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

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Abstract

IL-1 β and TNF- α are proinflammatory cytokines that affect inflammatory responses and matrix degradation. Although interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) have been detected in the synovial fluids from patients such as 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-1 β 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-1 β 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-1 β 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-1 β 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 NF κ B inhibitors inhibited IL-1 β 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-1 β and/or TNF- α is one factor associated with inflammatory progression of the intracapsular pathological conditions of the TMJ.

Keywords :

interleukin-1 β ,
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 pathologic 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,

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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 pathological condition in the TMJ (9).

Interleukin-1 β (IL-1 β) 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-1 β 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-1 β 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-1 β 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-1 β 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-1 β 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-1 β 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, Gravesano, TI, Switzerland), 100 μ g/mL penicillin G (Meiji, Tokyo, Japan), 100 μ g/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-1 β (Pepro Tech, Rocky Hill, NJ, USA), 10 ng/ml TNF- α (Pepro Tech) or 0.1 ng/ml IL-1 β +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 $^{\circ}$ 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,

Table 1. Characteristics of patients with internal derangement

Patient No	Cell NO	Sex	Age	Diseased Side	ROM	Pain	Wilkes's Staging
1	TMJ1	F	23	L	+	+	II
2	TMJ2	F	18	R	++	+	III
3	TMJ3	F	26	R	++	+	III

F, female; R, right; L, left; ROM, range of motion; +, 30-34mm; ++, 25-29mm.

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-1 β 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 μ l cDNA. Amplification was performed using the DNA Engine Opticon 1 (Bio-Rad, Hercules, CA, USA), with preheating at 95 $^{\circ}$ C for 10 min, then 40 cycles of 94 $^{\circ}$ C for 15 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ 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 $\Delta\Delta C_T$ 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-1 β and/or TNF- α for the appropriate times, the culture supernatants were collected and stored at -80 $^{\circ}$ 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

Table 2. Primers used for PCR analysis of genes

Gene	Primers	Amplicon size(bp)
M-CSF	F : 5'-GCTGATGAGGGGAGACAGAG-3'	344
	R : 5'-CACCATGCGCTTCAGAGATA-3'	
GM-CSF	F : 5'-GTCCTCAGGTTCTCTTGA-3'	206
	R : 5'-CAGAAATGTTTGACCTCCAG-3'	
G-CSF	F : 5'-GGGAGACCTGATACTCTCAA-3'	201
	R : 5'-TTTCATTCTCTGCTGTAG-3'	
GAPDH	F : 5'-ATCGACTGTGGTCATGAG-3'	318
	R : 5'-ATCACCATCTTCCAGGAG-3'	

F, forward; R, reverse; M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte

macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; GAPDH,

glyceraldehyde-3-phosphate dehydrogenase.

manufacturer's protocol.

Inhibition of ERK, p38, JNK, and NF κ B

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 μ M) (Alexis Biochemicals, San Diego, CA, USA), SB203580 (p38 inhibitor, 10 μ M) (Alexis Biochemicals), SP600125 (JNK1/2 inhibitor, 10 μ M) (Biomol, Plymouth Meeting, PA, USA), or ammonium pyrrolidine dithiocarbamate (APDC) (NF κ B inhibitor, 10 μ M) (Calbiochem, Biochemicals, San Diego, CA, USA) for 15 min, followed by incubation with IL-1 β (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 $^{\circ}$ C until use.

Statistical analysis

Data are expressed as mean values $\pm$ 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

Fig. 1. Venn diagram of regulated genes in microarray analysis of synovial fibroblasts stimulated with IL-1 β and/or TNF- α . Gene expression profiles of the cells were compared between non-treated control and stimulation. A Venn diagram of upregulated (a) or down-regulated (b) genes in cells stimulated with IL-1 β , TNF- α , or co-stimulated with IL-1 β and TNF- α is shown.

Fig. 2. Effects of IL-1 β and/or TNF- α on CSF gene expression. Human synovial fibroblasts were treated or not (squares) with 0.1 ng/ml IL-1 β (triangles), 10 ng/ml TNF- α (circles), or 0.1 ng/ml IL-1 β +10 ng/ml TNF- α (diamonds) for 2, 4, or 8 h following which the gene expression of GM-CSF (a), M-CSF (b), or G-CSF (c) was examined using real-time PCR. Fold changes were calculated using the $\Delta\Delta\text{CT}$ method. Mean $\pm$ SD (n = 3). *p<0.05, **p<0.01 compared with the untreated control cells.

