Transforming growth factor-β1 increases C-C chemokine ligand 11 production in interleukin 4-stimulated human periodontal ligament cells

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Text pages: 16 (including 1 title page), Figures: 4

Running head: TGF-β1 and IL-4 induce CCL11 production

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Keywords: IL-4, TGF-β1, CCL11, STAT6, periodontal ligament cells

Abbreviations

CCL: C-C chemokine ligand
CCR: C-C chemokine receptor
ELISA: enzyme-linked immunosorbent assay
ERK: extracellular signal-regulated kinase
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
HPDLC: human periodontal ligament cells

IL: interleukin

JNK: c-Jun N-terminal kinase

MAPK: mitogen-activated protein kinases

STAT: signal transducer and activator of transcription

TGF-β1: transforming growth factor-β1

TLR: toll-like receptor

TNF: tumor necrosis factor

Abstract

Transforming growth factor (TGF)-β1 is a multifunctional cytokine, which can control certain functions of various kinds of cells. However, it is unclear whether TGF-β1 affects T-cell migration in periodontal lesions. The aim of this study was to examine the effects of TGF-β1 on the production of C-C chemokine ligand (CCL)11, which is a T-helper 2-type chemokine, in human periodontal ligament cells (HPDLC). Interleukin (IL)-4 induced CCL11 production, but TGF-β1 did not, in HPDLC. However, TGF-β1 enhanced CCL11 production in IL-4-stimulated HPDLC. Western blot analysis showed that the signal transducer and activator of transcription 6 (STAT6) pathway was highly activated in HPDLC that had been stimulated with both IL-4 and TGF-β1. Mitogen-activated protein kinase activation did not differ between the HPDLC treated with a combination of IL-4 and TGF-β1 and those treated with IL-4 or TGF-β1 alone. Moreover, a STAT6 inhibitor significantly inhibited CCL11 production in HPDLC that had been stimulated with IL-4 and TGF-β1. The current study clearly demonstrated that TGF-β1 enhanced IL-4-induced CCL11 production in HPDLC. The STAT6 pathway is important for CCL11 production in IL-4- and TGF-β1-treated HPDLC.

1. Introduction

Host responses to bacteria in periodontal lesions are involved in the progression of periodontal disease [Cekiti et al., 2014]. In particular, T cells are associated with the pathogenesis of chronic periodontal disease. Garlet reported that cytokines from T-helper (Th)1 cells and Th17 cells increased receptor activator of nuclear factor kappa-β ligand (RANKL) levels and induced bone resorption in periodontal lesions. He also suggested that cytokines from Th2 cells might antagonize the destructive events induced by Th1 and Th17 cells [Garlet, 2010]. On the other hand, it has been reported that Th2 cells are involved in the pathogenesis of periodontal disease. Myneni et al. demonstrated that *Tannerella forsythia*-induced Toll-like receptor (TLR)2 activation resulted in alveolar bone destruction in a mouse model. Furthermore, Th2 cell proliferation downstream of TLR2 activation was found to be associated with alveolar bone destruction caused by *Tannerella forsythia* [Myneni et al., 2011]. The role of Th2 cells in periodontal lesions is still disputed. However, it is important to elucidate the mechanism responsible for Th2 cell migration and accumulation in periodontal lesions because Th2 cells are obviously involved in the pathological process responsible for periodontal disease.

Th2 cells preferentially express C-C chemokine receptor type 3 (CCR3), and C-C chemokine ligand (CCL)11, which is a CCR3 ligand, is found in the gingival crevicular fluid of patients with periodontal disease [Sallusto et al., 1997; Thunell et al., 2010]. Therefore, we examined whether periodontal resident cells produce CCL11. As a result, we revealed that treatment

with interleukin (IL)-4 in combination with IL-1β or tumor necrosis factor (TNF)-α induced CCL11 production in human gingival fibroblasts [Hosokawa et al., 2013]. However, it is unclear whether other cytokines modulate CCL11 production.

Transforming growth factor (TGF)-β1 is a multifunctional cytokine, which modulates various functions in many different cell types [Grotendorst et al., 1997; Lawrence et al., 1996]. For example, TGF-β1 inhibits the proliferation of most cells. However, TGF-β1 enhances the growth of some kinds of mesenchymal cells [Lawrence et al., 1996]. TGF-β1 has immunosuppressive effects on certain types of cells, which involve the inhibition of proinflammatory cytokine production [Lawrence et al., 1996]. TGF-β1 also induces the production of extracellular matrix molecules [Lawrence et al., 1996]. Recently, it was reported that compared with healthy tissue, tissue affected by periodontal disease exhibits elevated TGF-β1 expression [Khalaf et al., 2014]. However, there have been few reports about the effects of TGF-β1 on chemokine production, and it is unclear whether TGF-β1 modulates CCL11 expression in periodontal ligament cells.

