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Retinoic acid-inducible gene-I is induced in gingival fibroblasts by lipopolysaccharide or poly IC: possible roles in interleukin-1β, -6 and -8 expression

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Retinoic acid-inducible gene-I (RIG-I) is a member of the DExH family of proteins, and little is known of its biological function in the oral region. We previously reported that interleukin 1β (IL-1β) induced RIG-I expression in gingival fibroblasts. In this study, we studied the mechanism of RIG-I expression induced by lipopolysaccharide (LPS) or double-stranded RNA (dsRNA) in gingival fibroblasts. We also addressed the role of RIG-I in the expression of IL-1β, IL-6 and IL-8 in gingival fibroblasts stimulated with LPS or dsRNA. We stimulated cultured human gingival fibroblasts with LPS or dsRNA, and examined the expression of RIG-I mRNA and protein. The effect of cycloheximide, a protein synthesis inhibitor, on RIG-I induction by these stimuli was examined. The expression of IL-1β, IL-6 and IL-8 in gingival fibroblasts transfected with RIG-I cDNA stimulated with LPS or dsRNA was examined. LPS or dsRNA induced the expression of mRNA and protein for RIG-I in concentration- and time-dependent manners. We also examined the localization of RIG-I, and found that it was expressed in cytoplasm. Cycloheximide did not suppress the LPS or dsRNA-induced RIG-I expression. Introduction of RIG-I cDNA into gingival fibroblasts resulted in enhanced expression of IL-1β, IL-6 and IL-8; moreover, overexpression of RIG-I stimulated with LPS or dsRNA synergistically increased expression of IL-1β, IL-6 and IL-8. RIG-I may have important roles in the innate immune response in the regulation of IL-1β, IL-6 and IL-8 expression in gingival fibroblasts in response to LPS and dsRNA.

Key words: chemokine; cytokine; dsRNA; lipopolysaccharide; retinoic acid-inducible gene-I

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Retinoic acid-inducible gene-I (RIG-I) is a member of the DExH box protein family, and its gene was identified as being induced by retinoic acid in a promyelocytic leukemia cell line (36). It is designated as a putative RNA helicase from its amino acid sequences. However, details of its biological function are not known. In vascular endothelial cells, the RIG-I protein is shown to regulate the expressions of factors involved in inflammatory reactions including cyclo-oxygenase-2 (COX-2) (12). In human hepatoma cells, RIG-I is essential for hepatitis C virus RNA-induced signaling to the interferon-β (IFN-β) signaling (8). We have previously reported that RIG-I was induced by interleukin-1β (IL-1 β) in cultured human gingival fibroblasts. In that study the overexpression of RIG-I upregulated the expression of other inflammatory mediators such as COX-2, RANTES (regulation upon activation of normal T-cell expressed and secreted) and galectin-9 (31). Lipopolysaccharide (LPS), localized in the cell wall of gram-negative bacteria has pathogenicity to trigger inflammation.

LPS participates in the destruction of periodontal tissue containing gingival tissue, periodontal ligament and alveolar bone, and is known to advance periodontal disease (10, 11, 21–23, 32, 33). Double-stranded RNA (dsRNA) is either present in viral genomes or generated during viral replication, and is involved in the cellular recognition of RNA viruses (1). It elicits various cellular responses similar to those provoked by viral infection (13, 34). The mechanisms by which viral infection leads to inflammation in the oral region is poorly understood. IL-1β functions as an extracellularly active cytokine and binds to specific plasma membrane receptors. In humans, IL-1β is mainly produced by monocytes, macrophages, keratinocytes and Langerhans cells. IL-1β plays a central role in many pathological states, including periodontal disease (30). It is also an important mediator of cell proliferation and differentiation processes although the secretory pathway is poorly understood (35). IL-6, a central pro-inflammatory cytokine, has been implicated in the progression of periodontitis, although its role remains unknown (2, 9). IL-6 is produced by numerous cell types, including macrophages, monocytes, fibroblasts, endothelial cells and smooth muscle cells. *In vivo* studies have shown low levels of constitutive IL-6 production by fibroblasts of different origins, including dermal (7), liver (myo) (38), duodenal (29), renal (19), foreskin (18), human periodontal ligament (26, 42), oral fibrotic submucosal, buccal mucosal (6) and normal human gingival (5, 16, 17, 25) origins. The chemokine IL-8 is small secreted protein that causes chemotactic migration of leukocytes by binding to specific receptors; it plays a major role in inflammatory processes (20). In human gingival fibroblasts, *Porphyromonas gingivalis*, which is a major oral pathogen and associated with chronic and severe periodontal diseases, induces IL-8 production and it preferentially recruits neutrophils (27).

The oral cavity is a potential reservoir of pathogenic microorganisms and viruses, but the specific mechanism by which those pathogens are recognized in innate immunity in the oral region is far from elucidated. Recently, it was reported that RIG-I is a key molecule in antiviral responses (44). IL-1β induced RIG-I in cultured human gingival fibroblasts and it is possible that RIG-I might play an important role in host defense in gingival fibroblast (31).

