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The Role of Low-Level Sodium Fluoride in Periodontal Inflammation

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Abstract: Fluoride has a wide range of physiological activities that may contribute to its beneficial effects against inflammation-related diseases. However, the molecular mechanisms underlying the anti-inflammatory activity of fluoride are not completely characterized, and many features remain to be elucidated. In this study, we investigated the molecular basis for the down-regulation of toll-like receptor 4 (TLR4) signal transduction by low-level fluoride (NaF) in periodontal ligament cells. In addition, the expression of asporin was investigated by quantitative real-time PCR as well as by immunohistochemical analysis of periodontal ligament cells, with or without fluoride treatment. \textit{P. gingivalis}-derived lipopolysaccharide (LPS) markedly elevated the mRNA and protein expression levels of DEC1, a regulator of TLR signaling. LPS-induced expression of TLR4, DEC1 and Notch1 was inhibited by low-level NaF. Asporin and peristin were expressed in periodontal ligament cells as expected. Treatment with LPS decreased the expression levels of asporin and peristin in periodontal ligament cells and treatment with low-level NaF increased those levels both \textit{in vivo} and \textit{in vitro}. These novel findings provide new insights into understanding the regulatory mechanisms of the TLR4 signaling pathway and the pharmacological role of low-level NaF in the inflammatory response against the development and progression of periodontal disease.

Key words: Fluoride, TLR4, DEC1, \textit{P. gingivalis}, Periodontal inflammation

Introduction

Inflammation is a process by which the human body attempts to counteract potentially harmful agents such as invading bacteria, viruses and other pathogens\textsuperscript{1,2}. The innate immune response to bacterial pathogens relies on the detection of pathogen-associated molecular patterns by pattern recognition molecules. Toll-like receptor 4 (TLR4) is one of the best characterized members of the family of mammalian TLRs that represents the most extensively studied class of pattern recognition molecules\textsuperscript{3}.

Periodontal diseases are known to be of microbial origin. Lipopolysaccharides (LPSs), also known as bacterial endotoxins, play a major role in the pathogenesis of progressive periodontal disease\textsuperscript{4,5}. \textit{In vivo} studies indicate that fibroblasts and macrophages secrete cytokines in the presence of LPS\textsuperscript{6,7}. We previously demonstrated that the neutralization of interleukin-1\textbeta{} (IL-1\textbeta{}) results in a reduced severity of inflammation\textsuperscript{8}. LPS also inhibits the differentiation of osteoblasts, stimulating alveolar bone resorption and inhibiting alveolar bone formation, thus resulting in the erosion of tooth-supporting bones\textsuperscript{9-11}. LPS stimulates the transcription of many genes in multiple signaling pathways. DEC1 is a transcriptional regulatory protein that can directly activate and repress the transcription of genes with a wide array of functional roles. We previously demonstrated that the LPS-stimulated expression of DEC1 in human periodontal ligament (PDL) cells and hypoxia enhances the virulence of LPS to induce the expression of DEC1 via a DEC1-dependent pathway\textsuperscript{12}. The rapid induction of these proteins in response to environmental stimuli suggests that DEC1 is protective against detrimental conditions.

Human PDL cells also play an essential role in periodontal regeneration owing to their ability to differentiate into cementoblasts and osteoblasts\textsuperscript{13,14}. Asporin, also known as periodontal ligament-associated protein 1 (PLAP1), is an extracellular matrix (ECM) protein that belongs to the class I small leucine-rich repeat proteoglycan/protein (SLRP) family\textsuperscript{15}. \textit{In vitro} studies suggest that asporin plays an important role in mineralization\textsuperscript{16}. Peristin is another important ECM protein and its multifaceted role has also been well documented\textsuperscript{17}.

Fluoride has been observed to stimulate osteoblast viability and function in humans\textsuperscript{18}. Sodium fluoride used to be considered the most potent agent to prevent osteoporosis in view of its pharmacological actions to enhance the recruitment of osteoblasts and matrix deposition\textsuperscript{19}. Fluoride serves as an effective prophylaxis against tooth caries\textsuperscript{20}. Fluoride influences cells \textit{in vitro}, with effective concentrations differing up to 1000-fold for different cell types\textsuperscript{21,22}. For any given cell type tested, low fluoride doses were mitogenic\textsuperscript{22,23}, whereas high concentrations (for odontoblasts and osteoblasts about 2 mM fluoride or above) provoked growth arrest and cell damage\textsuperscript{24}. Several intracellular signaling pathways react to fluoride, suggesting that gene expression may be selectively influenced in terms of inflammation. The activation of toll receptors is the first interaction directing the subsequent inflammatory cascade, which in progressive periodontal disease can lead to tooth loss.