The aim of this study was to investigate the effects of IL-4 and TGF-β1 on CCL11 production in human periodontal ligament cells (HPDLC), which are the major resident cells in periodontal tissues. Moreover, we examined the signal transduction pathways involved in CCL11 production, such as the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 6 (STAT6) pathways.

2. Materials and Methods

2.1. Cell culturing

HPDLC were obtained from Lonza Walkersville Inc. (Walkersville, MD, USA) and grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and antibiotics (penicillin G: 100 units/ml, streptomycin: 100 μg/ml) at 37°C in humidified air with 5% CO_2. The cells were used between passage numbers 5 and 10.

2.2. CCL11 production in HPDLC

HPDLC were stimulated with recombinant human TGF-β1 (0.01, 0.1, 1, or 10 ng/ml; PeproTech, Rocky Hill, NJ, USA) and/or recombinant human IL-4 (10 ng/ml; PeproTech) for 24 hours. The HPDLC culture supernatants were collected, and their CCL11 concentrations were measured in triplicate with DuoSet enzyme-linked immunosorbent assays (ELISA) (R&D systems, Minneapolis, MN, USA). All assays were performed according to the manufacturer's instructions, and cytokine levels were determined using the standard curves prepared for each assay. In selected experiments, HPDLC were cultured for 1 hour in the presence or absence of AS1517499, a STAT6 inhibitor (100 nM; Axon Medchem, Groningen, The Netherlands), prior to their incubation with TGF-β1 and IL-4. We used 3 samples in this experiment, and performed the ELISA in triplicate for each sample.

2.3. Western blot analysis

To confirm whether IL-4 with or without TGF-β1 induced the phosphorylation of signal transduction molecules in HPDLC, Western blot analysis was performed. HPDLC that had

been stimulated with IL-4 (10 ng/ml) with or without TGF-β1 (10 ng/ml) for 15, 30, or 60

minutes were washed once with cold phosphate-buffered saline, before being incubated on

ice for 15 minutes with cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA)

supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). After the

removal of debris via 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 4-20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, before being

electrotransferred to a polyvinylidene difluoride membrane. The phosphorylation of p38

MAPK, extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and

STAT6 in HPDLC were assessed using a phospho-p38 MAPK rabbit monoclonal antibody

(Cell Signaling Technology), a phospho-ERK rabbit monoclonal antibody (Cell Signaling

Technology), a phospho-JNK rabbit monoclonal antibody (Cell Signaling Technology), a

phospho-STAT6 rabbit monoclonal antibody (Cell Signaling Technology), a p38 MAPK

rabbit monoclonal antibody (Cell Signaling Technology), an ERK rabbit monoclonal

antibody (Cell Signaling Technology), a JNK rabbit monoclonal antibody (Cell Signaling

Technology), a STAT6 rabbit monoclonal antibody (Cell Signaling Technology), or a

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit monoclonal antibody (Cell

Signaling Technology), according to the manufacturer's instructions. The protein bands were

incubated with the horseradish peroxidase-conjugated secondary antibody (Sigma) and then

visualized using the ECL detection system (GE Healthcare, Uppsala, Sweden). We

performed Western blot analysis using 3 samples, and we obtained similar results for each sample.

2.4. Statistical analysis

All experiments were repeated 3 times and produced similar results in each case. The significance of differences between groups was assessed by one-way analysis of variance (ANOVA) in the experiments shown in Figure 1 and Figure 4. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. The effects of IL-4 and/or TGF-β1 on CCL11 production in HPDLC

We first examined the effects of 24 hours' stimulation with TGF-β1 on CCL11 production in HPDLC. TGF-β1 (10 ng/ml) alone did not induce CCL11 production. So, we investigated whether TGF-β1 is able to modulate CCL11 production induced by another cytokine. We previously reported that IL-4 induced CCL11 production in human gingival fibroblasts [Hosokawa et al., 2013]. So, we focused on IL-4 in this study. Treating the HPDLC with 10 ng/ml IL-4 induced significant CCL11 production, but treating them with 1 ng/ml IL-4 only caused them to produce a small amount of CCL11. TGF-β1 significantly enhanced CCL11 production in IL-4-stimulated HPDLC in a dose-dependent fashion (Fig. 1).

