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Short communication

Retinoic acid-inducible gene-I is induced by interleukin-1β in cultured human gingival fibroblasts

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Retinoic acid-inducible gene-I (RIG-I) is a member of the DExH box family protein, and details of its biological function are not known. We have studied the mechanism of the interleukin-1β (IL-1β)-induced RIG-I expression in human gingival fibroblasts in culture. We also addressed the possibility of enhanced expression of COX-2, RANTES and galectin-9 in fibroblasts overexpressed RIG-I. We stimulated cultured human gingival fibroblasts with IL-1β and examined the expression of RIG-I mRNA and protein by reverse transcriptase-mediated polymerase chain reaction and Western blot analysis. The effect of cycloheximide, a protein synthesis inhibitor, on the IL-1β-induced expression of RIG-I was examined. The expression of COX-2, RANTES, galectin-9 and monocyte chemoattractant protein-1 in gingival fibroblasts transfected with RIG-I cDNA was also examined. IL-1β stimulated the expressions of mRNA and protein for RIG-I, in cultured fibroblasts, in a time- and concentration-dependent manner. Cycloheximide did not suppress the IL-1β-induced RIG-I expression. Introduction of RIG-I cDNA into fibroblasts resulted in enhanced expression of COX-2 mRNA, and slightly enhanced the expression of mRNA for RANTES and galectin-9. In contrast, RIG-I overexpression did not alter the level of mRNA for monocyte chemoattractant protein-1. We conclude that IL-1β stimulates RIG-I expression in human gingival fibroblasts.

Key words: COX-2; galectin-9; human gingival fibroblasts; interleukin-1β; RIG-I; RANTES

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Retinoic acid-inducible gene-I (RIG-I) is a member of DExH box family protein, and was identified as a gene induced by retinoic acid in a promyelocytic leukemia cell line (21). 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, RIG-I was shown to regulate the expressions of factors involved in inflammatory reactions including cyclooxygenase-2 (COX-2) (13). In the present study, we have examined whether interleukin-1 (IL-1) induces RIG-I expression in human gingival fibroblasts in culture. We also examined the role of RIG-I in the regulation of other inflammatory factors, such as COX-2, regulated upon activation of normal T-cell expressed and secreted (RANTES) and galectin-9, by introducing RIG-I cDNA into cultured cells.

RANTES is a small protein and one of the factors, along with other chemokines such as monocyte chemoattractant protein-3 (MCP-3), MCP-4 and eotaxin, which attract human eosinophils. RANTES and MCP-1 are both β chemokines secreted by monocytes/macrophages and a variety of other cell types (20). The cells, activated by RANTES, in turn elicit a series of responses leading to the generation of superoxide anion, cationic protein and lysosomal enzyme (18). RANTES is also active in other types of cells (2, 3). It mediates the trafficking and homing of lymphoid cells, and activation of T cells by RANTES leads to diverse effects including proliferation and release of pro-inflammatory cytokines (17). Increased RANTES

expression has been associated with a wide range of inflammatory disorders (4–6, 8, 19); however, little is known about the role of RANTES in oral diseases.

Galectins are animal lectins that have a conserved specific sequence motif of β-galactoside-binding domains (10). Galectin-9 was isolated as an eosinophil chemoattractant generated by activated T lymphocytes (11, 12, 16), and may regulate eosinophil traffic in immune responses and allergic reactions. In addition to lymphocytes, it is produced by various cells such as monocyte/macrophages and mouse embryonic cells (12, 22). Asakura et al. (1) showed that interferon (IFN)-γ-induced galectin-9 expression in fibroblasts mediates eosinophil adhesion to the cells. However, very little is known about galectin-9 in gingival fibroblasts.