were expressed in synovial fibroblasts. The expression of these genes was compared between non-treated control cells and IL-1 β 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-1 β 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-1 β + 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-1 β , TNF- α , and co-stimulation with IL-1 β +TNF- α but there was less overlap in the down-regulated 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-3a), 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-1 β or TNF- α stimulation, and was ranked number 2 in IL-1 β +TNF- α co-stimulation. M-CSF was also in the top 35 upregulated genes by each stimulator being ranked 27 in IL-1 β stimulation, 22 in TNF- α stimulation, and 34 in IL-1 β + TNF- α co-stimulation. G-CSF was ranked 28 in IL-1 β + TNF- α co-stimulation, but was not in the top 35 genes upregulated by IL-1 β or TNF- α .

Time course of CSF gene expression

CSF gene expression was upregulated by IL-1 β 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-1 β +TNF- α co-stimulation, followed by IL-1 β , 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

Fig. 3. Time course of the protein production of CSFs. Human synovial fibroblasts were treated or not (squares) with 0.1 ng/ml IL-1 (triangles), 10 ng/ml TNF- α (circles), or 0.1 ng/ml IL-1 β +10 ng/ml TNF- α (diamonds) for 8, 24, or 48 h following which the protein level of GM-CSF (a), M-CSF (b), and G-CSF (c) in the media was measured using ELISA. Mean $\pm$ SD (n=4). * p <0.05, ** p <0.01 compared with the untreated control cells.

increased over the first 4 h, it then decreased over the next 4 h (Fig. 2 (b)). After 8 h co-stimulation with IL-1 β +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-1 β +TNF- α was less than the fold increase in GM-CSF or in G-CSF. Furthermore, co-stimulation with IL-1 β +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-1 β , TNF- α , or IL-1 β +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-1 β +TNF- α co-stimulation, followed by IL-1 β , 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-1 β and IL-1 β +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-1 β and TNF- α doses. Synovial fibroblasts were incubated with concentrations of IL-1 β 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-1 β or TNF- α concentration dependent manner (Fig. 4). GM-CSF and G-

CSF protein production was higher with IL-1 β 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-1 β 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 NF κ B inhibitor, APDC. Synovial fibroblasts pre-treated with these inhibitors for 15 min were stimulated with IL-1 β 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-1 β 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-1 β 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.

Fig. 4. Effects of IL-1 β and TNF- α concentration on the protein production of CSFs. Human synovial fibroblasts were treated with IL-1 β at concentrations ranging from 0.01 to 1 ng/ml or with TNF- α at concentrations ranging from 1 to 100 ng/ml for 24 h, following which the protein level of GM-CSF ((a) and (b)), M-CSF ((c) and (d)), and G-CSF ((e) and (f)) in the media was measured using ELISA. Mean $\pm$ SD (n=4). * p <0.05, ** p <0.01 compared with the untreated control cells.

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-1 β 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

Fig. 5. Effect of inhibitors of the MAPK and NF κ B signaling pathway on IL-1 β and TNF- α induced CSF production. Human synovial fibroblasts were pretreated with 40 μ M PD98059, 10 μ M SB203580, 10 μ M SP600125 or 10 μ M APDC for 15 min. The cells were then stimulated with 0.1 ng/ml IL-1 β or 10 ng/ml TNF- α for 3 h (a) and (b) or 8 h (c) and (d) following which the protein levels of GM-CSF (a) and (b) and M-CSF (c) and (d) in the media were measured using an ELISA. Mean $\pm$ SD (n=4). * p <0.05, ** p <0.01 in comparison of the stimulated and non-treated cells.

(16). 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 pathology of TMJ. We therefore investigated the gene expression and production of CSFs in TMJ synovial fibroblasts stimulated with IL-1 β and/or TNF- α .

The increase in M-CSF gene expression and protein production following IL-1 β + 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 *Csf1lop/Csf1lop* 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

