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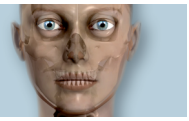

ORIGINAL ARTICLE

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The effect of IL-17 on the production of proinflammatory cytokines and matrix metalloproteinase-1 by human periodontal ligament fibroblasts

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Structured Abstract

Objectives – To investigate the effects of IL-17 on IL-6, IL-1β, and matrix metalloproteinase (MMP-1) production, and to compare the MMP-1 production between the individual and combined effects of IL-1β and IL-6 in human periodontal ligament fibroblasts (HPDLF).

Materials and Methods – Human periodontal ligament fibroblasts were cultured with IL-17 for 0.5, 1, 4, 24, 48, and 72 h, and were cultured with IL-1β, IL-6/sIL-6R, or a combination of IL-1β and IL-6/sIL-6R for 24 h. To measure the mRNA levels of *IL-6*, *IL-1β,* and *MMP-1*, total RNA was extracted from the cultured HPDLF, and a real-time PCR analysis was performed. The protein levels of IL-6, IL-1β, and MMP-1 in supernatants were measured using enzyme-linked immunosorbent assays (ELISAs).

Results – IL-17 significantly increased the expression of *IL-6* and *MMP-1* mRNA and protein, while IL-17 transiently increased the expression of *IL-1β* mRNA. The combination of IL-1β and IL-6/sIL-6R induced significantly higher levels of MMP-1 protein than IL-1β alone.

Conclusions – IL-17 upregulated the production of IL-6 and MMP-1 sequentially in HPDLF. IL-6/sIL-6R may enhance the effects of IL-1β on MMP-1 production. The present results suggest that IL-17 induces MMP-1 production not only directly, but also indirectly by promoting IL-6 production, thus resulting in the degradation of collagens in the PDL.

Key words: IL-17; IL-1β; IL-6; matrix metalloproteinase-1; periodontal ligament cells

Introduction

Orthodontic tooth movement is mediated by the remodeling of the periodontal ligament (PDL) and alveolar bone in response to mechanical loading. The transduction of mechanical forces to the cells of the osteoblast and/or fibroblast lineages triggers a biological response, which is regulated by a variety of inflammatory cytokines (1–3). The expression of IL-1β and IL-6 was detected in the PDL of rats after the application of orthodontic force by *in situ* hybridization (4). It was also reported that the concentrations of interleukin (IL)-1β and IL-6 were increased in the gingival crevicular fluid (GCF) during orthodontic tooth movement (5). IL-1β and IL-6, inflammatory mediators that induce bone resorption (6, 7), may play important roles in bone resorption during orthodontic tooth movement.

IL-1β upregulates matrix metalloproteinase (MMP)-1 in human periodontal ligament fibroblasts (HPDLF), and this can lead to the breakdown of periodontal ligament collagens (8). In HPDLF, mechanical force causes a significant increase in the *MMP-1* mRNA expression (9). Furthermore, some studies have reported high levels of *MMP-1* mRNA expression in the human periodontal ligament (2) and an increase in MMP-1 protein expression in the human GCF during tooth movement (10). These studies suggest the involvement of MMP-1 in the degradation of the PDL during orthodontic tooth movement. IL-1β, IL-6, and MMP-1 are thought to play important roles in PDL degradation and bone resorption.

IL-17 is produced by Th17 cells, a subset of T helper cells (11). High levels of IL-17 were found in the synovial fluids of patients with rheumatoid arthritis (12) and in the periodontal tissue of patients with periodontitis (13). The previous studies showed IL-17$^{-/-}$ mice significantly reduced infection-stimulated bone resorption compared with wild-type animals while interferon (IFN)-γ$^{-/-}$ and tumor necrosis factor (TNF)-α$^{-/-}$ mice did not. This result suggests IL-17 plays a crucial role in bone destruction in periodontal lesions (14). IL-17 is also an important mediator of orthodontically induced inflammatory root resorption (15). It is expected that IL-17 plays a crucial role in orthodontic tooth movement, which is mediated by the remodeling of PDL and alveolar bone. The effects of mechanical stress on IL-17 expression have been reported; the compressive force induces the expression of *IL-17* mRNA in MC3T3-E1 cells (16). The IL-17 and IL-17R proteins were detected in the PDL tissue of the rats subjected to an orthodontic force. Th17 cells also appeared in the PDL exposed to an orthodontic force (17). IL-17 was shown to stimulate the secretion of IL-6 and IL-8 by synovial fibroblasts and gingival fibroblasts (13, 18–20). IL-17 also induces MMP-1 and MMP-3 production by synovial fibroblasts (21). These reports (13–21) suggest that the IL-17 produced in response to mechanical stress may promote the production proinflammatory cytokines and extracellular matrix degradation in PDL.