3.2. The effects of IL-4 and/or TGF-β1 on MAPK phosphorylation in HPDLC

We previously reported that MAPK are associated with CCL11 production in human gingival fibroblasts [Hosokawa et al., 2013]. Therefore, we examined MAPK activation in IL-4-

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and/or TGF-β1-stimulated HPDLC. Figure 2 shows that stimulation with IL-4 or TGF-β1 alone activated the p38 MAPK, ERK, and JNK pathways in HPDLC. The levels of phosphorylated p38 MAPK and phosphorylated JNK between the HPDLC stimulated with both IL-4 and TGF-β1 (hereafter referred to as the IL-4/TGF-β1-stimulated HPDLC) and those stimulated with the IL-4 or TGF-β1 alone. On the other hand, the ERK phosphorylation level of the IL-4/TGF-β1-stimulated HPDLC was lower than that seen in the TGF-β1-stimulated HPDLC, although similar levels of ERK phosphorylation were observed in the IL-4-stimulated HPDLC and IL-4/TGF-β1-stimulated HPDLC. Judging from the Western blot data, we consider that MAPK pathways are not involved in the enhancement of CCL11 production in IL-4/TGF-β1-stimulated HPDLC because TGF-β1 stimulation did not enhance the level of MAPK phosphorylation induced by IL-4.

3.3. The effects of IL-4 and/or TGF-β1 on STAT6 activation in HPDLC

Next, we examined whether IL-4 and/or TGF-β1 stimulation affected the activation of STAT6 in HPDLC because it is known that IL-4 activates the STAT6 pathway in some types of cells [Takeda et al., 2000]. Figure 3 shows that IL-4 induced STAT6 phosphorylation in HPDLC after 15 minutes' stimulation although TGF-β1 did not induce STAT6 activation. On the other hand, compared with that seen after stimulation with IL-4 alone treatment with a combination of IL-4 and TGF-β1 enhanced the level of STAT6 phosphorylation in the HPDLC at all time points.

3.4. The effects of a STAT6 inhibitor on CCL11 production in HPDLC that had been

stimulated with IL-4 and TGF-β1

Figure 3 clearly shows that stimulation with both IL-4 and TGF-β1 enhanced STAT6 activation. Therefore, we hypothesized that the STAT6 pathway might control CCL11 production in IL-4/TGF-β1-stimulated HPDLC. Figure 4 shows that AS1517499 (a STAT6 inhibitor) significantly reduced CCL11 production in IL-4/TGF-β1-stimulated HPDLC. Moreover, AS1517499 inhibited CCL11 production in IL-4-stimulated HPDLC (data not shown).

4. Discussion

In this study, we revealed that TGF-β1 increased CCL11 production in IL-4-stimulated HPDLC. Matsukura et al. reported that TGF-β1 did not induce CCL11 production in human airway smooth muscle cells. However, they found that TGF-β1 enhanced CCL11 production in IL-4- or IL-13-treated human airway smooth muscle cells [Matsukura et al., 2010]. Wenzel et al. also reported that treatment with a combination of TGF-β1 and IL-13 synergistically increased CCL11 expression in human airway fibroblasts [Wenzel et al., 2002]. Judging from our data and those of previous reports, the number of Th2 cells in inflammatory lesions might markedly increase when both IL-4 and TGF-β1 are present because CCL11 production by host cells increases in such circumstances. A previous study detected elevated levels of TGF-β1 in periodontal lesions [Khalaf et al., 2014]. Therefore, the number of Th2 cells might markedly increase whenTh2 cells in periodontal lesions produce IL-4, and hence, IL-4 might be a key cytokine for Th2 cell accumulation.

We showed that treatment with a combination of IL-4 and TGF-β1 did not result in an increase in MAPK phosphorylation in HPDLC compared with stimulation with IL-4 alone (Fig. 2). In addition, MAPK inhibitors reduced CCL11 production in HPDLC stimulated with IL-4 alone or IL-4 and TGF-β1 (data not shown). Therefore, MAPK activation was not found to be associated with the enhanced CCL11 production seen in HPDLC treated with a combination of IL-4 and TGF-β1. El Mabrouk et al. reported that IL-4 did not affect p38 MAPK or ERK phosphorylation in TGF-β1-treated human chondrocytes although JNK phosphorylation was inhibited by IL-4 treatment [El Mabrouk et al., 2008]. Xie et al. reported that IL-4 inhibited TGF-β1-stimulated ERK activation in human airway muscle cells [Xie et al., 2005]. Previous studies, including ours, have shown that the effects of IL-4 on MAPK activation in TGF-β1 are dependent on the type of cell or the cytokine concentration [El Mabrouk et al., 2008; Xie et al., 2005]. Our previous study demonstrated that CCL11 production in human gingival fibroblasts was positively regulated by p38 MAPK, ERK, and JNK [Hosokawa et al., 2013]. However, the level of MAPK phosphorylation in IL-4-treated HPDLC was not affected by TGF-β1. So, we consider that STAT6 activation is more important for enhancing CCL11 production than for modulating MAPK activation.