Therefore, in the present study, we have examined whether LPS or dsRNA induces RIG-I expression in cultured human gingival fibroblasts. We also examined the role of RIG-I in the regulation of other inflammatory factors, such as IL-1β, IL-6 and IL-8, by introducing RIG-I cDNA into cultured cells and stimulating them with LPS or dsRNA.

Materials and methods

Reagents

Culture dishes and fetal calf serum were from Asahi Techno Glass (Tokyo, Japan). Synthetic dsRNA, polyinosinic–polycytidylic acid (poly IC) and LPS from *Escherichia coli* were obtained from Sigma (St Louis, MO). Antibiotic–antimycotic, oligo(dT)$_{12\text{-}18}$, Superscript^{TM} II, Trizol reagent and α-minimal essential medium were purchased from Invitrogen Corp (Carlsbad, CA). Cycloheximide (CHX) was purchased from Wako (Osaka, Japan). Recombinant ribonuclease inhibitor Rnasin^{TM} was from Promega Corporation (Madison, WI). The specific primers were synthesized by Hokkaido System Science (Sapporo, Japan). NE-PER^{TM} Nuclear and Cytoplasmic Extraction Reagents were obtained from Pierce (Rockford, IL). Horseradish peroxidase-labeled anti-rabbit immunoglobulin G antibody was supplied by Kirkegaard Perry (Gaithersburg, MD). A SuperSignal West Pico Chemiluminescent Substrate was purchased from Pierce. Effectene was from Qiagen (Hilden, Germany). LightCycler Fast Start DNA Master SYBR Green I and LightCycler-Primer Set-IL-1β, IL-6, IL-8 and porphobilinogen deaminase (PBGD) were from Roche Diagnostics (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Pierce.

Cell culture

Human gingival fibroblasts were isolated from healthy gingival tissues of five patients who underwent minor oral surgery at Hirosaki University Hospital. All patients gave written informed consent before providing the samples. The cells from each donor responded to agonists in a similar way and the variation was minimal. This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine, Hirosaki, Japan. Gingival tissues were washed in phosphate-buffered saline, pH 7.4 (PBS) and cut into small pieces, which were cultured in α-minimal essential medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamicin (80 μg/ml), for 2 weeks in an atmosphere of 95% air and 5% CO_2 at 37°C. When cells growing out from the explants had reached confluence, they were subcultured and experiments were performed on confluent cultures from third to sixth passages. Fibroblasts were stimulated with a series of concentrations of LPS (10 pg/ml to 100 ng/ml) and poly IC (1–50 μg/ml) for up to 48 h. In experiments using CHX, fibroblasts were incubated with 500 ng/ml CHX for 1 h before stimulation with 10 ng/ml LPS and 10 μg/ml poly IC for 6 h.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cell using trizol reagent. Single-stranded cDNA for a PCR template was synthesized from 1 μg total RNA using a primer oligo(dT)$_{12\text{-}18}$ and the Superscript^{TM} II reverse transcriptase under the conditions indicated by the manufacturer. Primers for RIG-I and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: RIG-I-F (5′-GCA TAT TGA CTG GAC GTG GCA-3′), RIG-I-R (5′-CAG TCA TGG CTG CAG TTC TGT C-3′), GAPDH-F (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′), GAPDH-R (5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′). The reaction conditions were one cycle at 94°C for 1 min, 30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min; then one cycle at 72°C for 1 min. The products were analysed on a 2% agarose gel stained with ethidium bromide. The expected sizes of the PCR products for RIG-I and GAPDH were 644 and 696 bp, respectively.

Western blotting

Cells were washed with cold PBS, and lysed using cell lysis buffer (20 mM PBS, pH 7.4, containing 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 0.01% protease inhibitor cocktail). The cell debris was pelleted by centrifugation and the supernatant was used as a sample. The nuclear and cytoplasmic fractions were separated from the cells using NE-PER^{TM} Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol. All samples were boiled in reducing sodium dodecyl sulfate sample buffer and electrophoresed on a polyacrylamide gel. Protein was then transferred to a polyvinyldifluoride membrane and the membrane was blocked by incubating in Superblock blocking buffer for 1 h. The membrane was incubated with an anti RIG-I antibody in Superblock for

1 h at room temperature as described previously (12). The membrane was washed with PBS containing 0.05% Tween-20 and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin G antibody for 40 minutes. Immunodetection was performed using a SuperSignal WestPico chemiluminescent substrate and the membrane was exposed to an X-ray film.

Transfection with RIG-I complementary DNA

To examine the biological effect of RIG-I expression in human gingival fibroblast, the cells were transfected with RIG-I cDNA. The coding region of the full-length RIG-I cDNA was cloned into mammalian expression vector pcDNA3 as described (12). RIG-I cDNA was transfected into human gingival fibroblasts using an Effectene reagent. After a 24-h incubation, fibroblasts were stimulated with 10 ng/ml LPS and 10 μg/ml poly IC. After a further 6 h of incubation, total RNA was extracted. The cells transfected with green fluorescent protein (GFP)-RIG-I cDNA (6) were examined under a confocal microscopy (IX70; OLYMPUS, Tokyo, Japan).