The purposes of this study were to investigate the effects of IL-17 on IL-6, IL-1β, and MMP-1 production and to examine the individual and combined effects of IL-1β and IL-6 on MMP-1 production in HPDLF.

Materials and methods

Cell cultures

Human periodontal ligament fibroblasts collected from two persons were purchased from Cell Research Corporation (Singapore). HPDLF were cultured in a complete medium consisting of Dulbecco's modified Eagle's medium (Life Technologies Japan, Tokyo) containing 10% FBS (Life Technologies Japan, Tokyo) and 100 μg/ml streptomycin (Life Technologies Japan, Tokyo). The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Passage 6–10 HPDLF were transferred to 100-mm culture dishes (Greiner, Tokyo), at a density of 7×10^6 cells per dish, for the experiments. No morphological change of HPDLF was observed under the microscope during the passages. The each period of growth to confluence was almost stable during subculture. For the experiments, at 70% confluency, the complete medium was replaced with medium containing

1% bovine serum albumin. After an overnight incubation, the HPDLF were treated with recombinant human IL-17 (R&D Systems, Minneapolis, MN, USA), IL-1β, or IL-6/sIL-6R (Pepro Tech, Rocky Hill, NJ, USA). HPDLF were stimulated for 0.5, 1, 4, 24, 48, or 72 h with 10 ng/ml of IL-17; 0 ng/ml of IL-17 at each time point served as a control.

Human periodontal ligament fibroblasts were stimulated for 24 h with IL-1β (0.25, 2.5, or 25 ng/ml), IL-6 (1, 10 or 100 ng/ml)/sIL-6R (100 ng/ml), or with a combination of IL-1β (2.5 ng/ml) and IL-6 (10 ng/ml)/sIL-6R (100 ng/ml). Samples of conditioned medium were collected and stored at −30°C for an enzyme-linked immunosorbent assay (ELISA). The cells were collected and stored at −80°C for real-time PCR.

Real-time PCR

Total RNA was isolated from HPDLF cells using the RNeasy Mini kit (QIAGEN, Tokyo). First-strand cDNA synthesis was performed using the High Capacity RNA-to-cDNA Master Mix (Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions. Real-time PCR was performed using the Fast SYBR Green Master Mix (Life Technologies) as follows: 95°C for 15 s followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. The specificity of the PCR products was verified using a melting curve analysis from 60°C to 72°C. All gene-specific primers used in this study are listed in Table 1. To control variability in amplification because of differences in starting mRNA concentrations, GAPDH was used as internal control. The mRNA expression levels of IL-6, IL-1β, and MMP-1 were expressed as ratios to that of GAPDH in HPDLF.

Measurement of the IL-6, IL-1β, and MMP-1 levels by ELISA

The total protein concentration in the culture supernatants from cultured HPDLF was estimated using a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The levels of IL-6, IL-1β, and MMP-1 proteins were analyzed using commercial sandwich ELISA kits (R&D Systems). The levels of IL-6, IL-1β, and MMP-1 proteins were divided by the total protein.

Statistical analysis

For all experiments, the data were expressed as the means ± standard error. Statistical analysis was based on four experiments in duplicate performed, respectively. Data from different groups were compared by the Mann–Whitney U-test and one-way analysis of variance (ANOVA). A p-value < 0.05 was considered to be significant.

Results

Effect of IL-17 on the IL-6 mRNA level and the IL-6 secretion

The *IL-6* mRNA expression was significantly increased after 1, 4, 24, 48, and 72 h of stimulation with IL-17 ($p < 0.05$; Fig. 1). The HPDLF secreted a significant amount of IL-6 protein after 4, 24, 48, and 72 h incubations with IL-17 ($p < 0.05$), and IL-17 increased the IL-6 secretion in a time-dependent fashion after 24 h.