Culture dishes and fetal calf serum (FCS) were from Asahi Techno Glass (Tokyo, Japan). Recombinant human IL-1β (r(h)IL-1β), antibiotic-antimycotic, oligo(dT)$_{12-18}$, Superscript™ II, Trizol reagent and α-MEM were from Invitrogen Corp (Carlsbad, CA). Cycloheximide was purchased from Wako (Osaka, Japan). Recombinant ribonuclease inhibitor RNasin™ was from Promega Corporation (Madison, WI). The specific primers were synthesized by Hokkaido System Science (Sapporo, Japan). Horseradish peroxidase (HRP)-labeled anti-rabbit IgG antibody was from Kirkegaard Perry (Gaithersburg, MD). A SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL). Effectene was from Qiagen (Hilden, Germany).

Human fibroblasts were isolated from healthy gingival tissues of patients who underwent minor oral surgery at the Hirosaki University Hospital. All the patients gave fully informed consent before providing the samples. Gingival tissues were washed in phosphate-buffered saline (PBS), pH 7.4, and cut into small pieces, which were cultured in α-MEM containing 10% FCS, 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 at the third to the sixth passage. Fibroblasts were stimulated with a series of concentrations (10 pg/ml to 10 ng/ml) of r(h)IL-1β for up to 24 h. In experiments using cycloheximide , fibroblasts were incubated with 500 ng/ml cycloheximide for 1 h prior to stimulation with 5 ng/ml IL-1β for 8 h.

Total RNA was isolated from the cells using a Trizol reagent. First-strand cDNA was synthesized from 1 μg of total RNA using primer oligo(dT)$_{12-18}$ and Superscript™ II reverse transcriptase following the manufacturer's instructions. Primers for RIG-I, COX-2, RANTES, galectin-9, MCP-1 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′), COX-2-F (5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′), COX-2-R (5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′), RANTES-F (5′-CTA CTC GGG AGG CTA AGG CAG GAA-3′), RANTES-R (5′-GAG GGG TTG AGA CGG CGG AAG C-3′), galectin-9-F (5′-GAG ATG GCC TTC AGC AGT TCC-3′), galectin-9-R (5′-CGC CTA TGT CTG CAC ATG GGT-3′), MCP-1-F (5′- AAA CTG AAG CTC GCA CTC TCG C-3′), MCP-1-R (5′-ATT CTT GGG TTG TGG AGT GAG T−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 1× (94°C, 1 min); 30× (94°C, 1 min; 58°C, 1 min; 72°C, 1 min); and 1× (72°C, 10 min). The products were analyzed on a 2% agarose gel which was stained with ethidium bromide. The expected size for the PCR products for RIG-I, COX-2, RANTES, MCP-1 and GAPDH were 644 bp, 305 bp, 318 bp, 353 bp and 696 bp, respectively. There are three isoforms of galectin-9: short, medium, and long types (12). The expected sizes for the PCR products for these isoforms are 942 bp, 978 bp and 1074 bp, respectively. Because all of these primer pairs were designed from different exons, the products with the expected size were amplified from single-strand cDNA but not from the contaminating genomic DNA.

For Western blot analysis, cells were washed twice with cold PBS, and lysed using cell lysis buffer (20 mM PBS, pH 7.4, containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 0.01% protease inhibitor cocktail). The cell debris was pelleted by centrifugation and the supernatant was used as a sample. All samples were boiled in reducing SDS sample buffer and electrophoresed on a polyacrylamide gel. Proteins were then transferred to a PVDF membrane and the membrane 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 (13). The membrane was washed with PBS containing 0.05% Tween 20 and incubated with HRP-labeled anti-rabbit IgG antibody for 40 min. Immunodetection was performed using a SuperSignal WestPico chemilumi-