Quantitative RT-PCR

A glass capillary was filled with 6 μl water, 2 μl LightCycler-Primer Set-IL-1β, IL-6, IL-8 and PBGD, 2 μl of LightCycler Fast Start DNA Master SYBR Green I, and 10 μl reverse-transcribed cDNA (10 ng). Capillaries were cloned, centrifuged and placed into the rotor of a LightCycler (Roche Diagnostics). Denaturation program, amplification and quantification were defined by the manufacturer's protocol. Amplification and detection of target DNA were performed as follows: an initial 10-min incubation at 95°C for FastStart *Taq* DNA polymerase activation, followed by 30 cycles of denaturation at 95°C for 15 s, primer and probe annealing at 60°C for 10 s and extension at 72°C for 10 s, with monitoring of fluorescence during the annealing phase. This was followed by a melting program of temperature increase from 65 to 95°C at 0.1°C/s with continuous monitoring of the fluorescence. Data were analysed on the software LC RUN Version 5.23.

ELISA for IL-1β, IL-6 and IL-8

The conditioned medium or cell lysate of fibroblasts was stored at −20°C until use. The level of IL-1β, IL-6 and IL-8 proteins were determined using an ELISA kit.

Statistics

Values were expressed as mean ± SD, and statistical significance was analysed by Student's *t*-test or Welch's *t*-test. All probability (*P*) values were based on two-tailed tests, and $P < 0.05$ was considered to be significant.

Results

RIG-I is induced in gingival fibroblasts stimulated with LPS

LPS induced the expression of RIG-I mRNA and protein in gingival fibroblast in a concentration-dependent manner (Fig. 1). The expression of RIG-I mRNA reached maximal level after 4–6 h of stimulation and decreased thereafter (Fig. 2A); the RIG-I protein production

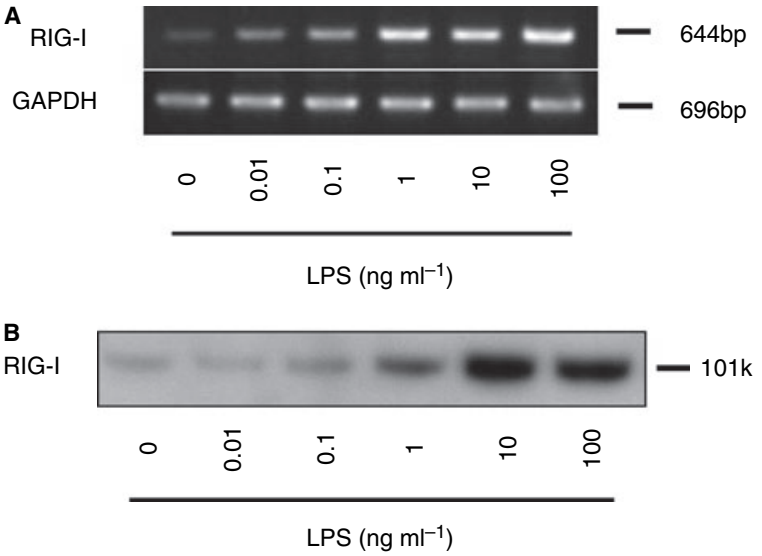

Fig. 1. Concentration-dependent expression of RIG-I in gingival fibroblasts stimulated with LPS. (A) Gingival fibroblasts were stimulated with 0.01–100 ng/ml LPS for 6 h and cells were subjected to total RNA extraction. Single-stranded cDNA was synthesized from 1 μg total RNA, and the specific cDNAs for RIG-I and GAPDH were amplified by PCR. (B) The expression of RIG-I protein in gingival fibroblasts stimulated with 0.01–100 ng/ml LPS for 24 h. After the stimulation, the cells were washed twice with cold PBS and lysed. Lysates were subjected to Western blot analysis for RIG-I. The data represent one of three experiments with similar results.

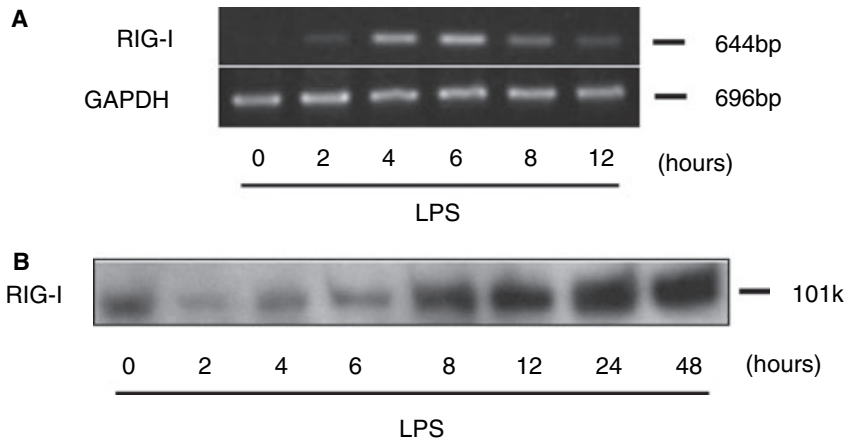

Fig. 2. Time–course of the expression of RIG-I in gingival fibroblasts stimulated with LPS. (A) Gingival fibroblasts were stimulated with 10 ng/ml LPS for up to 12 h, and the cells were subjected to total RNA extraction. RT-PCR analysis was performed. (B) The expression of RIG-I protein in gingival fibroblasts stimulated with 10 ng/ml LPS for up to 48 h. Western blot analysis for RIG-I was performed as described in Fig. 1. The data shown are representative of three experiments.

lagged several hours behind mRNA expression (Fig. 2B).