Effect of IL-17 on the IL-1β mRNA expression and IL-1β secretion

IL-17 significantly increased the *IL-1β* mRNA expression level after 1, 4, and 24 h of stimulation

Table 1. **Primers used for the real-time polymerase chain reaction**

Gene	Forward	Reverse	Product size
IL-6	5′-TACCCCCAGGAGAAGATTCC-3′	5′-TTTTCTGCCAGTGCCTCTTT-3′	139
IL-1β	5′-GGACAAGCTGAGGAAGATGC-3′	5′-TCGTTATCCCATGTGTCGAA-3′	118
MMP-1	5′-TGCTCATGCTTTTCAACCAG-3′	5′-GGTACATCAAAGCCCCGATA-3′	125
GAPDH	5′-CAACGAATTTGGCTACAGCA-3′	5′-AGGGGTCTACATGGCAACTG-3′	211

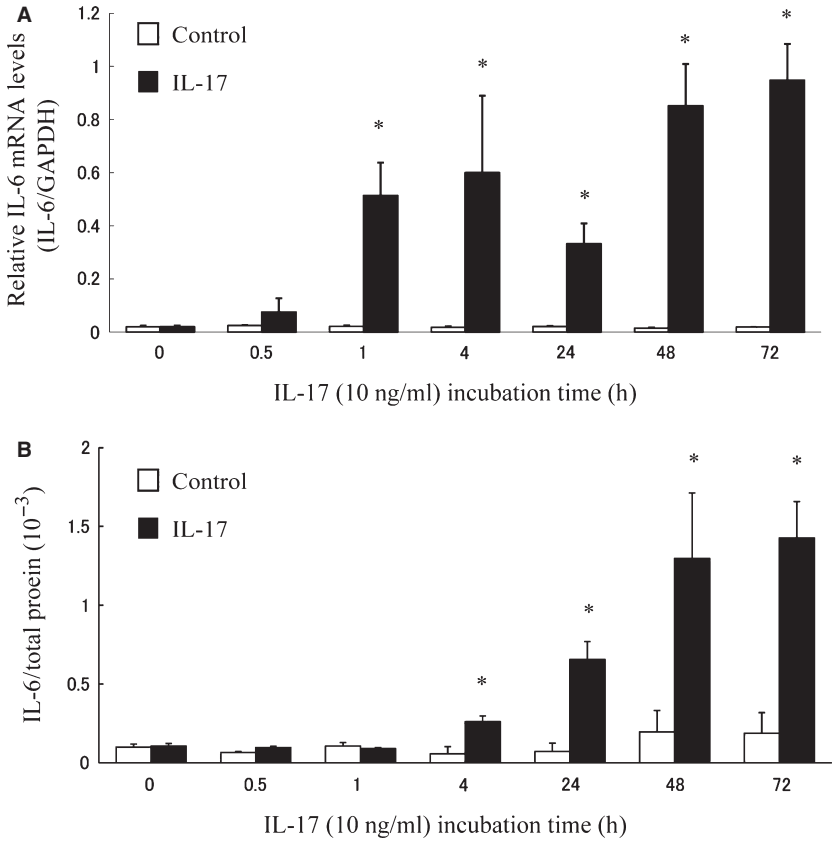

Fig. 1. The effects of IL-17 on the *IL-6* mRNA level (A) and the IL-6 protein level (B) in HPDLF. HPDLF were stimulated with IL-17 (10 ng/ml) for 0.5–72 h. Data are expressed as the means ±SE of four experiments performed in duplicate. *$p < 0.05$ vs. control Mann–Whitney U-test.

with IL-17 ($p < 0.05$), while the levels of *IL-1β* mRNA after 0.5, 48, and 72 h were not significantly different compared with the controls (Fig. 2). IL-1β could not be detected in HPDLF culture supernatants after the stimulation with IL-17.