The primary goal of this study was to determine whether gene expression could be altered by fluoride treatment. To this end, we tried to elucidate the molecular basis for the down-regulation of TLR4 signal transduction by treatment of PDL cells and tissues with low-level NaF. Here, we show that DEC1 is essential for mediating the anti-inflammatory activity of low-level NaF in LPS-treated PDL.
Materials and Methods

**Cell culture**

Human immortalized PDL cells were obtained from Professor Takashi Takata (Hiroshima University, Japan) and were cultured in Dulbecco’s modified Eagles medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (FBS; Biowest, Riverside, MO, USA). The PDL cells were seeded in 60-mm plastic tissue culture dishes and were incubated in 5% CO2 at 37°C. When the cells reached sub-confluence, they were harvested and sub-cultured. PDL cells at the fourth passage were used in these experiments. LPS from *P. gingivalis* was added to the PDL cell cultures for 24 h to evaluate the effects of treatment with bacteria. The concentration of *P. gingivalis* LPS used (500 nM) was adopted from our previous study.25

**Preparation of bacteria**

*P. gingivalis* ATCC 33277 was grown in brain heart infusion broth supplemented with 5 mg/ml yeast extract, 5 μg/ml hemin and 0.2 μg/ml vitamin K1, as described previously.27 Bacterial cells were grown under anaerobic conditions (85% N2, 10% H2, and 5% CO2) at 37°C for 24 h. LPS from *P. gingivalis* ATCC 33277 was obtained from *P. gingivalis* according to the manufacturer’s instructions (iNtRON Biotechnology, Kyungki-Do, Korea).28

**RNA extraction and Quantitative Real Time-PCR (QRT-PCR)**

Total RNA was extracted from PDL cells using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) as described previously.25 A TURBO DNase-free™ Kit (Applied Biosystems, Foster City, CA, USA) was used to remove contaminating DNA from the RNA preparations. First-strand cDNAs were synthesized from 1 μg total RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), according to the manufacturer’s protocol. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out in the absence of reverse transcriptase in order to identify whether residual genomic DNA contaminated the RNA samples. The relative expression levels of target mRNAs, compared to the level of β-actin RNA, were analyzed by real time PCR with the corresponding TaqMan MGB probes (Hs01566750_m1 for periostin and Hs01060665_g1 for β-actin) using a QuantStudio 6 Real Time PCR System (Applied Biosystems). The thermal cycling conditions used were according to the TaqMan Fast Universal PCR protocol.

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**Experimental periodontitis**

The animals used in this study have been described in our previous report.27 Briefly, eighteen 3-week-old male Sprague-Dawley rats (CLEA Japan, Tokyo, Japan) were obtained and housed in cages for 2 weeks to acclimatize before starting the experimental period. The rats were given sulfamethoxazole (1 mg ml−1) and trimethoprim (200 μg ml−1) in their drinking water for 4 days to reduce any original oral microorganisms, followed by a 3-day antibiotic-free period before starting the oral challenge with bacteria. Rats were divided into the following three groups of 6 rats each. Group A received only 5% carboxymethyl cellulose (CMC) (control group). Groups B (Pg group) and C (Pg + NaF group) were orally challenged with *P. gingivalis* ATCC 33277 with 0.5 ml (1.0 × 108 cells per ml) of the bacterial suspension in 5% CMC by oral gavage at 8, 10 and 12 days. Group C was then treated with 500 μM fluoride in the drinking water after the 3 *P. gingivalis* treatments. All rats were sacrificed 30 days later, and horizontal alveolar bone loss was measured using a morphometric method. The experimental procedures of this study were reviewed and approved by the Committee of Ethics on Animal Experiments of the Kanagawa Dental College.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded gingival tissue sections were immunostained using a CSA II System (DAKO, Carpenteria, CA, USA), in accordance with the manufacturer’s instructions. Sections were initially immersed in Target Retrieval Solution (DAKO) at 95°C for 12 min, and then were cooled for 30 min. Endogenous peroxidase activity was blocked with REAL Peroxidase-Blocking Solution (DAKO) for 30 min. Antibodies against aspirin (1:75; Abcam, Cambridge, MA, USA), DEC1 (1:75; Novus, Littleton, CO, USA), periostin (1:75; Abcam, Cambridge, MA, USA), *P. gingivalis* (1:100; a kind gift from Prof. Kazuyuki Ishihara), TLR4 (1:50; Abcam, Cambridge, MA, USA) and Notch1 (1:75; Abcam, Cambridge, MA, USA) were used as primary antibodies and were incubated overnight at 4°C. A secondary antibody conjugated to peroxidase (Nichirei Biosciences, Tokyo, Japan) was then incubated at room temperature for 25 min. After rinsing with PBS, all specimens were color developed with a 3,3’-diaminobenzidine tetrahydrochloride (DAB) chromogen kit (DAKO), counterstained with hematoxylin and then examined by light microscopy. The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAB exposure conditions.

