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CpG oligonucleotides induce an immune response of odontoblasts through the TLR9, MyD88 and NF-κB pathways

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ABSTRACT

Odontoblasts are the first-line defense cells against invading microorganisms. Toll-like receptors (TLRs) play a crucial role in innate immunity, and TLR9 is involved in the recognition of microbial DNA. This study aimed to investigate whether odontoblasts can respond to CpG DNA and to determine the intracellular signaling pathways triggered by CpG DNA. We found that the mouse odontoblast-like cell line MDPC-23 constitutively expressed TLR9. Exposure to CpG ODN induced a potent proinflammatory response based on an increase of IL-6 and TNF-α expression. Pretreatment with an inhibitory MyD88 peptide or a specific inhibitor for TLR9, NF-κB or IκBα markedly inhibited CpG ODN-induced IL-6 and TNF-α expression. Moreover, the CpG ODN-mediated increase of κB-luciferase activity in MDPC-23 cells was suppressed by the overexpression of dominant negative mutants of TLR9, MyD88 and IκBα, but not by the dominant negative mutant of TLR4. This result suggests a possible role for the CpG DNA-mediated immune response in odontoblasts and indicates that TLR9, MyD88 and NF-κB are involved in this process.

1. Introduction

Dental caries and tooth pulp infection are common chronic infections in humans. Innate immunity is the first line of defense against infection in the dentin–pulp complex. Odontoblasts are located at the pulp–dentin interface and extend into the dentin. Thus, they are the first cells encountered by microorganisms or their products in the tooth and may play an important role in the innate immune defense of the tooth. Specific conserved components of microorganisms include lipopolysaccharides (LPSs), lipoteichoic acid, bacterial DNA and flagellin [1–3]. Some evidence has indicated that LPSs and lipoteichoic acid elicit an immune response in odontoblasts [4–6]. However, there is currently no information on the direct biological effects of bacterial DNA on odontoblasts.

The immunostimulatory activity of bacterial DNA is characterized by a high content of unmethylated cytidine–phosphate–guanosine (CpG) motifs, which are prevalent in bacterial but not mammalian genomic DNA [7]. CpG DNA is recognized by TLR9. Upon recognizing CpG DNA, intracellular TLR9 recruits other signaling molecules, such as myeloid differentiation marker 88 (MyD88), and activates MAPKs and NF-κB through the MyD88/IRAK/TRAF6/TAK1 kinase cascade, followed by the activation of transcription factors like NF-κB or AP-1. The activation of NF-κB or AP-1 drives the induction of several proinflammatory cytokines and chemokines [8].

Synthetic oligonucleotides containing unmethylated CpG motifs (CpG ODN) can mimic the ability of bacterial DNA to activate immune cells such as macrophages, B-cells, NK-cells and dendritic cells to produce cytokines through TLR9 [9–12]. Bacterial DNA or CpG ODN also stimulates non-immune cells, including epithelial cells and endothelial cells [13–15]. Similar to epithelial cells and endothelial cells, odontoblasts are also the first-line defense cells of hosts. However, there is no report regarding the effect of CpG DNA on odontoblasts and the intracellular molecular mechanisms triggered by CpG DNA. The purpose of the current study was to determine the CpG DNA-mediated immune response and intracelluar signaling pathways in odontoblast cells.

In the present study, we show that TLR9 is expressed in the mouse odontoblast-like cell line MDPC-23, a spontaneously immortalized cell line derived from fetal mouse molar dental papillae [16]. CpG ODN induces proinflammatory cytokine expression through the activation of TLR9. Furthermore, we investigate the role of the MyD88 and NF-κB signaling pathways in the regulation of CpG ODN-induced proinflammatory cytokine expression in MDPC-23 cells.

2. Material and methods

2.1. Plasmids and reagents

Plasmids encoding dominant-negative forms of MyD88 and TLR4 were kindly provided by Koanhoi Kim (School of Medicine

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and Medical Research Institute, Pusan National University, Busan, Republic of Korea). Plasmids encoding the dominant-negative form of TLR9 were purchased from Invivogen (San Diego, CA). IκBαSR, a nondegrading mutant of IκBα, was kindly provided by Harikrishna Nakshatri (School of Medicine, Indiana University, Indianapolis, USA). The pκB-luciferase reporter plasmid was a generous gift from Chih-Ho Lai (School of Medicine, China Medical University, Taiwan). Oligodeoxynucleotides containing the unmethylated bacterial CpG motif with a phosphorothioate backbone (mouse-specific ODN 1668 or CpG ODN), control oligodeoxynucleotides with an inverted CpG motif (ODN 1720) and human-specific ODN 2006 were purchased from Sangon (Shanghai, China). The sequences were as follows: ODN 1668, 5′-TCC ATG ACG TTC CTGATG CT-3′; ODN 1720, 5′-TCC ATG AGC TTC CTGATG CT-3′; ODN 2006, 5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′. ODN 1668, ODN 1720 and ODN 2006 were dissolved in endotoxin-free sterile distilled deionized H_2O according to the manufacturer's recommendations and were used at the indicated concentrations. The nuclear factor-κB-specific (NF-κB specific) inhibitor pyrrolidine dithiocarbamate (PDTC) was obtained from Sigma Chemical Company (St. Louis, MO, USA). Chloroquine, Bay 117082, MyD88, and the control inhibitory peptide were purchased from Invivogen (San Diego, CA).

