Interleukin-1β and Tumor Necrosis Factor-α Synergistically Induce Expression of Colony Stimulating Factors in Synovial Fibroblasts from the Human Temporomandibular Joint

Suguru Watanabe,1 Naomi Ogura,2, 3 Miwa Akutsu,2 Mutsumi Kawashima,2, 3 Toshio Hattori,2 Teruo Yano,1 Ko Ito,2, 3 and Toshirou Kondoh2, 3

1Nihon University Graduate School of Dentistry at Matsudo, Maxillofacial Surgery, Matsudo, Chiba 271–8587, Japan
2Department of Maxillofacial Surgery, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271–8587, Japan
3Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271–8587, Japan

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Abstract
IL-1β and TNF-α are proinflammatory cytokines that affect inflammatory responses and matrix degradation. Although interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) have been detected in the synovial fluids from patients with temporomandibular joint disorders (TMD), there is little known about the molecular mechanisms of inflammatory conditions of TMD. To identify putative factors associated with temporomandibular joint (TMJ) inflammation, we investigated IL-1β and/or TNF-α-responsive genes of synovial fibroblasts from patients with TMD using microarray analysis. Granulocyte macrophage colony stimulating factor (GM-CSF) was one of the genes whose expression was strongly up-regulated in synovial fibroblasts by IL-1β and/or TNF-α. The gene expressions of macrophage colony stimulating factor (M-CSF) and Granulocyte colony stimulating factor (G-CSF) were also up-regulated by IL-1β and/or TNF-α. Gene expression and protein production of GM-CSF and G-CSF, but not of M-CSF, were synergistically increased in synovial fibroblasts stimulated with IL-1β and TNF-α. M-CSF protein was only detected in the conditioned medium of the non-stimulated control in which GM-CSF and G-CSF were not detected. In addition, MAPK and NFκB inhibitors inhibited IL-1β and TNF-α stimulated production of GM-CSF and M-CSF. CSFs act on hemopoietic cells as growth factors and activation/differentiation factors. These results suggest that expression of CSFs in synovial fibroblasts stimulated by IL-1β and/or TNF-α is one factor associated with inflammatory progression of the intracapsular pathological conditions of the TMJ.

Keywords:
interleukin-1β,
tumor necrosis factor-α,
colony stimulating factor,
temporomandibular joint

Introduction
The temporomandibular joint (TMJ) is the articulation that is formed between the mandibular condyle and the temporal bone, and it plays an important role in functions such as the jaw motion that is involved in speaking, chewing, and swallowing. Temporomandibular joint disorders (TMD), which are defined as a subgroup of craniofacial pain problems that involve the TMJ, masticatory muscles and associated head and neck musculoskeletal structures, are characterized by limited mandibular motion, joint noise, and arthralgia. The recent insights obtained from arthroscopic (1, 2) and magnetic resonance imaging findings (3), histological studies (4) and synovial fluid analysis (5) may contribute to understanding the intracapsular pathological conditions of the TMJ, such as internal derangement (ID), disc displacement (DD), fibrous adhesions, synovial inflammation (synovitis) and osteoarthritis (OA). These entities are not mutually exclusive and may coexist (1–5).

Synovitis, an inflammatory disorder of the synovial membrane, frequently accompanies ID and/or OA in the TMJ (6) and has been suggested to be a key feature of intracapsular pathological conditions of the TMJ (7). The synovial membrane lines all of the intra-articular structures,
except for the articular cartilage of the eminence, fossa and mandibular condyle, and the articular disc (3). The lining layer of synovial tissue is composed of fibroblast-like and macrophage-like cells and overlies loose connective tissue of the synovial sublining that contains blood vessel sublining fibroblasts and leukocytes. Synovial fibroblasts that are producing a number of putative mediators of inflammation and tissue degradation (4,5,8) and other immune cells communicate with one another in a unique inflammatory microenvironment (4). An understanding of the molecular mechanisms that underlie the activities of these factors may contribute to an understanding of the pathogenesis of TMD; however, little is known about the molecular mechanisms that underlie the development of the pathologic condition in the TMJ(9).

Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are proinflammatory cytokines that affect cell proliferation, inflammatory responses and matrix remodeling (10, 11). Elevated expression of IL-1β and TNF-α in the joint is known to result in activation of inflammatory and degradative pathways in synovial cells, which in turn contribute to the progression of rheumatoid arthritis (RA) (11, 12). In previous studies, levels of IL-1β and TNF-α were also found to be elevated in synovial fluid obtained from TMD patients with OA and ID (5, 13). Our previous study demonstrated that IL-1β or TNF-α induced the gene expression and protein production of many inflammatory mediators such as chemokines and cytokines in synovial fibroblasts from patients with OA and/or ID of the TMJ (14).

Here, we performed an oligonucleotide microarray analysis of human synovial fibroblasts treated with IL-1β and/or TNF-α. The genes encoding granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) were found to be highly upregulated in IL-1β and/or TNF-α-treated synovial fibroblasts. The colony-stimulating factor (CSF) family includes CSF-1/M-CSF, CSF-2/GM-CSF, and CSF-3/ granulocyte colony-stimulating factor (G-CSF). CSFs were originally defined in vitro as hemopoietic growth factors (15), but have since been shown to act on mature myeloid populations as pro-survival and/or activation/differentiation factors (16). Additionally, CSFs are also reported to have many pro-inflammatory functions and function as key factors in inflammatory conditions of joints such as RA (17–19). We also investigated the effects of stimulation of pro-inflammatory cytokines such as IL-1β and/or TNF-α on the gene expression and production of CSFs in synovial fibroblasts from TMJ patients.

**Materials and Methods**

**Cell culture and ethics statement**

Human synovial tissue was obtained from three female patients with ID who underwent arthroscopy of the TMJ (age range: 18–26 years). Patient characteristics and symptoms are summarized in Table 1. All patients provided written informed consent for the surgery and for the use of their tissue specimens for research purposes. The isolation, primary culture, and experimentation with synovial fibroblasts were performed according to the guidelines established by the Institutional Review Board of the Nihon University School of Dentistry at Matsudo (Ethics Committee Registration Number: EC10-037).

Human synovial fibroblasts were isolated from the synovial tissues of these patients using the out-growth method of Ogura et al (20). The culture medium used was Ham’s F12 (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Technologies, Gravessano, TI, Switzerland), 100 μg/mL penicillin G (Meiji, Tokyo, Japan), 100 μg/mL kanamycin sulfate (Meiji), and 250 ng/mL Fungizone (Gibco, Grand Island, NY, USA). The medium was changed twice per week. For all experiments, passage 6 to 10 synovial fibroblasts were used.

**Total RNA extraction**

Confluent-stage synovial fibroblasts were cultured for 24 h in 2% FBS-containing medium and were then not treated (control) or treated with 0.1 ng/ml IL-1β (Pepro Tech, Rocky Hill, NJ, USA), 10 ng/ml TNF-α (Pepro Tech) or 0.1 ng/ml IL-1β + 10 ng/ml TNF-α for 2, 4, or 8 h. Harvested cells were homogenized with 1 ml TRIZOL reagent (Thermo Fisher Scientific, MA, USA). Total RNA was extracted using the Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction (AGPC) method (21) and was stored in ethanol at -80°C until use.

**DNA microarray analysis**

Total RNA samples were run on an RNA 6000 Nano Gel System (Agilent Technologies Inc., Santa Clara, CA, USA) using the Agilent 2100 Bioanalyzer (Agilent) for RNA quality determination. Total RNA samples were profiled on a Human Genome Focus Array (Affymetrix, Santa Clara,
CA, USA) according to the manufacturer’s instructions.

Raw data from nine GeneChips were loaded into the GeneSpring GX13 software program (version 13; Agilent Technologies). The data were normalized using the median of raw data from each array as a reference, and were then analyzed. Changes in gene expression were determined by comparing controls with IL-1β and/or TNF-α treatment (average 3 samples).