RIG-I is induced in gingival fibroblasts stimulated with poly IC

poly IC also induced the expression of RIG-I mRNA and the RIG-I protein production in gingival fibroblasts in a concentration-dependent manner (Fig. 3). The poly IC-induced expression of RIG-I mRNA reached a maximum after 4–6 h of stimulation and decreased thereafter; the RIG-I protein production lagged several hours behind mRNA expression (Fig. 4).

Effect of CHX on RIG-I mRNA expression

To clarify the mechanism of LPS- or poly IC-dependent activation of RIG-I, we investigated the effect of CHX. CHX had no effect on the expression of RIG-I mRNA stimulated with LPS or poly IC (Fig. 5).

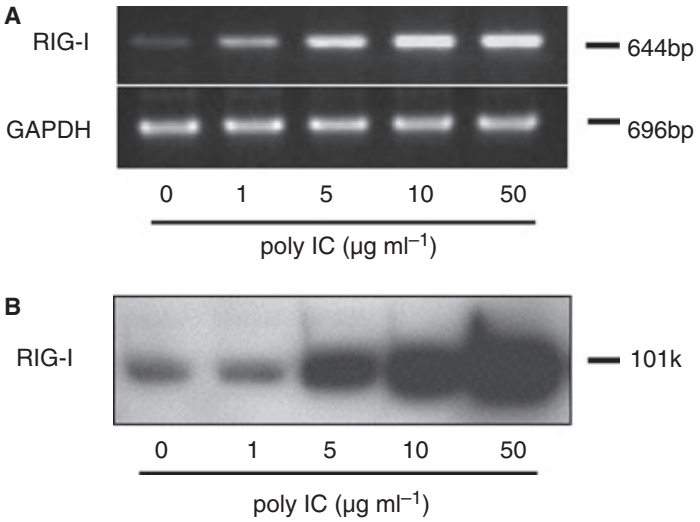

Fig. 3. Concentration-dependent expression of RIG-I in gingival fibroblasts stimulated with poly IC. (A) Gingival fibroblasts were stimulated with 1–50 μg/ml poly IC for 6 h, and the cells were subjected to total RNA extraction. RT-PCR analysis was performed. (B) The expression of RIG-I protein in gingival fibroblasts stimulated with 1–50 μg/ml poly IC for 24 h. Western blot analysis for RIG-I was performed as described in Fig. 1. The data are representative of three experiments.

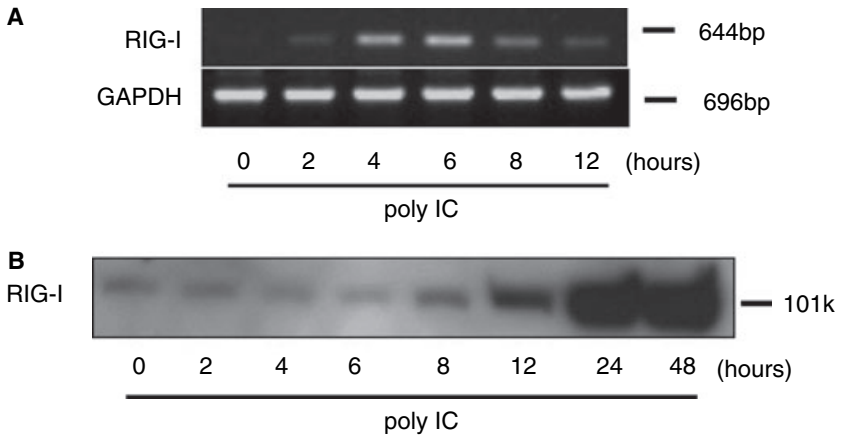

Fig. 4. Time–course of the expression of RIG-I in gingival fibroblasts stimulated with LPS. (A) Gingival fibroblasts were stimulated with 10 μg/ml poly IC for up to 12 h, and the cells were subjected to total RNA extraction. RT-PCR was performed. (B) The expression of RIG-I protein in gingival fibroblasts stimulated with 10 μg/ml poly IC for up to 48 h. Western blot analysis for RIG-I was performed as described in Fig. 1. The data are representative of three experiments.

RIG-I is expressed in the cytoplasm of gingival fibroblasts

We also examined the localization of RIG-I in gingival fibroblasts. RIG-I protein was detected in the cytoplasmic fraction by Western blot analysis (Fig. 6A). When the GFP-RIG-I construct was transfected into gingival fibroblasts, the fluorescence was observed in the cytoplasm (Fig. 6B).