Effects of IL-17 on the MMP-1 mRNA expression level and MMP-1 secretion

The *MMP-1* mRNA expression tended to increase in a time-dependent fashion after 24 h of stimulation with IL-17, and IL-17 significantly

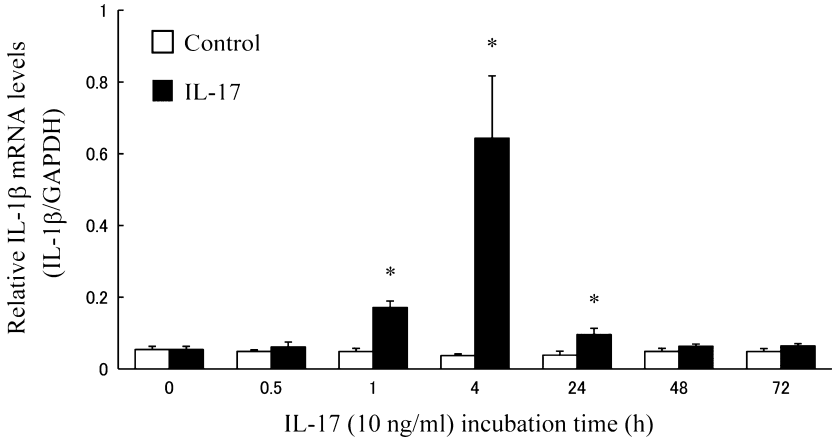

Fig. 2. The effects of IL-17 on the expression of *IL-1β* mRNA HPDLF were stimulated with IL-17 (10 ng/ml) for 0.5–72 h. The data are expressed as the means ±SE of four experiments performed in duplicate. *$p < 0.05$ vs. control Mann–Whitney U-test.

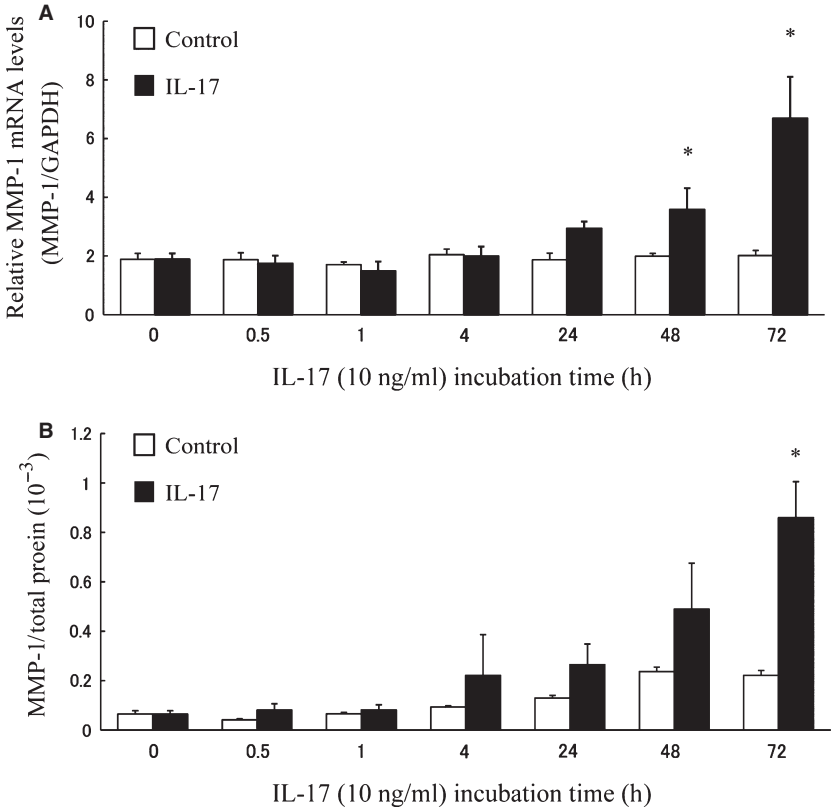

Fig. 3. The effects of IL-17 on the *MMP-1* mRNA level (A) and the MMP-1 protein level (B) in HPDLF. HPDLF were stimulated with IL-17 (10 ng/ml) for 0.5–72 h. The data are expressed as the means ±SE of four experiments performed in duplicate. *$p < 0.05$ vs. control Mann–Whitney U-test.

increased the *MMP-1* mRNA expression after 48 h and 72 h ($p < 0.05$; Fig. 3). The MMP-1 protein levels tended to increase in a time-dependent fashion after 24 h of stimulation with IL-17, and the HPDLF secreted a significant amount of MMP-1 protein after 72 h ($p < 0.05$).