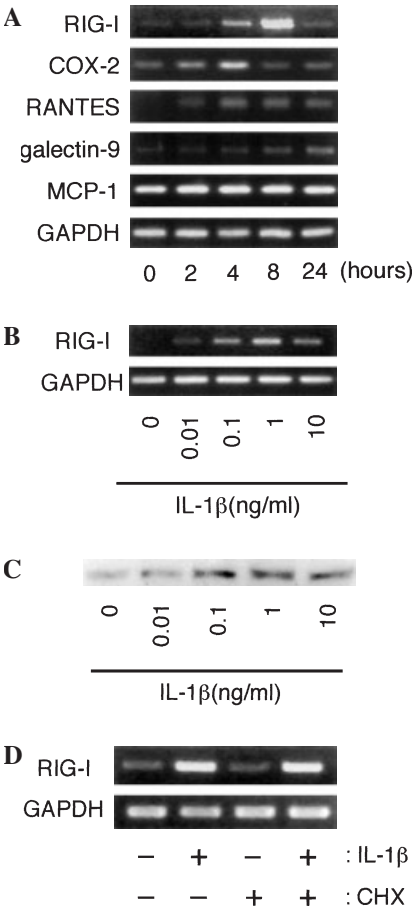

Fig. 1. Expression of RIG-I in gingival fibroblasts stimulated with IL-1β. A) RIG-I is expressed in gingival fibroblasts stimulated with IL-1β in a time-dependent fashion. Fibroblasts were treated with 5 ng/ml IL-1β for up to 24 h, and the cells were subjected to total RNA extraction. Single-strand cDNA was synthesized from 1 μg total RNA, and the specific cDNAs for RIG-I, COX-2, RANTES, galectin-9, MCP-1 and GAPDH were amplified by PCR. B) Concentration-dependent induction of RIG-I in gingival fibroblasts stimulated with IL-1β. The cells were incubated with 0.01–10 ng/ml IL-1β for 8 h, and total RNA extracted. RT-PCR analysis was performed as described in (A). C) Expression of RIG-I in gingival fibroblasts stimulated with IL-1β. Concentration-dependent expression of RIG-I protein in fibroblasts stimulated with IL-1β for 8 h. After the stimulation, the cells were washed twice with cold PBS and lysed. Western blot analysis for RIG-I protein was performed. D) Effect of cycloheximide on the IL-1β-induced RIG-I expression in gingival fibroblasts. Cells were treated with 500 ng/ml cycloheximide for 1 h, and then stimulated with 5 ng/ml IL-1β for 8 h. The analysis for RIG-I mRNA was performed by RT-PCR as described in (A).

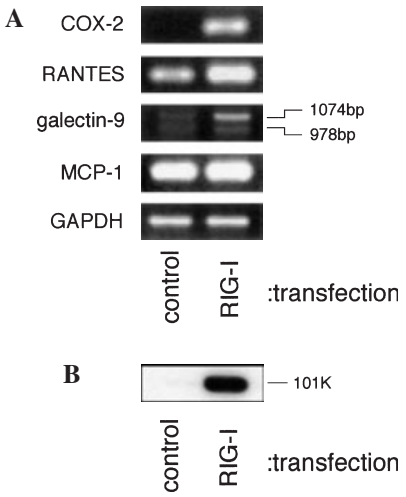

Fig. 2. A) RIG-I enhances expression of COX-2, RANTES and galectin-9. RIG-I cDNA was transfected into fibroblasts, and after 24 h incubation, total RNA was extracted. RT-PCR analysis for COX-2, RANTES, and galectin-9 was performed as described in Figure 1. B) Cell lysate was prepared and subjected to Western blot analysis for RIG-I.

nescent substrate and the membrane was exposed to an X-ray film.

The coding region of the full-length RIG-I cDNA was cloned into mammalian expression vector pcDNA3 as described (13). RIG-I cDNA was transfected into human gingival fibroblasts using an Effectene reagent. After 24 h incubation, total RNA was extracted and RT-PCR analysis for COX-2, RANTES, galectin-9, MCP-1 and GAPDH was performed as described above.