Rank	IL-1 β			TNF- α			IL-1 β +TNF- α		
	Gene	GenBank ID	Fold	Gene	GenBank ID	Fold	Gene	GenBank ID	Fold
1	CCL20	NM_004591	430.0	CCL20	NM_004591	322.2	CCL20	NM_004591	860.1
2	CXCL3	NM_002090	150.4	IL-8	AF043337	76.7	GM-CSF	M11734	274.9
3	GM-CSF	M11734	107.4	GM-CSF	M11734	38.0	CXCL3	NM_002090	235.1
4	IL-8	AF043337	89.8	ICAM1	NM_000201	32.1	IL8	AF043337	130.6
5	CXCL1	NM_001511	59.5	CXCL3	NM_001565	31.1	BCL2A1	NM_004049	116.6
6	CXCL2	M57731	50.1	CXCL10	NM_001565	27.8	CXCL1	NM_001511	75.3
7	IL-6	NM_000600	40.1	BCL2A1	NM_004049	24.5	CXCL2	M57731	70.0
8	PTGS2	NM_000963	37.8	GCHI	NM_000161	21.9	PTGS2	NM_000963	63.7
9	BCL2A1	NM_004049	37.3	IL17RB	NM_018725	21.9	CXCL10	NM_001565	55.9
10	CXCL10	NM_001565	28.7	CX3CL1	U84487	21.6	IL-1 β	M15330	53.0
11	LIF	NM_002309	24.5	TNFAIP2	NM_006291	18.0	IL-6	NM_000600	50.8
12	ICAM1	NM_000201	24.4	CXCL1	NM_001511	15.1	ICAM1	NM_000201	45.7
13	CCL7	NM_006273	23.5	LIF	NM_002309	14.5	SERPINB2	NM_002575	38.5
14	CCL8	A1984980	20.5	CLEC2D	NM_013269	13.9	LIF	NM_002309	35.5
15	GCHI	NM_000161	19.4	IL-6	NM_000600	13.9	MMP1	NM_002421	34.1
16	IL17RB	NM_018725	18.4	PTGS2	NM_000963	13.7	MMP3	NM_002422	32.5
17	CXCL6	NM_002993	16.1	CXCL2	M57731	13.3	CCL7	NM_006273	29.5
18	IL-1 β	M15330	15.4	GPR56	AL554008	12.9	IL17RB	NM_018725	28.2
19	MMP3	NM_002422	12.2	TNFAIP3	NM_006290	12.8	GCHI	NM_000161	27.4
20	TNFAIP2	NM_006291	12.1	CCL5	NM_002985	12.0	INHBA	M13436	22.2
21	CLEC2D	NM_013269	12.0	CD83	NM_004233	11.8	CCL8	A1984980	21.4
22	LYN	A1356412	11.2	M-CSF	M37435	9.4	MYB	NM_005375	20.1
23	CX3CL1	U84487	11.1	STX11	AF071504	8.8	CXCL6	NM_002993	20.3
24	MMP1	NM_002421	10.9	IL1RN	U65590	8.8	GPR56	AL554008	19.7
25	GOS2	NM_015714	10.9	NKX3-1	AF247704	8.7	CLEC2D	NM_013269	18.8
26	BIRC3	U37546	9.1	CCL7	NM_006273	8.7	MAP3K8	NM_005204	17.5
27	M-CSF	M37435	9.0	MMP1	NM_002421	8.1	CCL5	NM_002985	16.9
28	TNFAIP3	NM_006290	9.0	CXCL6	NM_002993	8.1	G-CSF	NM_000759	15.9
29	MYB	NM_005375	8.4	MMP3	NM_002422	7.9	TNFAIP2	NM_006291	15.7
30	STX11	AF071504	8.3	NEUROGI	NM_006161	7.9	BIRC3	U37546	15.0
31	MAP3K8	NM_005204	8.1	BIRC3	U37546	7.9	AREG	NM_001657	14.6
32	IER3	NM_003897	7.9	BDKRB1	NM_000710	7.6	GOS2	NM_015714	13.7
33	TNFRSF1B	NM_001066	7.8	TRAF1	NM_005658	7.5	CX3CL1	U84487	13.4
34	SERPINB2	NM_002575	7.6	ADORA2A	NM_000675	7.4	M-CSF	M37435	13.3
35	BDKRB1	NM_000710	7.5	SYCP1	X95654	7.3	CD83	NM_004233	13.2

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-1 β 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-1 β and TNF- α . A previous study showed that IL-1 α 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-1 α cross-induction of TNF receptors may contribute to the biochemical mechanisms underlying the synergistic stimulation of G-CSF and GM-CSF production by IL-1 α and TNF- α . In our microarray analysis, IL-1 β induced type II TNF receptor expression in synovial fibroblasts although type I TNF receptor was expressed constitutively (data not shown). Therefore, IL-1 β cross-induction of type II TNF receptor may be one mechanism underlying the synergistic stimulation of G-CSF and GM-CSF production by IL-1 β and TNF- α in synovial fibroblasts from TMJ. However, there was no synergistic effect of IL-1 β 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-1 α 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-1 α 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-1 β and TNF- α may result in an increase in mRNA stability via RNA-binding proteins and/or microRNA, through an as yet uncharacterized mechanism.