**Real-time polymerase chain reaction (PCR)**

cDNA was synthesized using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with a DyNAmo SYBR Green qPCR kit (Thermo Fisher Scientific). The PCR mixture contained 20 pmol forward and reverse primers and 2 μl cDNA. Amplification was performed using the DNA Engine Opticon 1 (Bio-Rad, Hercules, CA, USA), with preheating at 95 °C for 10 min, then 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Amplicons were directly detected by measuring the increase in fluorescence caused by SYBR Green binding, using the DNA Engine Opticon 1. Table 2 lists the primer sequences used for this analysis. Analyzed genes were examined for their relative expression to their respective control by using the ΔΔCT method (22). All analyses were performed in three replicates. The results were confirmed by three independent experiments.

**Enzyme-linked immunosorbent assay (ELISA)**

Synovial fibroblasts were plated in 24-well plates (5 × 10^4 cells per well) with Ham’s F12 medium containing 10% FBS. Confluent cells were cultured for 24 h in the same medium containing 2% FBS. After treatment with the appropriate dose of IL-1β and/or TNF-α for the appropriate times, the culture supernatants were collected and stored at -80 °C until use. M-CSF, GM-CSF, and G-CSF levels in the conditioned medium were measured using an ELISA kit (R&D Systems, McKinley, MN, USA), according to the manufacturer’s protocol.

**Inhibition of ERK, p38, JNK, and NFkB**

Synovial fibroblasts were plated in 24-well plates (5 × 10^4 cells per well) with Ham’s F12 medium containing 10% FBS. Confluent cells were cultured for 24 h in medium containing 2% FBS. The cells were not pretreated (control) or were pretreated with PD98059 (ERK1/2 inhibitor, 40 μM) (Alexis Biochemicals, San Diego, CA, USA), SB203580 (p38 inhibitor, 10 μM) (Alexis Biochemicals), SP600125 (JNK1/2 inhibitor, 10 μM) (Biomol, Plymouth Meeting, PA, USA), or ammonium pyrrolidine dithiocarbamate (APDC) (NFκB inhibitor, 10 μM) (Calbiochem, Biochemicals, San Diego, CA, USA) for 15 min, followed by incubation with IL-1β (0.1 ng/ml) or TNF-α (10 ng/ml). After 3 h or 8 h, the culture supernatants were collected and stored at -80 °C until use.

**Statistical analysis**

Data are expressed as mean values ± standard deviations (SD). The statistical significance for multiple comparisons was assessed using one-way ANOVA. Post hoc analyses were carried out using the Student-Newman-Keuls (SNK) Multiple Comparison Test. Statistical significance is indicated in the graphs as P-values.

**Results**

**Gene expression profiling**

Of the 8133 genes on the DNA microarray, 5737 genes
were expressed in synovial fibroblasts. The expression of these genes was compared between non-treated control cells and IL-1β and/or TNF-α-treated cells. Regulated genes were defined as genes that displayed greater than 2-fold changes in expression between non-treated control and treated cells. IL-1β treatment regulated 564 genes, upregulating 283 genes and downregulating 281 genes. TNF-α treatment regulated 576 genes, upregulating 315 genes and downregulating 261 genes. IL-1β + TNF-α co-stimulation regulated 670 genes, upregulating 398 genes and downregulating 272 genes. There was a lot of overlap in the genes that were upregulated with IL-1β, TNF-α, and co-stimulation with IL-1β+TNF-α but there was less overlap in the downregulated genes (Fig. 1). Table 3 lists the top 35 upregulated genes in each stimulation group. The gene that was most strongly upregulated by all of the stimulators was CCL20 (MIP-3α), and a number of chemokine superfamily members were found in the top 35 upregulated genes, consistent with our previous reports (13).

GM-CSF was ranked number 3 in IL-1β or TNF-α stimulation, and was ranked number 2 in IL-1β+TNF-α co-stimulation. M-CSF was also in the top 35 upregulated genes by each stimulator being ranked 27 in IL-1β stimulation, 22 in TNF-α stimulation, and 34 in IL-1β + TNF-α co-stimulation. G-CSF was ranked 28 in IL-1β + TNF-α co-stimulation, but was not in the top 35 genes upregulated by IL-1β or TNF-α.