We first examined the time-dependent expression of RIG-I mRNA in fibroblasts stimulated with IL-1β as shown in Fig. 1A. RT-PCR analysis revealed the expression of RIG-I mRNA in fibroblasts stimulated with IL-1β. RIG-I mRNA levels reached the maximum 8 h after the stimulation with 5 ng/ml IL-1β. COX-2 mRNA expression reached its maximal level 4 h after stimulation, and decreased thereafter. IL-1β-induced expression of mRNA for RANTES and galectin-9 was slightly enhanced in a time-dependent manner. MCP-1 mRNA was basally expressed in fibroblasts. The expression of GAPDH, used as a control housekeeping gene, was also confirmed. IL-1β enhanced the expression of RIG-I mRNA in a concentration-dependent manner, as shown in Fig. 1B. Western blot analysis showed that the concentration-dependent RIG-I protein expression agreed with the mRNA expression (Fig. 1C).

Next, to clarify the mechanism of the IL-1β-dependent activation of RIG-I, we investigated the effect of cycloheximide , an inhibitor of protein synthesis, on the expression of RIG-I induced by IL-1β. Pretreatment of fibroblasts with 500 ng/ml cycloheximide had no effect on the levels of IL-1β induced RIG-I mRNA expression in fibroblasts (Fig. 1D), and IL-1β may act directly on the expression of RIG-I.

We examined whether overexpression of RIG-I induced COX-2, RANTES, galectin-9, MCP-1 mRNA expression (Fig. 2A). Introduction of RIG-I cDNA resulted in enhanced expression of COX-2 mRNA, and slightly enhanced the expression of the mRNAs for RANTES and galectin-9 in this model. In contrast, RIG-I did not alter expression of the mRNA for MCP-1 and GAPDH, a housekeeping gene that is basally expressed in fibroblasts. Overexpression of RIG-I protein in transfected cells was confirmed by Western blot analysis (Fig. 2B).

The RIG-I cDNA was previously cloned as a gene induced by retinoic acid during the differentiation of an acute promyelocytic leukemia cell line (21). In the present study, we found that IL-1β induced the expression of RIG-I mRNA and protein in fibroblasts. We previously observed lipopolysaccharide-induced expression of RIG-I in cultured human endothelial cells (13). Also a porcine homolog of RIG-I was identified as a putative porcine RNA helicase induced by virus (RHIV-1), which was induced in alveolar macrophages by infection with porcine reproductive and respiratory syndrome virus (24). These findings suggest that RIG-I has important functional roles in activated and inflamed fibroblasts. Next we examined the possibility that RIG-I may influence expression of other genes that are induced in a regulated fashion in fibroblasts, by factors such as IL-1β. Overexpression of RIG-I in fibroblasts resulted in selectively enhanced expression of mRNA for COX-2, RANTES, and galectin-9. This result suggests that RIG-I may play some pathophysiological role in immune and inflammatory reactions in fibroblasts, at least in part, by regulating the expression of these genes.

We have previously reported that RIG-I is induced in endothelial cells stimulated with lipopolysaccharide and regulates expression of COX-2 (13). The results of the present study in gingival fibroblasts support the previous observation made in endothelial cells.

IL-1 has an important role in a variety of processes such as inflammatory reactions (7), cell proliferation and bone destruction (9). IL-1 is generated by many types of cells including lymphocytes, monocytes/macrophages and fibroblasts (14). It stimulates the target cells to synthesize active neutral proteases such as plasminogen activator, matrix metalloproteinases (MMPs) and elastase, which contribute to the increased rate of collagen degradation in inflammatory diseases (15). RIG-I may contribute to these pathophysiological functions of IL-1.

Recently, it was reported that RIG-I is a key molecule in antiviral responses (23). Therefore, RIG-I may play an important role in the host defense in gingival fibroblasts.

We conclude that IL-1β stimulates RIG-I expression in human gingival fibroblasts, and the overexpression of RIG-I up-regulated the expression of other inflammatory mediators such as COX-2, RANTES and galectin-9. This may be an important observation with respect to the progress of the mechanism of the immunity to infection and the host defense system in oral area.

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