Human Dental Pulp Cells Express Cellular Markers for Inflammation and Hard Tissue Formation in Response to Bacterial Information

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Abstract

Introduction: Lipopolysaccharide (LPS) is a major component of the outer membranes of gram-negative bacteria associated with deep dental caries and pulpitis. When bacteria invade dentinal tubes and dentin is continually destroyed, tertiary dentin is formed by preexisting odontoblasts. However, the relationship between LPS and tertiary dentin formation remains unclear. We investigated whether LPS stimulation induces the formation of hard tissue in human dental pulp cells (hDPCs). **Methods:** Immortalized hDPCs were cultured, and *Escherichia coli*–derived LPS (1 μg/mL) was incorporated into the culture medium. Samples were obtained after 0, 1, 3, 7, 14, and 21 days, and messenger RNA expression of *IL-1β*, *IL-6*, *Wnt5a*, *Runx2*, *ALP*, and alkaline phosphatase (ALP) activity was investigated. **Results:** Quantitative real-time polymerase chain reaction revealed higher messenger RNA expression levels of *IL-1β* and *IL-6* in the LPS group on 1 day ($P < .05$). The expression levels of dentinogenesis-related markers including *Wnt5a*, *Runx2*, and *ALP* were higher in the LPS group (2.0-, 4.7- and 10.0-fold, respectively) than that in the control group at 14 days ($P < .01$). ALP activity was significantly stronger in the LPS group than in the control group at 21 days ($P < .01$). Treatment of Box5, an antagonist of Wnt5a, showed a decreased expression of *Runx2* and *ALP* ($P < .05$). **Conclusions:** These results indicate that LPS stimulation induces the gene expression of inflammatory cytokines and hard tissue formation through Wnt5a signaling pathways in hDPCs. *(J Endod 2018;■:1–5)*

Key Words

Cytokines, hard tissue formation, human dental pulp cells, lipopolysaccharide, Wnt5a

Significance

This study aids in the understanding that bacterial component stimulation induces not only the expression of inflammatory cytokines but also the formation of tertiary dentin.

Dental pulp is a highly specialized mesenchymal tissue that is characterized by being surrounded by rigid mineralized dentin (1). Dental pulp contains several types of cells such as odontoblasts, fibroblasts, vascular endothelial cells, stem cells, and its precursor cells (2). Various environmental stimuli, including mechanical, physical, and chemical injuries as well as dental caries, may irritate the dentin/pulp complex. When dental pulp tissue is stimulated, tertiary dentin is reactively formed through a number of signaling pathways (3, 4).

Dental caries is an endogenous microbiological infection of gram-positive and -negative bacteria and results in the decalcification and destruction of enamel and dentin. When dental caries deeply invades dentin, dental pulp is stimulated and forms tertiary dentin, implicating bacteria and its components in dentin formation. We previously reported that dental pulp cells stimulated by *Streptococcus mutans* and *Porphyromonas gingivalis* exhibited increased alkaline phosphatase (ALP) activity and bone sialoprotein expression (5). Lipopolysaccharide (LPS) is a major outer membrane component in gram-negative bacteria and has been detected in deep-seated dental caries and pulpitis (6). LPS stimulates toll-like receptor 2 and toll-like receptor 4 on the cell membranes of dental pulp cells and activates the nuclear factor kappa B (NF-κB) pathway, producing inflammatory cytokines such as interleukin (IL) 1 beta (IL-1ß) and IL-6 (7–9). However, the relationships among LPS, inflammatory cytokines, and hard tissue formation in dental pulp remain unclear.

Wnt is a secreted glycoprotein and has been identified and conserved in many biological species. To date, 19 different kinds of Wnt have been specified, whereas others remain unknown (10). The Wnt signaling pathway is involved in embryonic development, tissue development, and other biological processes (11–13). Several Wnt signaling pathways have been associated with dentinogenesis (14), and the Wnt5a signaling pathway has been shown to induce the differentiation of dental papilla cells into odontoblasts (15). Furthermore, a previous study showed that the Wnt5a signaling pathway regulated the proliferation of odontoblastlike cells derived from embryonic stem cells (16). LPS was recently shown to stimulate the NF-κB pathway, thereby enhancing Wnt5a expression in human dental pulp stem cells (hDPSCs) (17). However, it currently remains unclear whether the Wnt5a signaling pathway is involved in hard

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tissue formation in dental pulp cells. Moreover, there has been direct knowledge that the bacterial component induces hard tissue formation.

Based on previous findings and clinical phenomena, we hypothesized that LPS in dental plaque triggers not only the production of inflammatory cytokines via the NF-κB pathway but also the induction of tertiary dentin by the expression of Wnt5a. The objective of the present study was to investigate the influence of LPS stimulation on the expression of hard tissue formation markers via Wnt5a signaling pathways in hDPCs *in vitro*.

Materials and Methods

Cell Culture

We used hDPCs immortalized by transfection with a human telomerase transcriptase gene (HPD-hTERTs). These cells were provided courtesy of Professor Takashi Takata (Hiroshima University Graduate School, Hiroshima, Japan). The characteristics of HDP-hTERTs were described previously (18). Cells were cultured until they reached 70%–80% confluence in minimum essential medium alpha (Thermo Fisher Scientific Inc, Waltham, MA) with 10% fetal bovine serum (Biosera, Kansas City, MO) plus 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific Inc) in a humidified atmosphere of 5% CO_2 at 37°C. In order to induce differentiation into hard tissue formation cells, the medium was changed every 3 days.