**Time course of CSF gene expression**

CSF gene expression was upregulated by IL-1β and/or TNF-α stimulation for 4 h using DNA microarray analysis. We next examined the time course of CSF gene expression in treated synovial fibroblasts by using real-time PCR. The levels of all of the CSFs gene expression were upregulated to the highest level by IL-1β+TNF-α co-stimulation, followed by IL-1β, and then TNF-α stimulation. GM-CSF and G-CSF gene expression increased in a time-dependent manner (Fig. 2(a) and (c)). In contrast, although M-CSF gene expression
increased over the first 4 h, it then decreased over the next 4 h (Fig. 2(b)). After 8 h co-stimulation with IL-1β+TNF-α there was a huge increase in G-CSF expression in the treated cells compared to control. The fold increase in the level of M-CSF expression following 8 h co-stimulation with IL-1β+TNF-α was less than the fold increase in GM-CSF or in G-CSF. Furthermore, co-stimulation with IL-1β+TNF-α had a synergistic effect on the expression of GM-CSF and G-CSF, whereas it had an additive effect on M-CSF expression.

**CSF protein production**

We first examined the time course of CSF protein levels in the conditioned media from synovial fibroblasts treated with IL-1β, TNF-α, or IL-1β+TNF-α co-stimulation. GM-CSF and G-CSF protein levels were increased by all treatments in a time dependent manner (Fig. 3 (a) and (c)). G-CSF production was strongly increased at the later culture times (Fig. 3 (c)). The highest increases in GM-CSF and G-CSF protein levels were seen with IL-1β+TNF-α co-stimulation, followed by IL-1β, and then TNF-α. In contrast, the M-CSF protein level was similar for all treatments, and the level plateaued from 8 to 48 h during treatment with IL-1β and IL-1β+TNF-α co-stimulation (Fig. 3 (b)). For all CSFs, the patterns of protein production appeared to reflect the patterns of gene expression.

We next examined CSF protein production in response to various IL-1β and TNF-α doses. Synovial fibroblasts were incubated with concentrations of IL-1β ranging from 0.01 to 1 ng/ml or TNF-α ranging from 1 to 100 ng/ml for 24 h. CSF protein production occurred in an IL-1β or TNF-α-concentration dependent manner (Fig. 4). GM-CSF and G-CSF protein production was higher with IL-1β than with TNF-α-stimulation. M-CSF was the only CSF that was detected at the protein level in the conditioned medium of the non-stimulated control (Fig. 4 (c) and (d)). GM-CSF and G-CSF protein levels in this conditioned medium were below the ELISA detection levels (Fig. 4(a), (b), (e) and (f)).

**Effect of signaling inhibitors on M-CSF, GM-CSF, and G-CSF production**

To investigate the IL-1β or TNF-α signaling pathway involved in CSF production in synovial fibroblasts, we examined the effects of the MAPK family inhibitors, PD98059 (ERK1/2), SB203580 (p38), and SP600125 (JNK), and the NFκB inhibitor, APDC. Synovial fibroblasts pre-treated with these inhibitors for 15 min were stimulated with IL-1β or TNF-α for 3 h or 8 h. GM-CSF production was inhibited by PD98059, SB203580, SP600125, and APDC in synovial fibroblasts stimulated with IL-1β for 3 h, whereas it was inhibited by PD98059, SB203580, and APDC in the cells stimulated with TNF-α for 3 h (Fig. 5 (a) and (b)). M-CSF and G-CSF protein levels at 3 h were below the detection limits of the ELISA kit (data not shown). However, M-CSF production could be detected by ELISA after 8 h treatment, and M-CSF production was inhibited by PD98059, SP600125, and APDC treatment in both IL-1β and TNF-α 8 h-stimulated cells (Fig. 5 (c) and (d)). The effects of inhibitors on G-CSF could not be determined because the protein level of G-CSF after 8 h stimulation was again below the ELISA kit detection limit.
Discussion