Overexpression of RIG-I in gingival fibroblasts is related to the expression of IL-1β, IL-6 and IL-8 induced by LPS or poly IC

We transiently transfected RIG-I cDNA into gingival fibroblasts and confirmed that RIG-I was overexpressed in the cells (Fig. 7A,B).

We examined whether overexpression of RIG-I was related to the expression of IL-1β, IL-6 and IL-8 in gingival fibroblasts stimulated with LPS and poly IC. The expression of IL-1β, IL-6 and IL-8 mRNA was quantified by using LightCycler.

The expression of mRNA for IL-1β, IL-6 and IL-8 was induced by LPS or poly IC in control cells (Fig. 7C–E). Overexpression of RIG-I resulted in the expression of mRNAs for these cytokines and chemokine, and significantly enhanced the induction of their mRNA in response to LPS or poly IC (Fig. 7C–E). Expression of IL-1β mRNA was 135-fold increased by the overexpression of RIG-I (Fig. 7C).

We also examined the production of IL-1β, IL-6 and IL-8 proteins by these cells using ELISA. Overexpression of RIG-I significantly enhanced the expression of IL-1β protein in cell lysates stimulated with LPS or poly IC (Fig. 8B). In the condition medium the concentration of IL-1β was not altered, either by stimulation or RIG-I overexpression (Fig. 8A). Overexpression of RIG-I significantly enhanced the concentration of IL-6 protein in the conditioned medium or cell lysates; this agreed with the results of the real-time PCR (Fig. 8C,D). The concentration of IL-8 in the cell lysates was significantly increased by RIG-I overexpression but the effect of RIG-I overexpression was not clear in the conditioned medium (Fig. 8-E,F).

Discussion

Gingival fibroblasts are the predominant cells in periodontal tissue and are responsible for the synthesis and degradation of connective tissue. These cells can be stimulated to produce cytokines and

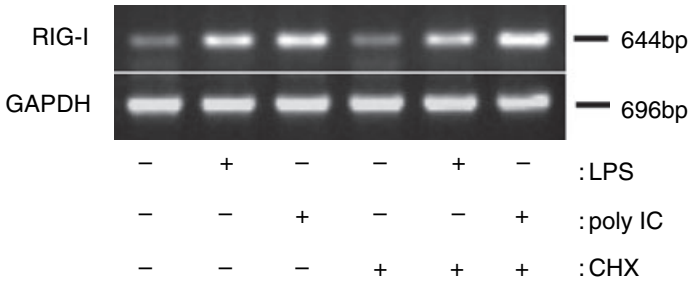

Fig. 5. Effect of CHX on the expression of RIG-I in gingival fibroblasts stimulated with LPS and poly IC. Cells were treated with 500 ng/ml CHX for 1 h, and then stimulated with 10 ng/ml LPS and 10 μg/ml poly IC for 6 h. The analysis for RIG-I mRNA was performed by RT-PCR. The data shown are representative of three experiments.

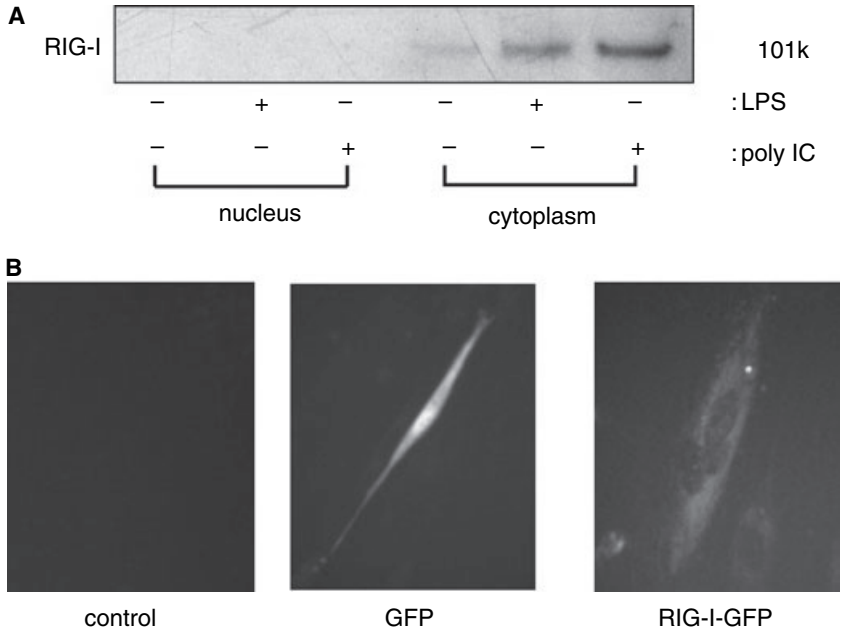

Fig. 6. RIG-I is expressed in cytoplasm in gingival fibroblasts. (A) Gingival fibroblasts were stimulated with 10 ng/ml LPS or 10 μg/ml poly IC for 24 h. The nuclear and cytoplasmic fractions were separated from cells, and Western blot analysis for RIG-I was performed as described in Fig. 1. (B) Transfection of gingival fibroblasts with GFP-RIG-I. Cells were transfected with GFP-RIG-I or control GFP cDNA. After the incubation for 24 h, the fluorescence was observed under confocal microscopy. The data shown are representative of three experiments.