Effects of IL-1β and IL-6 on the mRNA level and secretion of MMP-1

The *MMP-1* mRNA expression and MMP-1 secretion were significantly increased after 24 h of stimulation with IL-1β compared with the control ($p < 0.05$), while the *MMP-1* mRNA expression and MMP-1 secretion were not increased by IL-6/sIL-6R (Figs 4 and 5). The combination of IL-1β and IL-6/sIL-6R induced higher levels of *MMP-1* mRNA than treatment with IL-1β alone, although no significant variations were found (Fig. 4C). The level of protein induced by the combination of IL-1β and IL-6/sIL-6R was significantly higher than that induced by the stimulation of cells with IL-1β alone (Fig. 5C).

Discussion

The present study demonstrated that IL-17 sequentially stimulates IL-6 and MMP-1 production by HPDLF. The recent report showed that IL-17 upregulated IL-6 and MMP-1 production in HPDLF (17, 22). These results indicate that IL-17 may induce HPDLF to produce inflammatory cytokines and MMP-1, resulting in amplification of the inflammatory response and tissue degradation in the PDL.

The present study showed that the IL-1β protein in the culture supernatants was not detected at any point in time, although IL-17 transiently increased the *IL-1β* mRNA expression. It was reported that IL-17 stimulated IL-1β production in human monocytes/macrophages (13, 23) while IL-1β was not detected following the stimulation

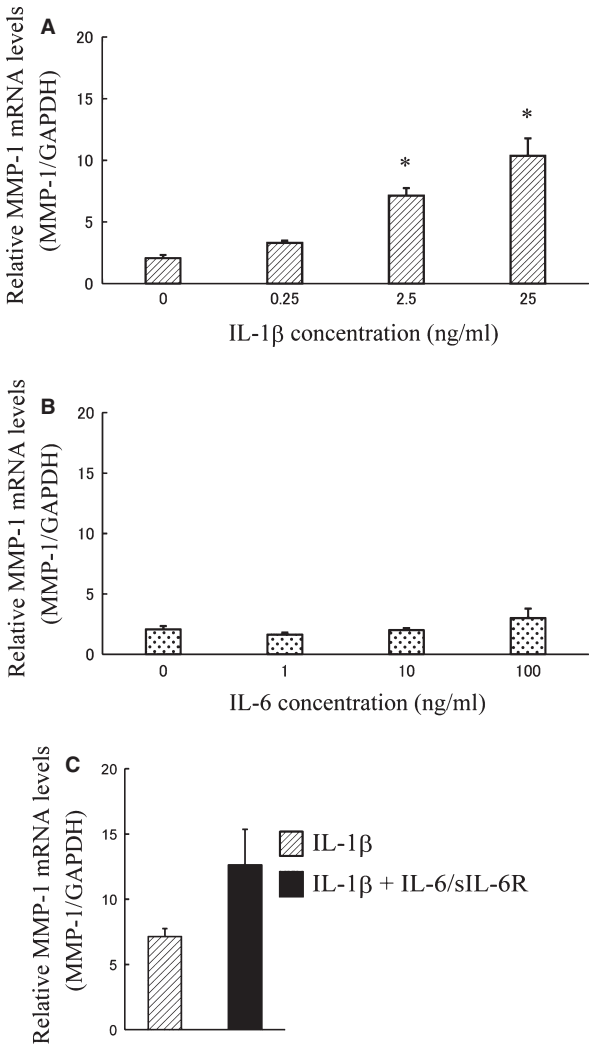

Fig. 4. Cytokine-induced expression of *MMP-1* mRNA in HPDLF. (A) The effects of IL-1β on the expression of *MMP-1* mRNA. (B) The effects of IL-6/sIL-6R on the expression of *MMP-1* mRNA. (C) The effects of IL-1β + IL-6/sIL-6R on the expression of *MMP-1* mRNA. HPDLF were stimulated with IL-1β, IL-6/sIL-6R, or IL-1β (2.5 ng/ml) + IL-6/sIL-6R (10 ng/ml) for 24 h. The data are expressed as the means ±SE of four experiments performed in duplicate. $*p < 0.05$ vs. 0 ng/ml one-way ANOVA.