We showed that TGF-β1 increased STAT6 phosphorylation in IL-4-stimulated HPDLC. Wenzel et al. reported that TGF-β1 did not induce STAT6 phosphorylation in human airway fibroblasts, although it enhanced CCL11 production in IL-13-stimulated human airway fibroblasts [Wenzel et al., 2002]. They also showed that TGF-β1 increased the level of

STAT6 phosphorylation in IL-13-stimulated human airway fibroblasts [Wenzel et al., 2002]. Our and Wenzel's findings indicate that TGF-β1 enhances STAT6 activation in Th2 cytokine-stimulated cells and might contribute to Th2-type inflammation because STAT6 promotes Th2-associated processes [Forbes et al., 2010].

We revealed that STAT6 is involved in CCL11 production in HPDLC. Fritz et al. reported that CCL11 production was abrogated in IL-4/oncostatin M-stimulated STAT6 knockout mouse lung fibroblasts [Fritz et al., 2006]. Hosoya et al. showed that STAT6 siRNA inhibited CCL11 production in IL-4/TNF-α-stimulated dermal fibroblasts [Hosoya et al., 2011]. Previous studies, including ours, have demonstrated that STAT6 activation is essential for CCL11 production in IL-4-treated cells. Myneni et al. recently reported that STAT6 is required for alveolar bone destruction caused by *Tannerella forsythia* because STAT6 knockout mice exhibited less bone loss than wild type mice [Myneni et al., 2011]. It is unclear whether CCL11 is involved in alveolar bone resorption. Therefore, we should investigate the role of CCL11 in alveolar bone resorption in periodontal lesions in the future.

Some previous studies have found that TGF-β1 is an immunosuppressive cytokine, which stimulates wound-healing processes in inflammatory lesions. For example, Chantry et al. reported that TGF-β1 inhibited IL-1β production in lipopolysaccharide-stimulated peripheral blood mononuclear cells [Chantry et al., 1989]. Musso et al. also found that TGF-β1 downregulated IL-6 production in human monocytes that had been stimulated with IL-1β [Musso et al., 1990]. In the current study, we revealed that TGF-β1 enhanced CCL11

production in IL-4-treated HPDLC. We consider that the effects of TGF-β1 are dependent on the cell type and the other stimulatory factors present. Further studies are necessary to clarify the role of TGF-β1 in periodontal lesions.

In summary, our results indicate the mechanism responsible for Th2 cell migration in periodontal lesions. Namely, TGF-β1 might contribute to the accumulation of Th2 cells in periodontal lesions by inducing CCL11 production. Therefore, TGF-β1 is associated with the pathogenesis of periodontal disease, as it induces Th2 chemokine production in resident periodontal cells. We also revealed that STAT6 is important for controlling CCL11 production in HPDLC. Myneni et al. recently reported that *Tannerella forsythia*-induced alveolar bone loss was significantly reduced in STAT6 knockout mice [Garlet et al., 2010]. Their findings and ours indicate that the STAT6 pathway is involved in the pathogenesis of periodontal disease. Therefore, it might be possible to treat periodontal disease by targeting STAT6. However, further investigations into STAT6 are necessary because its role in periodontal disease, except for its effects on CCL11 production in HPDLC, are still uncertain. Finally, this study showed that TGF-β1 increased IL-4-induced CCL11 production in HPDLC, and STAT6 is essential for CCL11 release in IL-4/TGF-β1-treated HPDLC.

Funding

Conflicts of interest

The authors confirm that they have no conflicts of interest.

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Figure legends

Fig. 1. Effects of IL-4 and/or TGF-β1 on CCL11 production in HPDLC

HPDLC were stimulated with IL-4 (10 ng/ml) and/or TGF-β1 (0.01, 0.1, 1, or 10 ng/ml), and the resultant supernatants were collected after 24 hours' incubation. The CCL11 levels of the supernatants were measured using ELISA. The results are shown as the mean and

standard deviation (SD) of one representative experiment performed in triplicate. The error

bars represent SD values. * *P*<0.05, significantly different from the IL-4-stimulated HPDLC

Fig. 2. Phosphorylation of p38 MAPK, ERK, and JNK in IL-4 and/or TGF-β1-stimulated HPDLC

The cultured cells were stimulated with IL-4 (10 ng/ml each) and/or TGF-β1 (10 ng/ml) for

15, 30, or 60 minutes. The cells were lysed in lysis buffer containing protease inhibitors, and

p38 MAPK, ERK, and JNK phosphorylation were assessed using Western blot analysis. The

levels of phospho-p38 MAPK, total p38 MAPK, phospho-ERK, total ERK, phospho-JNK,

and total JNK in the HPDLC were determined using Western blotting in 3 independent

experiments.

