IL-6 and IL-17 in the Gingival Crevicular Fluid During Orthodontic Root Resorption

Yoshihiro Yamaguchi, Takashi Nariyasu, Noriko Hayashi, Ryo Nakajima, Shoji Fujita, Masaru Yamaguchi, and Kazutaka Kasai

Department of Orthodontics, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

Correspondence to:
Masaru Yamaguchi
E-mail: yamaguchi.masaru@nihon-u.ac.jp

Abstract

Objectives: Root resorption is an unwanted effect of orthodontic tooth movement. An analysis of the cytokines in gingival crevicular fluid (GCF) is a potentially safer method of quantifying root resorption compared with conventional radiographic methods. This study aimed to quantify the levels of interleukin-6 (IL-6) and 17 (IL-17), released into the GCF during physiological root resorption and orthodontic tooth movement.

Materials and Methods: Subjects with severe root resorption (>1/3 of the original root length) were identified. A control group comprising subjects with no loss of the root structure or undergoing orthodontic treatment was also identified. Gingival crevicular fluid (GCF) was collected non-invasively from the mesial and distal sides of each of the four upper incisors by using filter paper strips. The eluted GCF was used for an enzyme-linked immunosorbent assay (ELISA) and a Western blot analysis. The antibodies used were specific for IL-6 and IL-17.

Results: The ELISA results with IL-6 and IL-17 antibodies showed statistically significant differences between the control group and the root resorption group. The Western blot analysis showed differential expression of IL-6 and IL-17 in the control and root resorption subjects.

Conclusions: These results confirm the presence of cytokines in the GCF of root resorbed subjects. The results highlight the potential for measuring IL-6 and IL-17 in the GCF as a biomarker to monitor root resorption.

Keywords: orthodontic tooth movement, root resorption, gingival crevicular fluid, IL-6, IL-17

Introduction

Orthodontically induced inflammatory root resorption (OIIRR) is an unavoidable pathological consequence of orthodontic tooth movement. It can be defined as an iatrogenic disorder that occurs, unpredictably, after orthodontic treatment, wherein the resorbed apical root portion is replaced with normal bone. The causes are reported to vary, and include the use of heavy force (1), the length of treatment, the type of root (2), genetic predisposition (3), and so on. However, the definite cause of resorption haves not yet been identified.

Many researchers demonstrated that inflammatory cytokines were increased in rats by the movement of molars. Alhashimi et al. (4) reported that induction of interleukin (IL)-1β and IL-6 were observed to reach a maximum on day 3 after the application of force. Bletsas et al. (5) reported that increased expression of IL-1α and tumor necrosis factor-alpha (TNF-α) was observed after 1 and 3 days of tooth movement. Yamaguchi (6) reported in vivo studies that showed the presence of the receptor activator of nuclear factor-kappa (RANK), receptor activator of nuclear factor-kappa ligand (RANKL), and osteoprotegerin (OPG) in the periodontal tissues during experimental tooth movement.

The GCF was first utilized by periodontists attempting to develop a diagnostic test for per-
iodontal diseases. This fluid is an osmotically-mediated transudate. The aqueous component is derived mainly from the serum; the constituents are derived from the serum, the gingival tissues through which the fluid passes, and the bacteria in the tissue and in the crevice (7). GCF was chosen because of its ready accessibility and because its collection poses minimal risk of harm to the patients. Orthodontic forces induce the movement of periodontal ligament fluids, and with them, any cellular and biochemical products produced from prior mechanical perturbation. During the course of orthodontic treatment, the forces exerted produce a distortion of the periodontal ligament extracellular matrix, resulting in alterations in the cellular shape and cytoskeletal configuration. Such events lead to the synthesis and presence of extracellular matrix components, tissue degrading enzymes, acids and inflammatory mediators in the deeper periodontal tissues, which induce cellular proliferation and differentiation and promote wound healing and tissue remodeling (8). The levels of inflammatory cytokines, such as IL-1 beta, IL-6 and RANKL are elevated in gingival crevicular fluid during human orthodontic tooth movement (9-11).