2.2. Cell lines and cultures

MDPC-23 cells, kindly provided by Prof. C.T. Hanks and Dr. Jacques E. Nor. (School of Dentistry, University of Michigan, MI, USA), were grown and maintained in α-MEM with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (Gibco, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Reverse transcription–PCR and quantitative real-time RT–PCR

Total RNA was extracted using the Trizol reagent (Invitrogen) and treated with DNase I (RNase-free, RQ1, Promega, Madison, WI). One microgram of total RNA was used as a template to make first-strand cDNA by oligo-dT priming using an Omniscript RT kit (Qiagen, Inc., Valencia, CA). Negative control samples (no first strand synthesis) were prepared by performing reverse transcription reactions in the absence of reverse transcriptase. The following synthetic gene-specific primer sets (Sangon, Shanghai) used for PCR: TLR9 (397 bp) 5′-AGCTAAAGGCCCTGACCAAT-3′ and 5′-CCAAAGCAG TCCCAAGAGAG-3′, and GAPDH 5′-CCCATCACCATC TTCCAGGAGC-3′ and 5′-CCAGTGAG CTTCCCGTTCAGC-3′. The PCR used 2 μl of first-strand cDNA, 0.5 μM of each mouse-specific primer and 25 μl of 2XRED Taq ReadyMix (Sigma, St. Louis, MO, USA) in a total volume of 50 μl. The cycling conditions were 94 °C for 5 min, followed by 25–30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The PCR-amplified products were analyzed in 2% agarose gels and stained with ethidium bromide.

Quantitative real-time RT–PCR analyses were accomplished using the iCycler iQ5 Real-Time PCR System (Bio-Rad) in the presence of the commercially available SYBR Green PCR Master Mix (Takara, Japan) in a 40-cycle PCR. The denaturing, annealing and extension conditions of each PCR cycle were 95 °C for 10 s, 60 °C for 20 s and 72 °C for 10 s, respectively. The mRNA levels of each target gene were normalized to the levels of β-actin and were represented as fold induction. The primer sequences of the real-time PCR were IL-6, 5′-CCAGAAAC CGCTATGAAGTTCC-3′ and 5′-TTGTCACCAGCATCAGTCCC-3′; TNF-α, 5′-TCTACTTTG GAGTCATTG-3′ and 5′-CAGAGTAAAGGGGTCAGAG-3′′; β-actin, 5′-GGCTGTATT CCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGC-CATGT-3′.

2.4. Transient transfection and luciferase assay

MDPC-23 cells at approximately 2×10^5 cells/well were plated in 24-well dishes and transfected 24 h later using the transfection reagent LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer's instructions. For each transfection, cells were co-transfected with 0.5 μg of the pκB-luciferase reporter plasmid, 0.1 μg of pRL-TK (Promega, Madison, WI) and either control vector (pcDNA3), DN-MyD88, DN-TLRs or IκBαSR plasmids. All plasmids were purified with the Endofree plasmid kit (Qiagen, Inc., Valencia, CA). At 24 h after transfection, the cells were treated with ODN 1668 (3 μM) or ODN 1720 (3 μM) for the indicated time periods. The luciferase activity was determined as previously described [17] and calculated as the degree of induction compared with an untreated control. Luciferase activity in the total cell lysate was measured with the dual luciferase reporter assay system (Promega, Madison, WI, USA). Luciferase activities were normalized based on Renilla luciferase expression from the pRL-TK control vector.

2.5. Cytokine assay

MDPC-23 cells were cultured in 24-well culture plates. After reaching 80% confluence, the cells were treated with various stimulators for the indicated times or pretreated with specific inhibitors as indicated, and then the culture supernatants were collected and stored at −80 °C until use. The concentrations of IL-6 and TNF-α in the culture supernatants were measured with a specific enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (R&D, Minneapolis, MN). Briefly, cell supernatants were diluted 1:10 in PBS. The diluted samples and recombinant mouse standard proteins were then applied to the wells. Unbound protein was removed by washing, and horseradish peroxidase-conjugated anti-IL-6 or anti-TNF-α antibody was added. After a color reaction with the substrate, the optical density was recorded at a wavelength of 450 nm with an automated ELISA reader (Bio-Rad Laboratories, Hercules, CA). The IL-6 and TNF-α concentrations were determined in relation to the standard curve generated with the recombinant IL-6 or TNF-α proteins.

2.6. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). The significance of differences between the experimental group and control were determined using Student's *t*-test. A *p*-value of less than 0.05 indicated statistical significance.

3. Results

3.1. Expression of TLR9 mRNA in mouse MDPC-23 cells

To investigate the expression of endogenous TLR9 in mouse odontoblasts, the presence of its mRNAs was assessed by RT–PCR in the mouse odontoblast cell line MDPC-23. RT–PCR analysis revealed constitutive expression of TLR9 transcripts (Fig. 1). In this experiment, mouse RAW 264.7 cells showed TLR9 mRNA expression as a positive control. In the absence of reverse transcriptase (RT), no expression of TLR9 was detected, which indicated that the samples were not contaminated with genomic DNA. Subsequent nucleotide sequence analyses of the RT–PCR products from MDPC-23 cells revealed 100% identity with the published mouse TLR9 cDNA sequence, confirming that the primers used in this study amplified the correct sequences.