Fig. 3. Phosphorylation of STAT6 in IL-4 and/or TGF-β1-stimulated HPDLC

The cultured cells were stimulated with IL-4 (10 ng/ml each) and/or TGF-β1 (10 ng/ml) for

15, 30, or 60 minutes. The cells were lysed in lysis buffer containing protease inhibitors, and

the phosphorylation of STAT6 was assessed using Western blot analysis. A representative

Western blot that indicates the phospho-STAT6, total STAT6, and GAPDH levels detected in

the HPDLC during three independent experiments is shown.

Fig. 4. Effects of a STAT6 inhibitor on CCL11 production in IL-4 and TGF-β1-stimulated HPDLC

The cells were pre-incubated with AS1517499 (100 nM) for 1 hour and then incubated with

IL-4 (10 ng/ml) and TGF-β1 (10 ng/ml). After 24 hours' incubation, the supernatants were

collected, and CCL11 production was assessed using ELISA. The results are shown as the

mean and SD of one representative experiment performed in triplicate. The error bars

represent SD values. *P<0.01 significantly different from the IL-4- and TGF-β1-stimulated
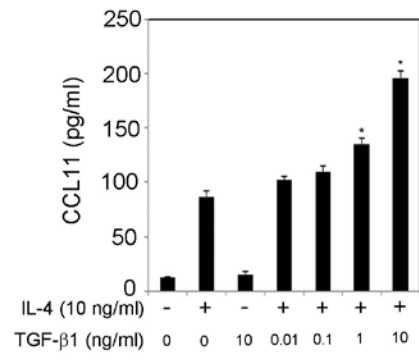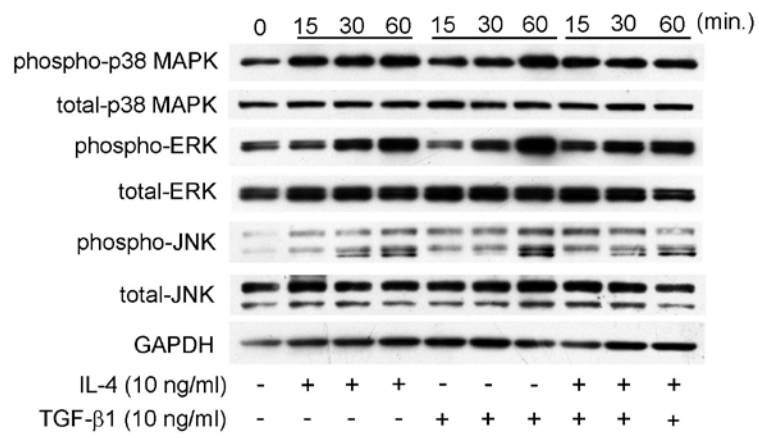
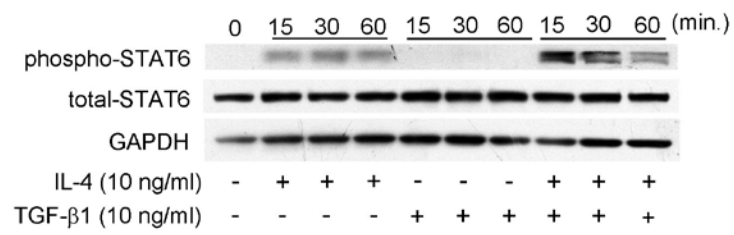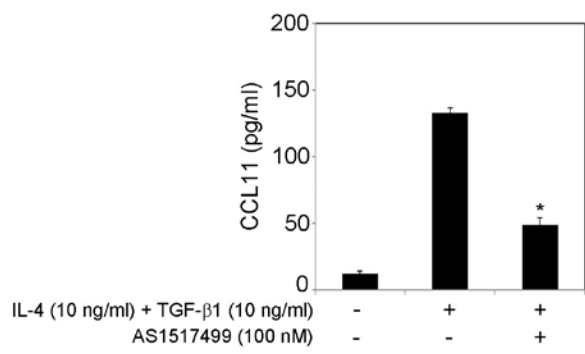HPDLC that were not treated with the inhibitor

Fig. 1

Fig. 2

Fig.3

IL-4 (10 ng/ml) + TGF-β1 (10 ng/ml)	−	+	+
AS1517499 (100 nM)	−	−	+

Fig. 4