IL-17, initially referred to as cytotoxic T lymphocyte-associated antigen-8, is an inflammatory cytokine that is produced exclusively by activated T cells (Th17 cells) (12). IL-17 has been shown to be an important mediator of autoimmune diseases, including rheumatoid arthritis (RA) (13), multiple sclerosis (14, 15) and allergic airway inflammation (16). Recently, IL-17 has been reported to induce osteoclastogenesis directly from monocytes alone (17). In addition, IL-17 induces RANKL production by osteoblasts, and was shown to be related to bone destruction in periodontitis (13, 18). Moreover, it has been shown that compressive force stimulates the expression of the IL-17 genes and their receptors in MC3T3-E1 cells, and also results in the induction of osteoclastogenesis (19). Therefore, IL-17 may positively contribute to alveolar bone remodeling during orthodontic tooth movement. However, little is known about the relationship between OIIRR and IL-17.

The purpose of this study was to determine the levels of IL-6 and IL-17 in the gingival crevicular fluid (GCF) of patients who showed radiographic evidence of root resorption.

Materials and Methods

Experimental subjects

Twenty subjects were selected from patients seeking treatment in the Department of Orthodontics at the Nihon University School of Dentistry at Matsudo. Two groups were set up, a control and a root resorption group. The control group included fifteen subjects (aged 25-34 years) who had been receiving treatment for at least 2.0 years with fixed appliances, and who had radiographic signs and had no radiographic evidence of root resorption. The root resorption group included five subjects (aged 27-32 years) who had been receiving treatment for at least 2.0 years with fixed appliances, and who had radiographic signs of severe root resorption of more than 1/3 of the original root length. Informed consent was obtained from each patient, and the project was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo (EC 10-019).

All subjects were in good general health with healthy periodontal tissues before the orthodontic treatment; the probing depths were ≤3 mm, and there was no radiographic evidence of periodontal bone loss. Subjects were excluded if they had had received antibiotic therapy during the treatment or if they had taken anti-inflammatory medication during the month preceding the start of the study.

GCF collection

Using the method described by Hoshino-Itoh et al. (20) GCF was collected from both the resorption and control groups. The GCF was collected from the interproximal surfaces of the upper and lower teeth by using filter paper strips (Peripaper, Oralfow, Smithtown, NY, USA) inserted 1-2 mm into the gingival sulcus for 60 seconds (s). After 1 minute (min) a second collection was performed. Care was taken to avoid mechanical injury to the
soft tissue. The contents were eluted into 1 × phosphate buffer saline (PBS) containing a protease inhibitor (0.1 mM phenylmethylsulphonyl fluoride) and were stored at −80 °C until further analysis. The volume of GCF on the paper strip was measured with a Periotron 8000 (Harco, Tustin, CA, USA) that had been calibrated with human serum. GCF collection was standardized so that the experimental and control sites and different subjects could be compared. After collection, the paper strips were stored at −30 °C.

For evaluation, the paper strips were placed individually in 100 μl of Tris buffer (12 mM Tris, containing 0.1 M NaCl and 0.05 per cent Tween 20) and then subjected to vortexing 3 times over a 30 min period. The strip was then removed and the eluate was centrifuged for 5 min at 3000 × g. The supernatants were separated and frozen at −30 °C for later use. The protein concentration in the extract was estimated by the Bradford (21) method using bovine serum albumin as a standard. The samples that total proteins were did not reach 134 μg/ml were removed previously by the Bradford method.

Enzyme-linked immunosorbent assay (ELISA)

The IL-6 and IL-17 levels in the GCF samples were determined by ELISA. A human IL-6 ELISA kit (R&D systems Co., MN, USA) and a human IL-17 ELISA kit (Gen-Probe, Besancon, France) were used according to the manufacturer’s guidelines. The minimum significant detection levels of the assay were 0.039 pg/ml for IL-6 and 3.0 pg/ml for IL-17.

Western blotting analysis

The IL-6 and IL-17 levels in the GCF samples were determined by a Western blotting analysis. The protein content of the samples was measured using the Bradford reagent (BIO-RAD, Tokyo, Japan) according to the manufacturer's protocol. The samples were boiled for 3 min with sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 3.3% SDS, 30% glycerol, 5% β-mercaptoethanol and 0.001% bromophenol blue) and the protein (10 μg) samples were then resolved by 10% SDS–polyacrylamide gel electrophoresis (PAGE) at 150 V for 1 hour (h). The proteins were electrotransferred from SDS gels onto an Amersham Hybond ECL membrane (GE Healthcare UK Ltd Amersham Place, Little Chalfont, Buckinghamshire, UK) for the immunoblot analyses. The blocking of nonspecific antigen–binding sites was performed with 5% nonfat dry milk in 150 mM NaCl, 50 mM Tris, pH 7.2, and 0.05% Tween 20 (TBST) buffer (Sigma Chemical Co., St. Louis, MO, USA). The membrane was incubated for 24 h with the anti–IL-6 rabbit monoclonal antibodies (Epitomics, Inc, Burlingame, CA, USA) diluted 1: 10,000 and anti–IL-17 mouse monoclonal antibodies diluted 1: 500 in 5% nonfat dry milk–TBST. Subsequently, the blots were incubated for 2 h with goat anti-rabbit IgG (H+L)-HRP conjugate (MP Biochemicals, LLC, Illkirch, France) diluted 1: 10000 and goat anti-mouse IgG (H+L)-HRP conjugate (BIO-RAD) diluted 1: 2500 in 5% nonfat dry milk–TBST, and then they were developed using an ECL system (GE Healthcare Limited).