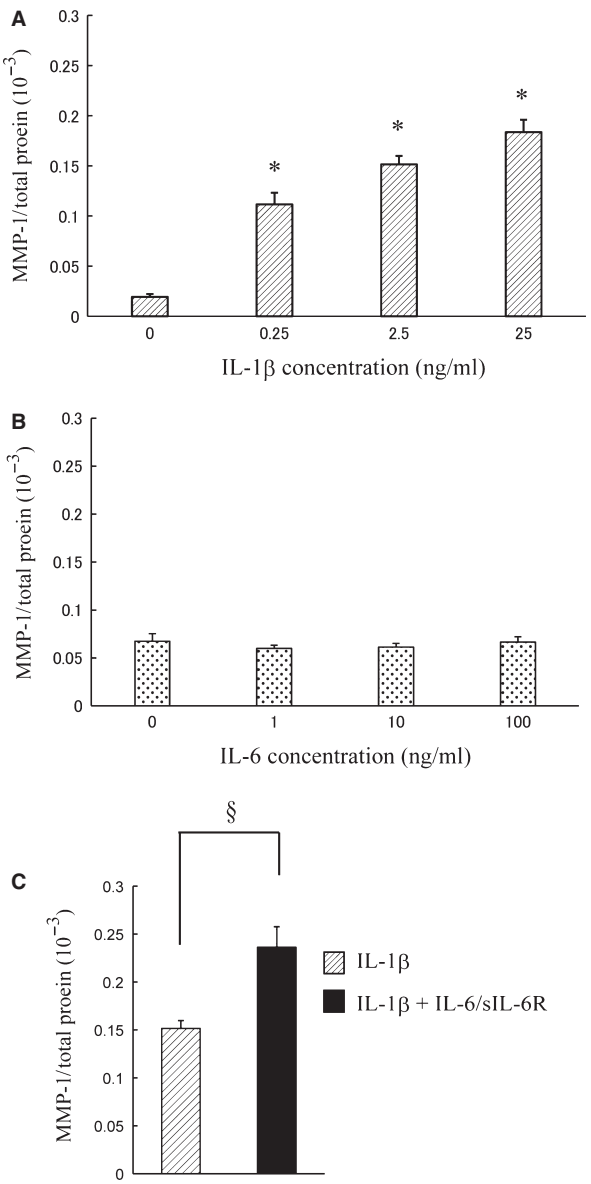

Fig. 5. Cytokine-induced secretion of MMP-1 in HPDLF. (A) The effects of IL-1β on the secretion of MMP-1. (B) The effects of IL-6/sIL-6R on the secretion of MMP-1. (C) The effects of IL-1β + IL-6/sIL-6R on the secretion of MMP-1. HPDLF were stimulated with IL-1β, IL-6/sIL-6R, or IL-1β (2.5 ng/ml) + IL-6/sIL-6R (10 ng/ml) for 24 h. The data are expressed as the means ±SE of four experiments performed in duplicate. $*p < 0.05$ vs. 0 ng/ml one-way ANOVA; $^{§}p < 0.05$ IL-1β vs. IL-1β + IL-6/sIL-6R Mann–Whitney U-test.

with IL-17 in synovial fibroblasts (24). Additionally, no report showed that IL-17 had any effect on IL-1β production in fibroblasts. Based on these previous reports and our present findings, it appears that IL-17 does not affect the IL-1β protein production in HPDLF, while IL-17 induces IL-1β protein expression in human immune cells.

The present results indicate that IL-17 promotes MMP-1 production later than IL-6 production. Beklen (13) reported that IL-17 induced IL-6 production from gingival fibroblasts, and IL-1β and TNF-α from macrophages. In that study, IL-1β and TNF-α increased MMP-1 more markedly than did IL-17, which indicates that IL-17 seems to exert a less potent direct regulatory effect on MMP-1 production, but may also function via the induction of other inflammatory cytokines, such as IL-1β and TNF-α. In this study, IL-17 could induce HPDLF to produce MMP-1 via the induction of IL-6. However, the

present results that IL-6/sIL-6R did not increase the *MMP-1* mRNA or protein levels, indicating that IL-6 did not affect the *MMP-1* mRNA expression or MMP-1 production. In addition, IL-1β was not detected after the stimulation with IL-17. These findings indicate that IL-1β has no effect on the MMP-1 production induced by IL-17 in HPDLF. Thus, it can be speculated that IL-17 might exert a direct regulatory effect on MMP-1 production in HPDLF.