factors which mediate inflammation (28). In this study, we examined the abilities of LPS or dsRNA to regulate the induction of RIG-I mRNA and protein synthesis in gingival fibroblasts. Innate defense against infections is activated by pattern recognition receptors (PRRs) that recognize molecular structures conserved on large groups of pathogens. RIG-I is a cytoplasmic RNA helicase that contains tandem motifs near its N terminus with limited homology to the caspase-recruiting domain (CARD) and a downstream DExD/H-box helicase domain. It was recently reported that RIG-I could be one of the intracellular PRRs and could participate in the recognition of dsRNA in cytoplasm. RIG-I is localized in cytoplasm in MCF-7 breast cancer cells (6). In this study, we found that RIG-I was mainly expressed in cytoplasm also in gingival fibroblasts. The helicase domain of RIG-I putatively binds synthetic and viral dsRNA, resulting in activation of signal transduction to nuclear factor-κB and IFN regulatory factor-3 (IRF-3) through signaling involving the N-terminal CARD-like homology domain (44). Yoneyama et al. reported that when viruses, particularly RNA viruses, infect cells, the genome starts to replicate and express encoded genes. Genome replication results in the generation of dsRNA, which can be recognized by the sensor molecules RIG-I. (43). Jesper et al. reported that myeloid cells utilize Toll-like receptor 7 (TLR7) and TLR8 in sensing single-strand RNA, and triggering of the antiviral response is dependent on RIG-I, TLR3 or dsRNA-activated protein kinase R. On the other hand, non-immune cells relied entirely on recognition of dsRNA through the RIG-I system. The mechanisms used by the host to recognize infecting viruses largely depends on cell-type specificity (14). However, the mechanisms, by which gingival fibroblasts recognize the infecting virus, are not reported. In the present study, we examined the effect of dsRNA on gingival fibroblasts, and report here that dsRNA induced the expression of RIG-I. This suggests that RIG-I may play an important role in the antiviral response in this cell type.

Recent results indicated that LPS from periodontal bacteria induced inflammatory reactions via CD14 and/or TLR in gingival tissue (40). Gingival fibroblasts obtained from patients with healthy or inflammatory gingiva expressed inflammatory molecules such as IL-1α, IL-1β, IL-6, IL-8, tumor necrosis factor-α, CD14, TLR2 and TLR4; and these proteins may contribute, directly or indirectly, to periodontal inflammation (41).

In this study, we stimulated gingival fibroblasts with LPS from *E. coli*. It has been shown that LPS from both *E. coli* and *Porphyromonas gingivalis* have similar characteristics; both are TLR4 ligands and upregulate TLR4 expression (24). Therefore, LPS from *E. coli* is considered to be an appropriate agonist for this study.

It is reported that expression of RIG-I is induced by LPS in vascular endothelial cells (12), and RIG-I is thought to play a role in anti-bacterial host defense mechanisms. However, in other cell types there are no reports on the relation between LPS and RIG-I. In this study, we demonstrated that LPS induced the expression of RIG-I in gingival fibroblasts. This suggests that RIG-I is involved in inflammatory responses in gingival fibroblasts not only against virus but also against bacteria.

LPS or poly IC induced the expression of RIG-I in cultured gingival fibroblasts. This result suggests that RIG-I may take part in the early phase of innate immune response in gingival fibroblasts, because gingival fibroblasts are exposed to many pathogenic microorganisms and viruses.

To clarify the signaling pathway of the LPS or poly IC-dependent activation of RIG-I, we investigated the effect of CHX, an inhibitor of protein synthesis, on the