Statistical methods

The values presented represent the means ± SD for each group. Mann–Whitney U-test was used to evaluate the statistical significant of differences between each pair of groups. A value of p < 0.01 was considered to indicate a significant difference.

Results

Clinical parameters

In all patients, plaque accumulation was minimal throughout the study, and the subjects’ gingival health was excellent. Furthermore, the probing depths remained less than 3 mm at all times throughout the experimental period, and there was no gingival bleeding on probing.

The mean volumes of GCF taken from paper strips were compared. There was no significant differences in the mean volumes of GCF between the root resorption group (Mean : 0.41 μl ±0.05 μl) and the non-root resorption group (Mean : 043 ±0.05 μl).
Fig. 1. The ELISAs determined the concentrations (pg/ml) of (A) IL-6 and (B) IL-17 in the GCF of controls and subjects with severe root resorption. The data are expressed as the means±SEM. *p<0.01, significantly different from the control (Mann-Whitney U test).

**ELISA**

The results from the ELISA performed with the IL-6 and IL-17 antibodies revealed a difference in the IL-6 and IL-17 concentrations between the control and the root resorption groups. The ELISA with the IL-6 antibody showed a significantly higher concentration in the resorption group than in the control group (Fig. 1A). The IL-17 concentration in the root resorption group was also significantly higher than that in the control group. The control group also showed a lower amount of protein in the GCF compared to the resorption group (Fig. 1B).

**Western blot analysis**

A Western blot analysis was performed to detect the presence of IL-6 and IL-17 in the control and resorption groups. Immunoblotting against IL-6 detected its expression in the samples from both groups. However, the intensity of the band in the resorption group was higher than that of the control group (Fig. 2). Immunoblotting against IL-17 also demonstrated that it was present at a higher level in the resorption group. The control subjects had less intense bands than the resorption cases (Fig. 2).

**Discussion**

During the process of root resorption, organic matrix proteins and cytokines are released into the gingival crevice. The objective of this study was to determine whether cytokines such as IL-6 and IL-17 could be used as biological markers for root resorption related to orthodontic treatment. The results from this study demonstrated that differences exist between the levels of these proteins in the GCF of subjects with severe root resorption confirmed by radiographs and subjects with no evidence of root...

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**Sample number**

1 2 3 4 5 6 7 8 9 10

**Western blot analysis**

(A) IL-6

(B) IL-17
resorption.

The results of this study demonstrated that the concentrations of IL-6 and IL-17 in the GCF were significantly higher in subjects with severe root resorption than those in the controls (Fig. 1). The results of the Western blot analysis of IL-6 and IL-17 showed that the levels of both cytokines were higher in the subjects with root resorption than those of the controls (Fig. 2).

IL-6 is a multifunctional cytokine, which plays an important role in osteoclastic bone resorption (22). Kurihara et al. (23) demonstrated that IL-6 stimulated the formation of early osteoclast precursors. Moreover, Adebanjo et al. (24) reported that IL-6 activates mature osteoclasts. The total amount of IL-6 in GCF samples is significantly increased in patients with chronic periodontal disease (25,26). Gastel et al. (27) reported that the IL-6 and IL-8 concentrations before orthodontic treatment were significant predictive factors for some potential inflammatory parameters during treatment. Zhang et al. (19) reported that compressive force induces the expression of IL-17 and its receptor in osteoblast–like cells, and that the IL-17 and IL-17R produced in response to compressive force may affect osteoclastogenesis. In another study, Zhang et al. (28) demonstrated that IL-17 induces the differentiation and function of osteoclasts via prostaglandin E2 (PGE$_2$) in osteoblasts. The total amount of IL-17 in GCF samples and in the culture supernatants of gingival cells are significantly increased in cases of periodontal disease. Kotake et al. (13) reported that IL-17 upregulates the RANKL on hOCPs and induce osteoclast differentiation and bone loss. Moreover, Yago et al. (17) demonstrated that IL-17 has been reported to induce osteoclastogenesis directly from monocytes alone. In addition, Liu et al. (29,30) reported that the PGE$_2$ and IL-6 signaling pathways stimulated osteoclastogenesis and Suzuki et al. (31) IL-6/sIL-6R directly induced RANKL expression in fibroblast–like synoviocytes. Furthermore, we demonstrated that IL-17 and IL-6 mediated osteoclastogenesis induced by orthodontic forces (32). Taken together, these findings and our present results suggest that increased IL-6 and IL-17 secretion in the GCF may activate odonto/osteoclastogenesis. Therefore, IL-6 and IL-17 may stimulate the differentiation of odontoclasts from osteoclast progenitor cells.