NF κ B and MAPK pathways play pivotal roles in regulating the expression of inflammatory mediators by IL-

1 β and TNF- α stimulated synovial fibroblasts. Our data regarding the effect of MAPK inhibitors and an NF κ B inhibitor suggest that the induction of GM-CSF production by IL-1 β or TNF- α occurs through ERK, p38 MAPK, and NF κ B activation in synovial fibroblasts derived from the TMJ (Fig. 5). JNK signaling was associated only with IL-1 β in GM-CSF production (Fig. 5). M-CSF production by IL-1 β or TNF- α occurs through ERK, JNK, and NF κ B activation (Fig. 5). Inhibition of p38 MAPK affected GM-CSF, but not M-CSF, production by IL-1 β -stimulation. A previous study showed that stabilization of GM-CSF mRNA by IL-1 α depended on p38 MAPK activation (30). M-CSF mRNA, whose production was not synergistically affected by IL-1 β 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-1 β or TNF- α but also some other stimuli induced by IL-1 β 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-1 β 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-1 β and/or TNF- α . Additionally, IL-1 β 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-1 β and/or TNF- α , and may help define new therapeutic targets for the inflammatory components of the TMJ.

References

- Kondoh T, Dolwick MF, Hamada Y, Seto K : Visually guided irrigation for patients with symptomatic internal derangement of the temporomandibular joint: a preliminary report. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 95: 544-551, 2003.
- Hamada Y, Kondoh T, Holmlund AB, Iino M, Nakajima T, Seto K: Visually guided temporomandibular joint irrigation in patients with chronic closed lock: clinical outcome and its relationship to intra-articular morphologic changes. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 95: 552-558, 2003.
- Santos KC, Dutra ME, Warmling LV, Oliveira JX: Correlation among the changes observed in temporomandibular joint internal derangements assessed by magnetic resonance in symptomatic patients. *J Oral Maxillofac Surg*, 71: 1504-1512, 2013.
- Suzuki T, Segami N, Nishimura M, Nojima T : Co-expression of interleukin-1 β and tumor necrosis factor alpha in synovial tissues and synovial fluids of temporomandibular joint with internal derangement: comparison with histological grading of synovial inflammation. *J Oral Pathol Med*, 31: 549-557, 2002.
- Kaneyama K, Segami N, Nishimura M, Suzuki T, Sato J: Importance of proinflammatory cytokines in synovial fluid from 121 joints with temporomandibular disorders. *Br J Oral Maxillofac Surg*, 40 : 418-423, 2002.
- Israel HA, Langevin CJ, Singer MD, Behrman DA : The relationship between temporomandibular joint synovitis and adhesions: pathogenic mechanisms and clinical implications for surgical management. *J Oral Maxillofac Surg*, 64: 1066-1074, 2006.
- Israel HA, Diamond B, Saed-Nejad F, Ratcliffe A: Osteoarthritis and synovitis as major pathoses of the temporomandibular joint: comparison of clinical diagnosis with arthroscopic morphology. *J Oral Maxillofac Surg*, 56: 1023-1028, 1998.
- Tominaga K, Habu M, Sukedai M, Hirota Y, Takahashi T, Fukuda J : IL-1 β , IL-1 receptor antagonist and soluble type II IL-1 receptor in synovial fluid of patients with temporomandibular disorders. *Arch Oral Biol*, 49: 493-499, 2004.
- Hattori T, Ogura N, Akutsu M, Kawashima M, Watanabe S, Ito K, Kondoh T: Gene Expression Profiling of IL-17A-Treated Synovial Fibroblasts from the Human Temporomandibular Joint. *Mediators Inflamm*. Epub ahead of print 29 Dec 2015. DOI: 10.1155/2015/436067.
- Bucala R, Ritchlin C, Winchester R, Cerami A: Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J Exp Med*, 173: 569-574, 1991.
- Field M: Tumour necrosis factor polymorphisms in rheumatic diseases. *QJM*, 94: 237-246, 2001.
- Kay J, Calabrese L: The role of interleukin-1 in the pathogenesis of rheumatoid arthritis. *Rheumatology (Oxford)*, 43:(Suppl 3): iii2-iii9, 2004.
- Akutsu M, Ogura N, Ito K, Kawashima M, Kishida T, Kondoh T: Effects of interleukin-1 β and tumor necrosis factor- α on macrophage inflammatory protein-3 α production in synovial fibroblast-like cells from human temporomandibular joints. *J Oral Pathol Med*, 42: 491-498, 2013.
- Hamada Y, Kondoh T, Holmlund AB, Yamamoto M, Horie A, Saito T, Seto K, Sekiya H: Inflammatory cytokines correlated with clinical outcome of temporomandibular joint irrigation in patients with chronic closed lock. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 102: 596-601, 2006.
- Whetton AD, Dexter TM: Myeloid haemopoietic growth factors. *Biochim Biophys Acta*, 989: 111-132, 1989.
- Hamilton JA: Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol*, 8 : 533-544, 2008.
- Darrieutort-Laffite C, Boutet MA, Chatelais M, Brion R, Blanchard F, Heymann D, Golf BL : IL-1 β and TNF α promote monocyte viability through the induction of GM-CSF expression by rheumatoid arthritis synovial fibroblasts. *Mediators Inflamm*. Epub ahead of print 17 Nov 2014. DOI: 10.1155/2014/241840.
- Lawlor KE, Campbell IK, Metcalf D, O'Donnell K, van Nieuwenhuijze A, Roberts AW, Wicks IP: Critical role for granulocyte colony-stimulating factor in inflammatory arthritis. *Proc Natl Acad Sci USA*, 101: 11398-11403, 2004.
- Campbell IK, Rich MJ, Bischof RJ, Hamilton JA: The colony-stimulating factors and collagen-induced arthritis: exacerbation of disease by M-CSF and G-CSF and requirement for endogenous M-CSF. *J Leukoc Biol*, 68: 144-150, 2000.
- Ogura N, Tobe M, Sakamaki H, Kujiraoka H, Akiba M, Abiko Y, Nagura H: Interleukin-1 beta induces interleukin-6 mRNA expression and protein production in synovial cells from human temporomandibular joint. *J Oral Pathol Med*, 31: 353-360, 2002.
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 162: 156-159, 1987.
- Livak KJ, Schmittgen TD: Analysis of relative gene expres-