Previous reports showed a relationship to exist between IL-17 and mechanical forces. For example, it was suggested that the effects of mechanical forces on the T-cell receptor (TCR) complex could initiate signaling in T cells (25), and TCR signaling could strengthen IL-17 expression (26). Taken together, these data led to the speculation that mechanical force can activate T cells, thus leading to IL-17 production. It was reported that a compressive force induced the expression of *IL-17* mRNA in MC3T3-E1 cells (16). IL-17 was shown to induce the production of bone-resorbing cytokines, such as IL-1β and TNF-α in human monocytes (13). Additionally, IL-17 was detected in the synovial tissues (12) and the PDL (17), which intervene between the hard tissues and play a role in buffering the mechanical forces. In support of this idea, Th17 cells were observed in the PDL tissue of rats subjected to an orthodontic force (17). The previous report showed that there were higher IL-17 levels in the GCF and in the supernatants from cellular cultures of gingival tissue from patients with periodontitis than in those from healthy subjects, thus suggesting a role for IL-17 in the pathogenesis of chronic periodontitis (27). The past and present findings led us to hypothesize that mechanical forces applied to the PDL induce IL-17-producing cells, such as Th17 cells, to produce IL-17. The IL-17 then regulates the degradation of the periodontal tissues under mechanical force, such as that due to orthodontic tooth movement by promoting the production of inflammatory mediators.

Irwin previously reported that the addition of IL-6/sIL-6R resulted in a significant upregulation of MMP-1 expression and collagenolytic activity, while IL-6 alone had no marked effect on MMP-1 expression (28). During IL-6 signaling, IL-6 first binds to the IL-6 receptor (IL-6R), and the complex of IL-6 and IL-6R associates with the signal-transducing membrane protein, gp130 (29, 30). The IL-6/sIL-6R complex associates with the gp130 expressed on the cells without IL-6R expression, such as osteoblasts and fibroblasts, and induces dimerization and initiates signaling (29, 31, 32). In the present study using fibroblasts, IL-6/sIL-6R did not affect the production of MMP-1. This suggests that IL-6 does not have a direct effect on the MMP-1 production. The differences in our results compared with those reported by Irwin (28) may have been caused by the differences in the kinds of cells used.

The present study elucidated that IL-6 enhances the effects of IL-1β on MMP-1 production. It is likely that IL-6 can promote the degradation of the periodontal ligaments via its synergistic effect with IL-1β on MMP-1 production. To the best of our knowledge, this is the first report to show this synergistic effect of IL-6 and IL-1β on MMP-1 production. However, a previous study reported that the addition of IL-6 and IL-1β synergistically increased MMP-3 and MMP-13 production from synovial cells by the inducing IL-1 receptor (IL-1R) as a result of IL-6 signaling (33). In the present study using HPDLF, the synergistic effect of IL-6 and IL-1β could also have been due to the induction of IL-1R by IL-6.

The present study suggests that the IL-17-induced IL-6 expression may enhance the effects of IL-1β on MMP-1 production. However, the IL-1β protein was not detected after the stimulation with IL-17 in HPDLF. IL-6 may therefore enhance the effects of the IL-1β produced by macrophages and osteoblasts in the PDL on the production of MMP-1. The present results suggest that IL-17 induces MMP-1 production not only directly, but also indirectly by promoting IL-6 production, thus resulting in the degradation of collagens in the PDL. IL-17, produced by Th17 cells, may play a role in the production of inflammatory cytokines and MMPs, and the immune cells in the PDL can regulate the production of inflammatory mediators in response to mechanical forces. Further investigations in regard to precisely how IL-17 regulates the

inflammatory mediators in HPDLF are expected to lead to a better understanding of orthodontic tooth movement.

Conclusion

The present study demonstrated that IL-17 sequentially stimulates IL-6 and MMP-1 production in HPDLF. The present study suggests that IL-6/sIL-6R may enhance the effects of IL-1β on MMP-1 production, while IL-6/sIL-6R does not directly affect the production of MMP-1.

Clinical relevance

We hypothesized that mechanical forces applied to the PDL induce IL-17-producing cells, such as Th17 cells, to produce IL-17, and that IL-17 regulates the degradation of the periodontal tissues in response to the tooth movement by promoting the production of these inflammatory mediators. The present study showed that IL-17 sequentially stimulates IL-6 and MMP-1 production in HPDLF and suggested that the IL-17-induced IL-6 expression may enhance the effects of IL-1β on MMP-1 production. Investigations of how IL-17 affects the production of inflammatory mediators will lead to the elucidation of the mechanism underlying the degradation of the PDL and bone resorption during orthodontic tooth movement.

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