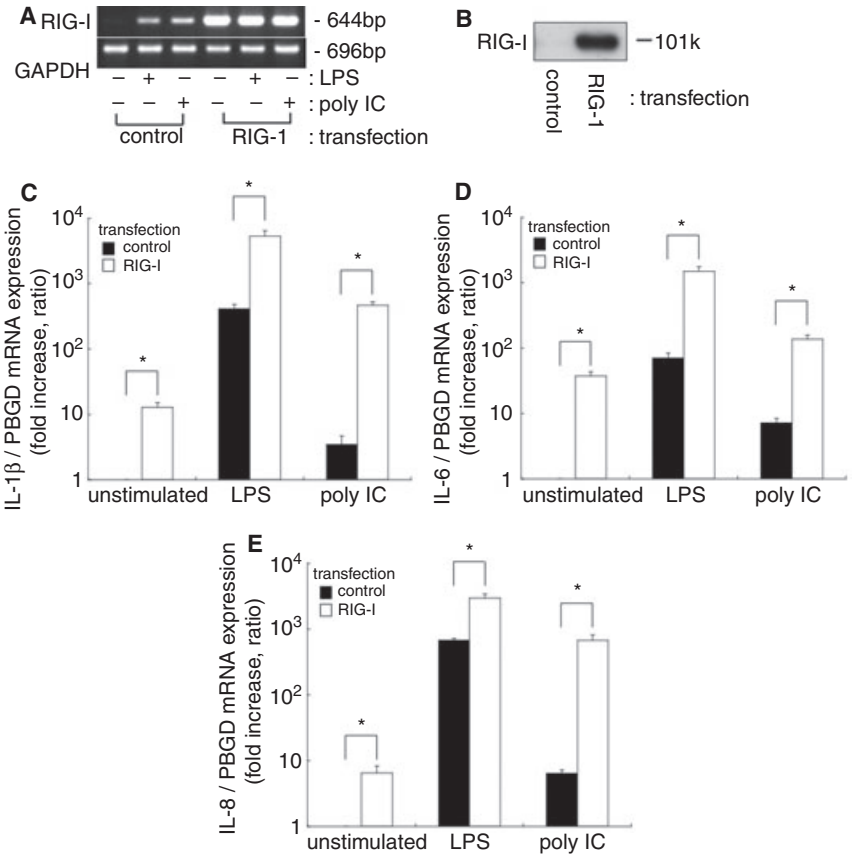

Fig. 7. Overexpression of RIG-I in gingival fibroblasts enhances the expression of IL-1β, IL-6 and IL-8 induced by LPS or poly IC. (A) RIG-I cDNA was transfected into gingival fibroblasts. After a 24-h incubation, the cells were stimulated with 10 ng/ml LPS or 10 μg/ml poly IC for 6 h. Total RNA was extracted and RT-PCR analysis for RIG-I was performed. (B) RIG-I cDNA was transfected in gingival fibroblasts. After a 24-h incubation, cell lysate was prepared and subjected to Western blot analysis for RIG-I. (C–E) RIG-I cDNA was transfected into gingival fibroblasts. After 24 h of incubation, the cells were stimulated with 10 ng/ml LPS or 10 μg/ml poly IC. RNA was extracted after a further 6 h of incubation, and the relative number of mRNA for IL-1β, IL-6 and IL-8 was quantified using a LightCycler system. The analysis for IL-1β, IL-6 and IL-8 mRNA represents the ratio of each mRNA against mRNA from unstimulated control cells. Means (±SD) of three different experiments are shown. Closed bar represents untransfected, and open bar represents transfected RIG-I cDNA. $*P < 0.05$, statistically significant difference between open bar compared with close bar.

expression of RIG-I induced by LPS or poly IC. Pretreatment of fibroblasts with CHX had no effect on the levels of LPS- or poly IC-induced RIG-I mRNA expression in fibroblasts, suggesting that newly synthesized protein is not required for RIG-I expression induced by LPS or poly IC and that either LPS or poly IC may directly induce the expression of RIG-I.

Cytokines and chemokines are important regulators of inflammatory reactions. Therefore, we examined the possibility that RIG-I may influence the expression of the pro-inflammatory cytokines and chemokine, IL-1β, IL-6 and IL-8. Introduction of RIG-I cDNA resulted in enhanced expression of IL-1β, IL-6 and IL-8. To examine the role of RIG-I in the reactions induced by LPS or poly IC, we introduced RIG-I into gingival fibroblasts and stimulated them with LPS or poly IC. Overexpression of RIG-I dramatically increased the expression of IL-1β, IL-6 and IL-8 induced by LPS or poly IC. In human nasal fibroblasts, poly IC induced IL-8 protein, but the production of IL-1β could not be detected (37). Otherwise, overexpression of RIG-I in gingival fibroblasts significantly enhanced the expression of IL-1β mRNA, protein in cell lysates in response to LPS or poly IC. However, IL-1β protein in the supernatant was not altered by RIG-I overexpression. This suggests that RIG-I is involved in IL-1β production, but perhaps not in its secretion mechanism. Another possibility is that IL-1β secreted by gingival fibroblasts may bind its receptor to the cell surface to work in an autocrine manner, and the concentration of IL-1β in the condition medium may not be altered. With respect to LPS-induced IL-1β, LPS is somewhat special in that it has been reported to act both as a priming stimulus and as a processing/secretion stimulus, but it does not appear to be an efficient trigger of both (4). This may partly support our results, but the mechanisms by which RIG-I enhances the production of IL-8 should be clarified. It is reported that gingival fibroblasts can act as accessory immune cells through the production of cytokines such as IL-6, and thereby contribute to periodontal destruction (39). Therefore, it is important to understand the mechanisms by which IL-6 production is regulated in this cell type. In the present study, we demonstrated that RIG-I overexpression dramatically enhances the induction of IL-6 by poly IC, but slightly enhances the induction of IL-6 by LPS. In mouse embryonic fibroblasts, which are deficient in RIG-I, RNA viruses, including Newcastle disease virus and Sendai virus, failed to upregulate IL-6, IFN-β and IFN inducible genes. These results indicate that RIG-I is essential for the induction of the IFN response upon viral infection in non-professional immune cells such as fibroblasts. In contrast, LPS induces the IL-6 in mouse embryonic fibroblasts that are deficient in RIG-I (15). Taken together, RIG-I may be an essential molecule in poly IC-induced IL-6 production, and can be an enhancer in LPS-induced IL-6 production in gingival fibroblasts. IL-8 is a chemokine, and has potent activity to induce the chemotaxis of neutrophils. Therefore, IL-8 produced by gingival fibroblasts in response to pro-inflammatory mediators may contribute to the maintenance of gingival inflammation (3). In the present study, overexpression of RIG-I in gingival fibroblasts results in the enhanced production of IL-8. This result suggests that RIG-I may be involved in physiological and pathological inflammation, at least in part, through the regulation of IL-8 production. Our results suggest that RIG-I may have important functional roles in the regulation of the local immune response, which produces a variety of cytokines and chemokines, including IL-1β, IL-6 and IL-8, in response to pathogenic microorganisms and viruses in the gingival fibroblasts.