**Statistical analysis**

Statistical analyses were performed by one-way ANOVA and Dunnett’s Test. A *P*-value of less than 0.05 is considered statistically significant.

Results

Figure 1. Effect of NaF on the TLR4-associated signaling pathway. Total RNA was isolated from each sample and was subjected to qRT-PCR analysis. TLR4, DEC1, and Notch1 mRNA levels were highly expressed in human PDL cells after treatment with LPS at 500 μM and low-level NaF significantly reduced their expression levels. High expression levels of aspirin and periostin were observed in the control group at 6 h and 24 h, while treatment with LPS reduced the expression of those genes and NaF abrogated that effect. Relative mRNA levels were calculated as a ratio to the housekeeping gene (β-actin). Each bar represents the mean ± SD for at least 3 independent experiments. *P < 0.05, compared with the control cells.
Figure 2. Role of low-level NaF in experimental periodontitis. Four µm thick sections of formalin-fixed, paraffin-embedded specimens were dep-araffinized and immunoreactivities were detected using a DAKO Envision Kit. (A) Hematoxylin and eosin (H-E) staining of *P. gingivalis* and NaF-treated rat upper jaw tissue sections showed less evidence of mononuclear cell infiltration compared to *P. gingivalis* only treated rats at 30 days after treatment. DEC1 showed more positive cells in the periodontal tissue from rats treated with *P. gingivalis* than from the *P. gingivalis* and NaF-treated rats. (B) TLR4 and Notch1 were abundantly expressed in the *P. gingivalis* challenged rat periodontal tissues. Immunohistochemical analysis revealed a higher expression of those genes in *P. gingivalis* treated rats compared to the control and *P. gingivalis* and NaF-treated rats. (C) Increased expression of asporin and periostin was observed in the control rats, but was decreased in the *P. gingivalis* group, while NaF treatment increased their expression in the periodontal tissues. Scale bars: 20 μm.
Effects of LPS and/or fluoride on the TLR4-associated signaling pathway

We used low-level NaF to investigate its potential to suppress the TLR4-associated signaling pathway. Given that fluoride disturbs the expression of LPS-induced TLR4, we focused on the PDL markers, asporin and peristin, and more particularly on the induction of the expression levels of the transcription factors DEC1 and Notch1 that lead to up-regulated TLR4 levels. We measured gene expression levels of DEC1 after a 6 or 24 h incubation period of PDL cells with or without fluoride. As shown in Fig. 1, fluoride reduced the LPS-induced up-regulation of DEC1 gene expression at 6 h and at 24 h in human PDL cells. Similar results were obtained for the expression of Notch1. To correlate the effects of fluoride at the transcript level with PDL markers, we performed qRT-PCR of human PDL cells treated with LPS with or without fluoride for 6 or 24 h. In the absence of LPS, we detected a high expression of asporin and peristin at 6 h and 24 h, while treatment with LPS reduced the expression of both those genes. Treatment with NaF reversed the inhibitory effects of LPS. Lastly, we observed that treatment with low level NaF abrogated the LPS-induced expression of TLR4 mRNA at 6 h and at 24 h.