- sion data using real-time quantitative PCR and the 2⁻(Delta Delta C(T))Method. *Methods*, 25: 402-408, 2001.
23. Manferdini C, Paoletta F, Gabusi E, Silvestri Y, Gambari L, Cattini L, Filardo G, Fleury S, Lisignoli G: From osteoarthritic synovium to synovial-derived cells characterization: synovial macrophages are key effector cells. *Arthritis Res Ther*. Epub ahead of print 4 April 2016. DOI: 10.1186/s13075-016-0983-4.
 24. Hamilton JA, Tak PP: The dynamics of macrophage lineage populations in inflammatory and autoimmune diseases. *Arthritis Rheum*, 60: 1210-1221, 2009.
 25. Ryan PC, Sleeman MA, Rebelatto M, Rebelatto M, Wang B, Lu H, Chen X, Wu CY, Hinrichs MJ, Roskos L, Towers H, Mckeever K, Dixit R: Nonclinical safety of mavrilimumab, an anti-GMCSF receptor alpha monoclonal antibody, in cynomolgus monkeys: relevance for human safety. *Toxicol Appl Pharmacol*, 279: 230-239, 2014.
 26. Kim YK, Kim SG, Kim BS, Lee JY, Yun PY, Bae JH, Oh JS, Ahn JM, Kim JS, Lee SY: Analysis of the cytokine profiles of the synovial fluid in a normal temporomandibular joint: preliminary study. *J Craniomaxillofac Surg*, 40: e337-e341, 2012.
 27. Hamilton JA, Achuthan A: Colony stimulating factors and myeloid cell biology in health and disease. *Trends Immunol*, 34: 81-89, 2013.
 28. Caldwell J, Emerson SG: Interleukin-1 alpha upregulates tumor necrosis factor receptors expressed by a human bone marrow stromal cell strain: implications for cytokine redundancy and synergy. *Blood*, 86: 3364-3372, 1995.
 29. Tebo J, Der S, Frevel M, Khabar KS, Williams BR, Hamilton JA: Heterogeneity in control of mRNA stability by AU-rich elements. *J Biol Chem*, 278: 12085-12093, 2003.
 30. Kawai T, Lal A, Yang X, Galban S, Manzan-Mamczarz, K, Gorospe M : Translational control of cytochrome c by RNA-binding proteins TIA-1 and HuR. *Mol Cell Biol*, 26: 3295-3307, 2006.
 31. Sica, Mantovani A: Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*, 122: 787-795, 2012.
 32. Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, Kastelein R, Kolk A, de Waal-Malefyt R, Ottenhoff TH : Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S A*, 101: 4560-4565, 2004.
 33. Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD: Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. *J Immunol*, 178: 5245-5252, 2007.