Despite the fact that gingival fibroblasts are easily exposed to pathogens in the oral cavity, they maintain a constancy. RIG-I may contribute to the constancy of gingival fibroblasts with regard to the recognition of pathogens to cause inflammation.

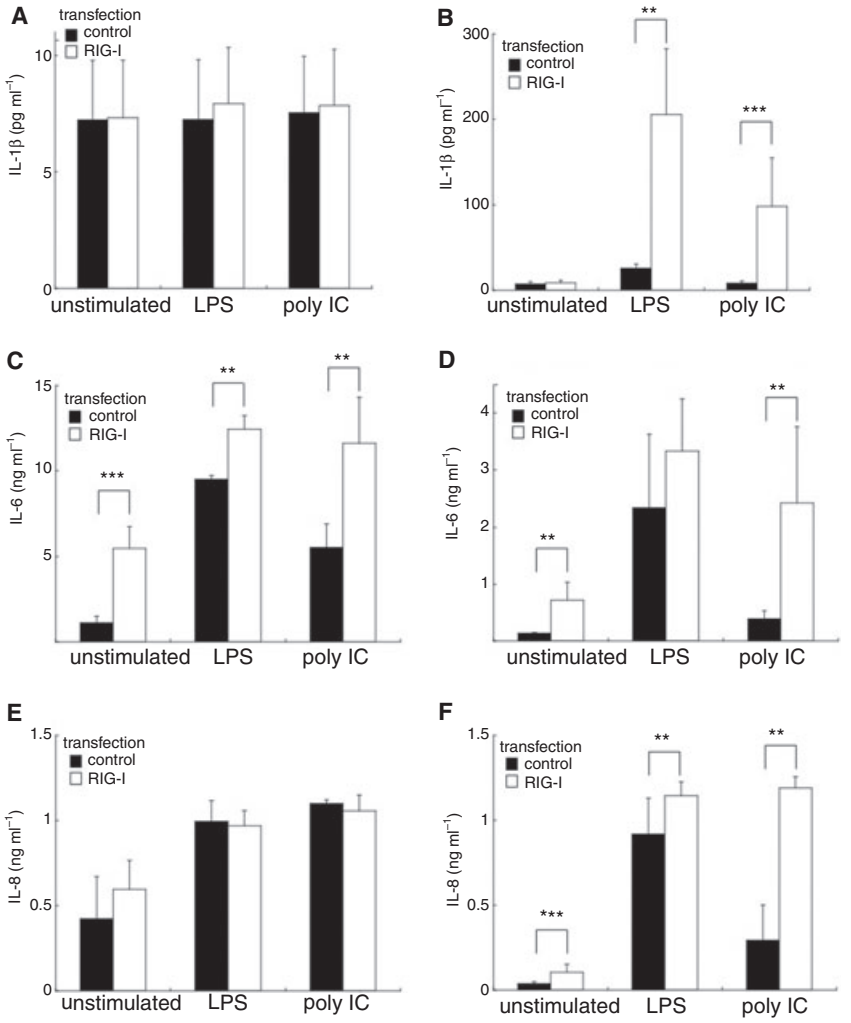

Fig. 8. Overexpression of RIG-I in gingival fibroblasts enhances the production of IL-1β, IL-6 and IL-8 protein induced by LPS or poly IC. RIG-I cDNA was transfected into gingival fibroblasts. After 24 h of incubation, the cells were stimulated with 10 ng/ml LPS or 10 μg/ml poly IC. The conditioned medium and cell lysate were collected after 12 h incubation, and IL-1β, IL-6 and IL-8 proteins were determined by ELISA. (A) The concentration of IL-1β protein in the medium. (B) The concentration of IL-1β protein in the cell lysate. (C) The concentration of IL-6 protein in the medium. (D) The concentration of IL-6 protein in the cell lysate. (E) The concentration of IL-8 protein in the medium. (F) The concentration of IL-8 protein in the cell lysate. $^{**}P < 0.001$, open bar significantly different from closed bar. $^{***}P < 0.01$, open bar significantly different from closed bar. Means (± SD) of six experiments are shown.

The details of the mechanisms by which RIG-I recognizes pathogens and enhances IL-1β, IL-6 or IL-8, remain to be determined.

In conclusion, we found that LPS and poly IC induced RIG-I mRNA and protein, which were related to the expression of IL-1β, IL-6 and IL-8, in gingival fibroblasts. RIG-I may be an important molecule in innate immunity of the oral region.

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