IL-17 is produced predominantly by activated Th17 cells (12). In addition, IL-17 can also be produced by other immune cells such as neutrophils and eosinophils (16,33). Further, we demonstrated that the increases of Th17 and IL-17 in the periodontal ligament by orthodontic force (32). The GCF component is derived mainly from the serum; the constituents are derived from the serum, the gingival tissues through which the fluid passes, and the bacteria in the tissue and in the crevice (7). Therefore, it is considered that IL-17 is derived from Th17 cells in capillaries and other immune cells in around periodontal tissues.

A recent study demonstrated that the concentrations of RANKL in the GCF were significantly higher in subjects with mild and severe root resorption than in controls (34). The RANK/RANKL system has been suggested to play an integral role in osteoclast activation during orthodontic tooth movement (6). For example, Shiotani et al. (35) observed RANKL in osteoblasts, osteocytes, fibroblasts, and osteoclasts during the application of orthodontic forces. The RANK/RANKL system may also regulate the natural process of root resorption in exfoliated primary teeth (36). Nakano et al. (37) demonstrated that the immunoreactivities for RANK/RANKL in root resorption lacunae in the rat molar were detected in odontoclasts exposed to an orthodontic force of 50 g on days 7 and 10, and that RANK/ RANKL may be involved in the process of root resorption resulting from the application of excessive orthodontic force.

Although no studies have demonstrated a clear relationship among IL-6, IL-17, and RANKL in OIIRR, previous studies reported that IL-17 stimulates IL-6 production in human epithelial cells, endothelial fibroblasts and RA synovial fibroblasts (38,39). Takahashi et al. (40) reported that IL-17 induced IL-6 production from human gingival fibroblasts
HGF) in a dose- and time-dependent fashion. Kar- 
makar et al. (41) concluded that the RANKL/
RANK/OPG pathway plays a critical role in regu-
late osteoclastogenesis in rheumatoid arthritis, and
that pro-inflammatory cytokines, including TNF-α,
IL-1, IL-6 and IL-17, are expressed by various cell
types in the inflamed synovium and exert effects on
osteoclastogenesis as well as on osteoclast function
and survival. Furthermore, Adamopoulos et al. (42)
reported that IL-17 upregulates the RANKL expres-
sion on hOCPs in vitro, leading to increased sensi-
tivity to RANKL signaling, osteoclast differentiation,
and bone loss. In addition, Liu et al. (29, 30) reported
that the PGE2 and IL-6 signaling pathways stimu-
lated osteoclastogenesis via their effects on the RANK/
RANKL/OPG system. Therefore, IL-6 and IL-17
may stimulate osteo/odontoclastogenesis via the
RANK/RANKL/OPG system, and this induction
may contribute to the inflammatory response asso-
ciated with the ensuing OIIRR. Further studies are
necessary to investigate the relationships among IL-
6, IL-17, and the RANK/RANKL/OPG system dur-
ing root resorption, including an increased number of
subjects for statistical analysis.

In the present study, immunoreactivities for IL-6
and IL-17 were detected in the GCF of patients
affected by severe root resorption. Therefore, these
factors may be significant predictive factors for
some potential inflammatory parameters during
orthodontic treatment.

Conclusions

In the present study, immunoreactivities for IL-6
and IL-17 were detected in the GCF of subjects with
severe root resorption. Collectively, the results of
this study demonstrate the potential of IL-6 and IL-
17 as analytical biomarkers for root resorption.
Therefore, these factors may be significant predic-
tive factors for some potential inflammatory param-
eters during orthodontic treatment.

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