Involvement of synovial cells in pathological conditions of the TMJ is poorly understood, because it is difficult to obtain synovial fibroblasts from the TMJ in sufficient quantities in order to study TMJ disorders. Thus, the TMJ is a small joint compared to the knee in humans. Furthermore, healthy control samples cannot be obtained to accord with medical ethics. In addition, no animal models exist that are completely accepted for investigation of TMJ disease. Cultured synovial fibroblasts from the knee have been extensively investigated in orthopedic studies that researched the mechanisms of the inflammatory response, immune reactions, and tissue degradation (11, 12, 17, 23). A cell culture method may therefore be useful for studying the responses and regulation of synovial cells that underlie the development of local pain, microcirculatory disturbances and tissue destruction in the TMJ. In this study, we isolated synovial fibroblasts from several patients with ID and/or OA of the TMJ and examined their gene expression profiles.

We found that GM-CSF and M-CSF were ranked high in the list of genes that were upregulated in synovial fibroblasts by IL-1β and/or TNF-α in microarray analysis (Table 3). Inflammatory cells such as macrophages and granulocytes, which are abundant in synovial tissue, are key cells in the physiopathology of not only OA and/or RA but also of ID and/or OA in the TMJ (24). CSFs have been shown to be present at elevated levels in the synovial fluid of RA patients and thus local proliferation of monocyte/macrophages within an inflamed lesion may contribute to the local tissue hyperplasia that is evident in inflammatory conditions.
CSFs can generate in vitro colonies of mature myeloid cells from bone-marrow precursor cells following the proliferation and differentiation of these cells (15). These CSFs can also act in vitro on mature myeloid cells and therefore might have broader roles in an immune response than acting simply as hematopoietic-cell growth factors (16). It has been suggested that such a ‘CSF network’ might explain the chronic nature of certain inflammatory lesions such as rheumatoid synovial joints, which is due to the increased CSF-dependent survival of inflammatory macrophages or granulocytes, leading to the increased number of these cells in the lesion.

Recently, an antibody against the GM-CSF receptor ‘mavrilimumab’ has shown clinical benefit in patients with rheumatoid arthritis (25). GM-CSF has also been detected in synovial fluids from TMJ with ID and/or OA (26), however, not much is known regarding the roles of CSFs in the pathiology of TMJ. We therefore investigated the gene expression and production of CSFs in TMJ synovial fibroblasts stimulated with IL-1β and/or TNF-α.

The increase in M-CSF gene expression and protein production following IL-1β + TNF-α co-stimulation for 8 h was less than that in GM-CSF and G-CSF. We also showed that M-CSF, but not GM-CSF or G-CSF, was expressed and produced in non-treated synovial fibroblasts (Figs. 2, 3 and 4). M-CSF is known to be constitutively and ubiquitously expressed, and data from Csf1op/Csf1op mice (which contain an inactivating mutation in the M-CSF gene) suggest that M-CSF is required for maintenance of certain tissue macrophage populations at the steady state (27). In our
Table 3. Top 35 upregulated genes by all stimuli

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study, M-CSF was also ubiquitously expressed in synovial fibroblasts from TMJ, and may be associated with maintenance of macrophage like cells in the synovial lining layer. This possibility awaits further investigation. However, M-CSF is also one of the crucial factors for osteoclast maturation (24). Therefore, the IL-1β and TNF-α induced upregulation of M-CSF production in synovial fibroblasts may induce osteoclast formation and bone resorption in the TMJ region.

