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TLR3 agonist enhances CC chemokine ligand 20 production in IL-1β-stimulated human gingival fibroblasts

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ABSTRACT

Viruses are related to the etiology of periodontitis. However, the role of viruses on Th17 cells infiltration in periodontitis lesions is unknown. Therefore, we examined the effects of TLR3 ligand on CCL20, which is related to Th17 cells migration, production in human gingival fibroblasts (HGFs). Polyinosinic-polycytidylic acid (Poly I:C), which is a TLR3 agonist, stimulation could moderately induce CCL20 production in HGFs. Poly I:C synergistically enhanced CCL20 expression from IL-1β-stimulated HGFs. Inhibitors of p38 MAPK, extracellular signal-regulated kinase (ERK), c-Jun N terminal kinase (JNK), and NF-κB significantly inhibited CCL20 production in Poly I:C/IL-1β-stimulated HGFs. Western blot analysis disclosed phosphorylation of p38 MAPK, JNK, and IκB-α were enhanced in Poly I:C/IL-1β-treated HGFs. These data suggested that virus infection is related to Th17 cells migration in periodontitis lesion to induce CCL20 production in HGFs via TLR3. Therefore, our results indicated that virus might be important pathogen in periodontal disease.

1. Introduction

Periodontitis is an inflammatory disease that effects tooth supporting structures, especially leading to alveolar bone destruction and tooth loss. Although the aetiology is diverse, it has been reported that plaque microbes are colligated with the initiation and progression of periodontal disease [1]. It is proposed that viral infection is related to the pathogenesis of periodontal disease [2–5]. For example, Thomasini et al. demonstrated that cytomegalovirus and human herpes virus 7 can be present at periodontitis-effected sites, and cytomegalovirus can be related to an inflammatory infiltrate with predominance of CD3-positiveT cells, whereas human herpes virus 7 can be associated with an infiltrate with predominance of CD4-positive T cells in periodontally diseased sites [2].

TLRs recognize pathogen-associated molecular patterns (PAMPS), which include common infectious agent's structures and nucleic acids that trigger activation of signaling pathways and production of immune mediators, such as cytokines [6]. It is certain that TLR3 could recognize double-stranded RNA (dsRNA) in virus [7]. Mahanonda et al. previously reported that human gingival fibroblasts (HGFs) functionally expressed TLR3, and a TLR3 agonist (polyinosinic-polycytidylic acid:poly I:C) stimulation could induce IL-8 production in HGFs [8]. This report means that virus dsRNA could stimulate HGFs via TLR3 in periodontal tissues.

CCL20 is a CC motif chemokine that functions as a chemoattractant to facilitate the recruitment of CCR6-expressing cells, including Th17 cells [9]. It has been reported that Th17 cells are involved in the exacerbation of some inflammatory diseases, such as rheumatoid arthritis [9] and periodontal disease [10]. We previously reported that proinflammatory cytokines, including IL-1β, TNF-α, and IL-17A, could induce CCL20 production in HGFs [11,12]. We also reported that CCL20 protein and mRNA expressions were seen in human periodontally diseased tissues though CCL20 expression was not found in human healthy periodontal tissues [13]. However, it is uncertain whether a TLR3 ligand could induce CCL20 production in HGFs or not.

The aim of this study was to examine the effect of a TLR3 ligand on CCL20 production in HGFs. Moreover, we examined the role of a TLR3 ligand on CCL20 production in IL-1β-stimulated HGFs because we found IL-1β could induce CCL20 production in HGFs. Furthermore, we investigated the effects of a TLR3 agonist and IL-1β stimulation on signal transduction pathways in HGFs, including MAPKs, and NF-κB.

2. Materials and methods

2.1. Gingival tissue biopsies and cell culture

We used HGFs that were isolated from three clinically healthy gingiva during routine distal wedge surgical procedures. The gingi-

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val specimens were cut into small pieces and transferred to culture dishes. The HGFs that grew from the gingiva were primarily cultured on 100 mm^2 uncoated plastic dishes in Dulbecco's modified Eagle's medium (DMEM: Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS: Gibco, Grand Island, NY, USA) and antibiotics (penicillin G: 100 units/ml, streptomycin: 100 μg/ml) at 37 °C in humidified air with 5% CO_2. Confluent cells were transferred and cultured for use in the present study. After three to four subcultures with trypsinization, the cultures contained homogeneous, slim, and spindle-shaped cells growing in characteristic swirls. The cells were used for experiments after five passages. Informed consent was obtained from all subjects participating in this study. The study was performed with the approval and compliance of the University of Tokushima Ethical Committee (No. 329).

2.2. CCL20 production in HGFs

The HGFs were stimulated with polyinosinic-polycytidylic acid (Poly I:C), a synthetic analog of dsRNA (10, 100, or 1000 ng/ml: InvivoGen, San Diego, CA) and IL-1β (10 ng/ml: Peprotech, Rocky Hill, NJ, USA) for 24 h. The supernatants from the HGFs were collected, and the CCL20 concentrations of the culture supernatants were measured in triplicate with ELISA. Human CCL20 DuoSet (catalog number: DY360, R&D systems, Minneapolis, MN, USA) was used for the determination. All assays were performed according to the manufacturer's instructions, and cytokine levels were determined using the standard curve prepared for each assay. In selected experiments, the HGFs were cultured for 1 h in the presence or absence of SB203580 (a p38 MAPK inhibitor, 20 μM: Santa Cruz Biotechnology, Santa Cruz, CA, USA), PD98059 (an extracellular signal-regulated kinase (ERK) inhibitor, 20 μM: Cayman Chemical, Ann Arbor, MI, USA), SP600125 (a c-Jun N terminal kinase (JNK) inhibitor, 20 μM: Enzo Life Sciences, Plymouth Meeting, PA, USA), or Bay11-7085 (a NF-κB inhibitor, 20 μM: BIOMOL international, Plymouth Meeting, PA, USA) prior to their incubation with Poly I:C and IL-1β. Inhibitors were dissolved in DMSO and diluted in the culture medium.