Fluoride reduces the decreased expression of P. gingivalis-induced inflammatory proteins

Hematoxylin and eosin (H-E) staining of upper jaw tissue sections showed reduced mononuclear cell infiltration in NaF-treated rats compared to P. gingivalis only treated rats at 30 days (Fig. 2A). Immunostaining with a P. gingivalis antibody or an anti-DEC1 antibody showed more positive cells in the PDL tissue from rats treated with P. gingivalis than rats also treated with NaF (Fig. 2A). Immunohistochemical staining showed that P. gingivalis treatment increased the expression of TLR4 and Notch1 in the infiltrating inflammatory cells (Fig. 2B). Compared with rats treated only with P. gingivalis, NaF-treated rats showed reductions in the expression of TLR4 and Notch1 proteins. Asporin and peristin expression appeared highest in PDL cells in the control group and was decreased in the P. gingivalis treated group, while NaF treatment increased their expression (Fig. 2C).

Discussion

The mechanisms by which bacteria mediate the intracellular pro-inflammatory response have been partially characterized. Genetic studies and gene transfer experiments indicate that the intracellular effects of bacteria may be mediated through TLR signaling. Because microbial products can activate cells via pattern-recognition receptors and since TLRs comprise a major class of those molecules, the possibility that supernatants containing LPS might activate a TLR was considered. Our data using immunohistochemistry showed a higher increase of TLR4 expression in PDL cells treated with LPS. In addition, we demonstrated that the induction of DEC1 and Notch1 by P. gingivalis LPS appears to be mediated by the TLR4 pathway.

Genetic and biochemical studies have suggested that TLR4 plays an important role in LPS signaling under physiological conditions. The importance of TLR4 in LPS signaling is further supported by the fact that TLR4-deficient mice are LPS hypo-responsive but respond normally to products of Gram-positive organisms25. We recently reported that mice lacking DEC1 are also hypo-responsive to LPS and are negatively associated with the magnitude of the inflammatory response26, which suggests that DEC1 also has a potential role in pro-inflammatory signaling27. However, it is not clear whether this negative DEC1 regulator is involved in the inhibitory effect of low-level NaF on inflammatory activities. Therefore, in this study, we investigated whether DEC1 mediates the inhibitory action of low-level NaF on TLR4 signaling. Our results indicate that the LPS-induced expression of DEC1, TLR4 and Notch1 is significantly inhibited by low-level NaF treatment, and that low-level NaF enhances the expression of asporin and peristin. Our previous studies supported the concept that the reduced expression of these genes is mediated through DEC128,29. These results confirm that DEC1 mediates the suppressive effect of low-level NaF on LPS-induced periodontal disease.

The molecular mechanism involved in the pathogenesis of NaF is poorly understood, so this study focused on exploring the different mechanisms involved in the effects of low-level NaF in responses to pathogens. TLRs play a fundamental role in the innate immune system by triggering proinflammatory signaling pathways and promoting the activation of leukocytes30. In vivo studies with P. gingivalis and in vitro studies with human PDL cells expressing TLR4 showed that the association of LPS with TLR4 is directly inhibited by low-level NaF, which suggests that the antimicrobial NaF can potentially deactivate cellular inflammatory processes. Accordingly, the outcome of the present study evidently proved that low-level NaF attenuates LPS-induced periodontal inflammation and is the first study to demonstrate the involvement of DEC1, TLR4, Notch1, asporin and peristin protein expression in NaF administration.

Peristin is an extracellular cell adhesion protein and its close association with TIMP-2 during development of the mouse mandible suggests that it has a role in ECM formation31. Peristin-deficient mice exhibit disturbances in the formation and remodeling of tissues, i.e. the maintenance of the PDL was impeded32. Asporin expression has also been found in diverse tissues that synthesize collagen, including the PDL, dentin, bone and cartilage33,34. Asporin can also promote mineralization in human dental pulp stem cells35 and can interact with calcium and collagen36. Type I collagen deposition37 and asporin expression38 can be modulated by fluoride, thus generating a regulatory loop of mineralization. In this study, the similar responses in the PDL suggests that the effector, fluoride, is a trigger that elicits a PDL specific action. Although the specific mechanism of action has not been elucidated, our previous studies and the in vitro and in vivo data reported here suggest that NaF acts directly on oral cells39,40.

In summary, we confirmed that low-level NaF-mediated effects on DEC1 regulate TLR4 signaling in PDL cells, which provides new insights into understanding the regulatory mechanisms of the TLR4 signaling pathway and the pharmacological role of low-level NaF in the inflammatory response against the development of periodontal disease.

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Conflict of Interest
The authors declare no conflict of interest.

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