The gene expression and protein production of GM-CSF and G-CSF, but not those of M-CSF, were synergistically increased in synovial fibroblasts treated with IL-1β and TNF-α. A previous study showed that IL-1α and TNF-α act in a synergistic manner to stimulate G-CSF and GM-CSF production by a clonally derived strain of bone marrow stromal cells (28). These results raise the possibility that IL-1α cross-induction of TNF receptors may contribute to the biochemical mechanisms underlying the synergistic stimulation of G-CSF and GM-CSF production by IL-1α and TNF-α. In our microarray analysis, IL-1β induced type II TNF receptor expression in synovial fibroblasts although type I TNF receptor was expressed constitutively (data not shown). Therefore, IL-1β cross-induction of type II TNF receptor may be one mechanism underlying the synergistic stimulation of G-CSF and GM-CSF production by IL-1β and TNF-α in synovial fibroblasts from TMJ. However, there was no synergistic effect of IL-1β and TNF-α on the expression and production of M-CSF in synovial fibroblasts. Synergistic production of cytokines by stimulators has been suggested to be associated with cytokine mRNA stability. Previous studies have shown that IL-1α promoted the stability of a subset of mRNAs that were unstable when transcriptionally induced by TNF-α (29). Stabilization of GM-CSF mRNA by IL-1α required ongoing protein synthesis, and depended on the activation of p38 MAPK. In addition, RNA-binding proteins such as HuR and tristetraprolin are characterized as binding to the 3’-untranslated regions of target mRNAs, and contribute the stability of mRNA (30). Furthermore, microRNA is also associated with mRNA stability. Therefore, the synergistic stimulation of GM-CSF and G-CSF production by IL-1β and TNF-α may result in an increase in mRNA stability via RNA-binding proteins and/or microRNA, through an as yet uncharacterized mechanism.

NFκB and MAPK pathways play pivotal roles in regulating the expression of inflammatory mediators by IL-1β and TNF-α stimulated synovial fibroblasts. Our data regarding the effect of MAPK inhibitors and an NFκB inhibitor suggest that the induction of GM-CSF production by IL-1β or TNF-α occurs through ERK, p38 MAPK, and NFκB activation in synovial fibroblasts derived from the TMJ (Fig. 5). JNK signaling was associated only with IL-1β in GM-CSF production (Fig. 5). M-CSF production by IL-1β or TNF-α occurs through ERK, JNK, and NFκB activation (Fig. 5). Inhibition of p38 MAPK affected GM-CSF, but not M-CSF, production by IL-1β-stimulation. A previous study showed that stabilization of GM-CSF mRNA by IL-1α depended on p38 MAPK activation (30). M-CSF mRNA, whose production was not synergistically affected by IL-1β and TNF-α in synovial fibroblasts, may not be stabilized by IL-1 in a p38 MAPK activation manner. Inhibition of G-CSF production in synovial fibroblasts could not be examined because the protein levels were under the limit of detection of the ELISA kit. As shown in Fig. 3, G-CSF production was low at early stimulation times such as up to 8 h stimulation, but subsequently its production was dramatically increased. Thus, not only IL-1β or TNF-α but also some other stimuli induced by IL-1β and TNF-α may be necessary for the expression and production of G-CSF.

Recently, macrophage populations have been broadly classified into M1 and M2 polarization states. M1 are classically activated macrophages that produce proinflammatory cytokines such as IL-1β and TNF-α, conversely M2 macrophages have anti-inflammatory functions and promote tissue repair (32). GM-CSF-treated monocytes/macrophages have been likened to M1 polarized macrophages (33). It has been suggested that GM-CSF-dependent M1 macrophage polarization may explain the proinflammatory function of GM-CSF.

G-CSF is less well studied than the other CSFs in the context of inflammation. G-CSF was suggested to be a biomarker of inflammatory arthritis, because G-CSF administration exacerbates collagen-induced arthritis in mice (18). It has been suggested that a central proinflammatory role for G-CSF in the pathogenesis of inflammatory arthritis may be to promote neutrophil trafficking into inflamed joints, in addition to G-CSF-induced neutrophil production (16).

CSFs play an important role in the differentiation of macrophages and in the infiltration of macrophages or neutrophils into inflammatory synovial membranes. In this study, CSFs were produced in synovial fibroblasts from
TMD patients stimulated with IL-1β and/or TNF-α. Additionally, IL-1β and TNF-α synergized to stimulate the gene expression and protein production of GM-CSF and G-CSF in synovial fibroblasts from TMD patients. This study may help to elucidate a novel pathway of cytokine interaction that may explain the apparently redundant roles of IL-1β and/or TNF-α, and may help define new therapeutic targets for the inflammatory components of the TMJ.

References
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