2.3. Western blot analysis

To confirm the Poly I:C and/or IL-1β-induced phosphorylation of signal transduction molecules, Western blot analysis was performed. HGFs stimulated with Poly I:C (1000 ng/ml) and/or IL-1β (10 ng/ml) were washed once with cold PBS, before being incubated on ice for 30 min with lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with Protease Inhibitor Cocktail (Sigma). After removal of debris by centrifugation, the protein concentrations of the lysates were quantified with the Bradford protein assay using IgG as a standard. A 20 μg protein sample was loaded onto a 4–20% SDS–PAGE gel, before being electrotransfered to a PVDF membrane. The activation of p38 MAPK, ERK, JNK, and inhibitor of nuclear factor κB (IκB)-α was assessed using anti phospho-p38 MAPK rabbit monoclonal antibody (Cell Signaling Technology), anti phospho-ERK rabbit monoclonal antibody (Cell Signaling Technology), anti phospho-JNK rabbit monoclonal antibody (Cell Signaling Technology), anti phospho-IκB-α mouse monoclonal antibody (Cell signaling Technology), anti p38 MAPK rabbit monoclonal antibody (Cell signaling technology), anti ERK rabbit monoclonal antibody, anti JNK rabbit monoclonal antibody (Cell signaling technology), or anti actin rabbit monoclonal antibody (Sigma) according to the manufacturer's instructions. Protein bands were visualized by incubation with the horseradish peroxidase-conjugated anti mouse or rabbit immunoglobulin G goat antibody (Sigma), followed by detection using a chemiluminescence reagent (ECL Western Blotting Detection Reagents: GE Healthcare, Uppsala, Sweden). We analyzed HGFs from three different donors separately.

2.4. Statistical analysis

Statistical significance was obtained using the Student's *t* test. We used the statview software. *P* values <0.05 were considered significant.

3. Results

3.1. Effects of Poly I:C and/or IL-1β on CCL20 production in HGFs

To reveal effects of Poly I:C and/or IL-1β stimulation on CCL20 production in HGFs, HGFs were exposed to Poly I:C (10, 100, 1000 ng/ml), IL-1β (10 ng/ml) with or without Poly I:C (10, 100, or 1000 ng/ml) for 24 h, and the CCL20 productions in the supernatant were measured using ELISA. High concentration of Poly I:C (1000 ng/ml) induced CCL20 production in HGFs (Fig. 1). On the other hand, low concentration of Poly I:C (100 ng/ml) enhanced IL-1β (10 ng/ml) induced CCL20 production in HGFs, though the single stimulation of Poly I:C (100 ng/ml) did not induce CCL20 in HGFs. Poly I:C (1000 ng/ml) synergistically increased CCL20 production in IL-1β-stimulated HGFs (Fig. 1).

3.2. Effects of chemical inhibitors of intracellular signaling pathways on CCL20 production from Poly I:C and IL-1β-stimulated HGFs

Our previous studies demonstrated that MAPKs [11,12] and NF-κB [11] pathways are related to CCL20 production in HGFs. Therefore, we examined the effects of a p38 MAPK inhibitor (SB203580), a MEK inhibitor (PD98059), a JNK inhibitor (SP600125), or a NF-κB inhibitor (Bay11-7085) on CCL20 production in Poly I:C and IL-1β-stimulated HGFs. Fig. 2 shows that SB203580, PD98059, SP600125, and Bay11-7085 significantly suppressed CCL20 production in Poly I:C and IL-1β-stimulated HGFs. The same concentration of DMSO did not change CCL20 production from Poly I:C and IL-1β-stimulated HGFs (data not shown). These data demonstrated that p38 MAPK, ERK, JNK, and NF-κB pathways are involved in CCL20 production in Poly I:C and IL-1β-stimulated HGFs.

3.3. Effects of Poly I:C and/or IL-1β stimulation on MAPKs and IκB-α phosphorylation in HGFs

Finally, to reveal effects of Poly I:C and/or IL-1β stimulation on the activation of intracellular signal transduction pathways in HGFs, HGFs were stimulated with Poly I:C (1000 ng/ml), IL-1β (10 ng/ml), or Poly I:C (1000 ng/ml) in combination with IL-1β (10 ng/ml) for 15, 30, or 60 min, HGFs were lysed, and western blot

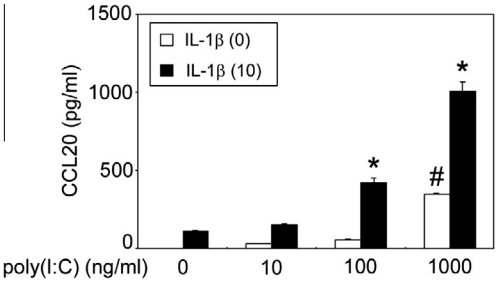

Fig. 1. Effects of Poly I:C and IL-1β co-treatment on HGFs CCL20 production. HGFs were stimulated for 24 h with Poly I:C, IL-1β, or mixtures of both substances at designated concentrations. The expression levels of CCL20 in the supernatants were measured using ELISA. Data are representative of three different HGFs samples from three different donors. The results are shown as the mean and SD of one representative experiment performed in triplicate. The error bars show the SD of the values. * = $P < 0.05$, significantly different from the IL-1β-stimulated HGFs, # = $P < 0.05$ significantly different from the non-stimulate HGFs.