of our RNA-silencing data affirms a prominent role of TLR4 in the activation of odontoblast responses to these bacteria. These results are shown in both hOD clones and are also supported by: (1) our previous findings in human odontoblasts maintained in natural teeth (Veerayutthwilai *et al.*, 2007); and (2) previous clinical data showing that the recovery of Gram-negative bacteria (e.g., *Prevotella*, *Porphyromonas*, and *Fusobacterium*) and their LPS from caries lesions was closely associated with pain and thermal hypersensitivity, which strongly indicates an extensive underlying pulpitis (Hahn *et al.*, 1991, 1993; Massey *et al.*, 1993; Khabbaz *et al.*, 2000; Hahn and Liewehr, 2007).

Using a different culture system, Paakkonen and colleagues reported an increase in inflammatory cytokine gene expression in the dental pulp, and in human odontoblasts treated with 1 ng/mL of TGF- β 1 for up to 24 hrs (Paakkonen *et al.*, 2007). We observed some similar results when treating our hOD cells with 0.05 ng/mL of TGF- β 1, but the effects were neither significant nor consistent. Differences in culture environment as well as dose-dependent effects of TGF- β 1 may contribute to this discrepancy. Nonetheless, the inhibitory effect of TGF- β 1 shown

Figure 4. A diagram illustrates the influence of Toll-like receptors (TLRs) and transforming growth factor- β 1 (TGF- β 1) on the interaction between human odontoblasts and carious bacteria. Odontoblasts respond to carious bacteria through the activities of TLR2 and TLR4. Gram-negative bacteria (e.g., *P. gingivalis*, *P. intermedia*, and *F. nucleatum*) stimulate both TLR2 and TLR4, consistent with their lipopeptide (LP) and lipopolysaccharide (LPS) cell-wall components, whereas Gram-positive bacteria (e.g., *S. mutans*, *L. casei*, and *E. faecalis*) activate only TLR2 via their LP and lipoteichoic acid (LTA) components. Odontoblasts greatly increase TLR expression and cytokine responses when exposed to the Gram-negative bacteria, *P. intermedia* and *F. nucleatum*, whose activation signal in odontoblasts occurs mainly through TLR4. TGF- β 1 is released from carious dentin and exerts anti-inflammatory action by inhibiting expression of TLR2, TLR4, and the inflammatory cytokines, IL-8 and TNF- α . The extent of pulpitis may be attributed to the balance between TLR-driven inflammatory signals and TGF- β 1 anti-inflammatory activities.

here agrees with the findings in TGF- β 1-null mice, which developed excessive inflammation in the dental pulp and periapical tissues, even in the absence of infection (D'Souza *et al.*, 1998). This uncontrolled extensive inflammation is also present in other vital organs, such as heart and lung (Kulkarni *et al.*, 1993, 1995). Loss of TLR2, TLR4, and TLR5 was also reported in epithelial dendritic cells, Langerhans cells (van der Aar *et al.*, 2007), which may be due to the exposure to TGF- β 1 during development. To our knowledge, this is the first report showing that TGF- β 1 inhibits the odontoblast expression of TLR2 and TLR4 and consequently reduces their inflammatory cytokine production in response to oral bacteria. Further, direct pulp-capping with TGF- β 1 improved the healing of pulp tissues and enhanced reparative dentin formation in mouse, dog, and goat teeth (Hu *et al.*, 1998; Tziafas and Papadimitriou, 1998; Zhang *et al.*, 2008). Taken together, these findings suggest the potential use of TGF- β 1 in the clinical treatment of pulpal inflammation.

In conclusion, these findings support and extend our previous results (Veerayutthwilai *et al.*, 2007) suggesting TLR4 as a major contributor to odontoblast inflammatory responses and a critical role of TGF- β 1 in maintaining homeostasis in the human tooth *in vivo*. The balance between TLR4 inflammatory signals and TGF- β 1 anti-inflammatory activities may play a key role in the development of pulpal inflammation.

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