Incorporation of LPS

HDP-hTERTs were seeded on 6-well plates at a density of 1×10^4 cells/mL and cultured in minimum essential medium alpha supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/mL), 10 mmol/L beta-glycerophosphate (Sigma-Aldrich, St Louis, MO), 50 μg/mL ascorbic acid (Wako Pure Chemicals Industries, Ltd, Osaka, Japan), and 10 nmol/L dexamethasone (Wako Pure Chemicals Industries, Ltd). After 24 hours, *Escherichia coli*–derived LPS (1 μg/mL *E. coli* O111:B4 L2630, Sigma-Aldrich) was incorporated into the culture medium. The culture medium was changed every 2 to 3 days, and LPS was added with every change. Samples were obtained after 0, 1, 3, 7, 14, and 21 days.

Addition of Wnt5a Antagonist

HDP-hTERTs were seeded in a 6-well plate at a density of 1×10^4 cells/mL and cultured in hard tissue induction medium. Wnt antagonist III, Box5 (Calbiochem, Billerica, MA), was added in a concentration of 1, 10, and 100 μg/mL at the same time as the addition of LPS. LPS and Box5 were also added when replacing the culture medium every 3 days, and samples were collected at 7 days.

Quantitative Real-time Polymerase Chain Reaction

The total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific Inc) according to the manufacturer's protocol. RNA (1 μg) was reverse transcribed into complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) analyses were performed with the ABI7500 Fast System (Applied Biosystems) using 2× TaqMan Fast Universal PCR Master Mix no AmpErase reagent (Applied Biosystems). The target genes were *IL-1ß* (Hs01555410_m1), *IL-6* (Hs00985639_m1), *Wnt5a* (Hs00998537_m1), *Runx2* (Hs00231692_m1), and *ALP* (Hs01029144_m1); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous control. Reaction conditions consisted of 40 cycles of polymerase chain reaction comprising denaturation at 95°C for 3 seconds and annealing and extension at 60°C for 30 seconds. The relative expression of genes of interest was estimated using the ΔΔ threshold cycle (Ct) method. Briefly, *GAPDH* was used to normalize the amount of target gene messenger RNA (mRNA). Its Ct value was subtracted from that of the target gene to obtain a ΔCt value. The difference (ΔΔCt) between the ΔCt values of samples for the target genes and that of the calibrator was assessed. qRT-PCR was performed in triplicate.

ALP Activity

HDP-hTERTs were incubated for 7, 14, and 21 days with or without LPS and lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40 [Sigma-Aldrich], 150 mmol/L NaCl, and 50 mmol/L Tris; pH = 7.4; containing protease inhibitors). Protein concentrations were measured using the Lowry method with bovine serum albumin as the standard. ALP activity was analyzed using Lab Assay ALP (Wako Pure Chemical Industries Ltd) following the manufacturer's instructions, and absorbance was measured at a wavelength of 405 nm using a microplate reader. ALP activity (U/μg protein) was defined as the release of 1 nmol *p*-nitrophenol per microgram of total protein.

Statistical Analysis

The significance of differences was evaluated using the Mann-Whitney *U* test, and significance was set at $P < .01$ or .05.

Results

Inflammatory Cytokine Expression

In order to evaluate inflammatory cytokine genes, we measured the expression of *IL-1ß* and *IL-6* mRNA using qRT-PCR.

IL-1β. The results obtained for *IL-1ß* are shown in Figure 1*A*. The expression of *IL-1ß* mRNA was detected in the LPS and control groups. Expression levels were significantly higher in the LPS group than in the control group at 1 day (6.7-fold, $P < .01$). However, no significant differences were noted at 3 days between the 2 groups.

IL-6. The expression of *IL-6* was detected in both groups, similar to *IL-1ß*, as shown in Figure 1*B*. Expression levels were significantly higher in the LPS group than in the control group at 1 day (11.6-fold, $P < .01$). Significant differences were also noted at 3 and 7 days (3.5-fold and 2.4-fold, respectively). The expression of *IL-6* was weaker in the LPS group than in the control group at 14 days (0.5-fold).

Expression of Hard Tissue Formation–related Genes

We investigated the expression of *Wnt5a*, *Runx2*, and *ALP* in order to evaluate hard tissue formation–related genes in HDP-TERTs.

Wnt5a. The expression levels of *Wnt5a* mRNA were significantly higher (2.0- to 4.0-fold) in the LPS group than in the control group at 7, 14, and 21 days after the incorporation of LPS ($P < .01$ at 21 days and $P < .05$ at 7 and 14 days) (Fig. 2*A*).

Runx2. The expression levels of *Runx2* were significantly higher (4.7-fold) in the LPS group than in the control group at 14 days ($P < .01$). However, no significant differences were observed at 7 or 21 days (Fig. 2*B*).

ALP. The expression of *ALP* gradually increased at 7 and 14 days (2.5-fold and 10-fold, respectively), and significant differences were observed ($P < .01$). In contrast, its expression was weaker in the LPS group than in the control group at 21 days (Fig. 2*C*).

ALP Activity

In order to confirm the expression of *ALP* mRNA, ALP activity was quantified using a modified colorimetric assay, with p-nitrophenyl phosphate acting as the substrate. Bacterial activation did not increase