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Tumor necrosis factor-α increases chemokine gene expression and production in synovial fibroblasts from human temporomandibular joint

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BACKGROUND: Synovitis, which is characterized by infiltration of inflammatory cells, often accompanies progression of clinical symptoms of the temporomandibular joint (TMJ). Synovial fibroblasts of the TMJ are believed to play important roles in progression of synovitis. The purpose of this study was to examine production and gene expression of chemokines by synovial fibroblasts stimulated by tumor necrosis factor-α (TNF-α).

METHODS: Protein levels of chemokines were measured by enzyme-linked immunosorbent assay (ELISA). Gene expression of chemokines was analyzed by real-time polymerase chain reaction (PCR).

RESULTS: Production of interleukin (IL)-8, growth-related oncogene (GRO)-α, monocyte chemoattractant protein (MCP)-1, and regulated upon activation normal T-cell expressed and secreted (RANTES) protein by synovial fibroblasts was increased by TNF-α. In contrast, stromal cell-derived factor (SDF)-1α, macrophage inflammatory protein (MIP)-1α and -1β were not detectable in conditioned media of synovial fibroblasts, with or without TNF-α treatment. Increases in gene expression of IL-8, GRO-α, MCP-1, and RANTES in response to TNF-α treatment were detected.

CONCLUSIONS: Increased protein production and gene expression of chemokines by synovial fibroblasts in response to TNF-α treatment appears to play an important role in recruitment of inflammatory cells into synovium and the progression of synovitis in the TMJ.

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Keywords: chemokine; synovial fibroblasts; temporomandibular joint; tumor necrosis factor-α

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Introduction

Intracapsular pathologic conditions of the temporomandibular joint (TMJ) are characterized by symptoms including limited mandibular movement, joint sound and pain, and generally involve disk displacement (DD)/internal derangement (ID), and/or osteoarthiritis (OA) of the TMJ. Synovitis, which often accompanies DD/ID and OA of the TMJ, is characterized by chronic inflammatory changes including hyperplasia of the synovial lining (1, 2), growth of small new blood vessels (2), and infiltration of inflammatory cells (2–5). Synovial cells produce a number of putative mediators of inflammation. However, involvement of synovial cells in pathologic conditions of the TMJ is poorly understood, because it has been difficult to obtain samples of synovial cells in sufficient quantities. Also, there are no animal models that are universally accepted for investigation of these diseases. In orthopedic studies, cultured fibroblast-like cells from the knee joint have been extensively used for investigation of the mechanisms of inflammatory response (6–8) and tissue degradation (9, 10). Our group has isolated and characterized synovial cells from the human TMJ (11–13).

Cytokines are mediators of cell–cell communication that play important roles in immune and inflammatory responses, wound healing, hematopoiesis, and maintenance of normal homeostasis. Chronic inflammatory diseases are often characterized by cyclic phases of cellular infiltration and tissue breakdown, which appear to be initiated and/or maintained partly by cytokine activity. Chemokines are low-molecular weight cytokines that are involved in regulation of leukocyte accumulation and activation in inflammatory tissue. All chemokines share certain primary structural similarities, including a conserved 4-cystein motif. Four chemokine subfamilies have been described based on the positions of certain cystein residues (CXC, CC, C, and CX_3C); CXC and CC are the two main subfamilies (14).

The CXC subfamily includes interleukin (IL)-8, growth-related oncogene (GRO), and stromal cell-derived factor (SDF). IL-8 and GRO-α are reported to exert proinflammatory effects, mainly via effects on neutrophils (14, 15). The CC subfamily includes monocyte chemoattractant protein (MCP), regulated upon activation normal T-cell expressed and secreted (RANTES), and macrophage inflammatory protein (MIP). CC chemokines appear to act on mononuclear cells with varying degrees of specificity (14, 16–18). Studies of chemokine expression have provided strong evidence for their involvement in rheumatoid arthritis (RA) (15, 19–22). Leukocytes recruited by chemokines are believed to play a key role in inflammatory and degenerative joint diseases by releasing degradative enzymes, various products of oxidative metabolism and inflammatory cytokines.

To assess the role of TMJ synovial fibroblasts in release of chemokines involved in inflammatory and degradative diseases of the TMJ, we analyzed their production and mRNA expression by synovial fibroblasts treated with tumor necrosis factor (TNF)-α; elevated levels of TNF-α have been found in synovial fluid obtained from the TMJ in cases of DD/ID and OA (23, 24).

Materials and methods

Cell culture

Human synovial tissue was obtained from two patients with ID who underwent arthrotomy of the TMJ (18-years-old female and 20-years-old female). The patients gave complete informed consent for the surgery and the use of their tissue in research. Isolation and primary cultures of synovial fibroblasts were performed according to the guidelines established by the Institutional Review Board of Nihon University School of Dentistry at Matsudo (EC03-003).

Synovial fibroblasts from the TMJ were prepared using the outgrowth according to the method of Ogura et al. (11). For the experiments, we used synovial fibroblasts from the sixth to eighth doubling passages.

ELISA

Synovial fibroblasts were plated at 5×10^4 cells per well in 24-well plates with Ham's F12 medium supplemented with 100 unit/ml of penicillin G (Banyu Pharmaceutical Co., Tokyo, Japan), 100 μg/ml of kanamycin sulfate (Sigma Chemical Co., St Louis, MO, USA), 250 ng/ml of fungizone (Flow Laboratories, McLean, VA, USA), 5 mM HEPES buffer (pH 7.2), and 10% fetal calf serum (FCS). The confluent-stage cells were cultured for 24 h in medium identical to the medium described above except that it contained 2% FCS, and were then treated with 10 ng/ml TNF-α (Pepro Tech EC Ltd, London, UK) or were left untreated. After incubation with or without TNF-α for 4, 8, 24 or 48 h, the culture supernatants were collected, centrifuged and kept at −80°C until used.

Levels of chemokines in conditioned media were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to protocols as recommended by the manufacturers. IL-8, MCP-1, RANTES, MIP-1α, and MIP-1β were purchased from PIERCE Endogen (Rockford, IL, USA), and GRO-α and SDF-1α were purchased from R&D Systems (Minneapolis, MN, USA).

Total RNA extraction

The synovial fibroblasts incubated with or without TNF-α for 4 h were homogenized using 1 ml of TRIZOL reagent (Life Technologies, Gaithersburg, MD, USA) and the FastPrep FP120 homogenizer (BIO 101, Vista, VA, USA), then added 200 μl of chloroform. The aqueous phase was transferred to a new tube, added chloroform. The aqueous phase was transferred a new tube, and then added isopropanol. Total cellular RNA was precipitated in isopropanol. The RNA precipitate was stored in ethanol at −80°C until used.

Real-time PCR

cDNA was synthesized using a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA). Briefly, cDNA synthesis was carried out at 42°C for 15 min in a final volume of 20 μl containing 2 μg of total RNA, 2.5 U/μl of MuLV Reverse Transcriptase, 5 mM $MgCl_2$, 1 mM NTP, 1 U/μl of RNase inhibitor, 2.5 mM Random Hexmers and oligo $d(T)_{16}$, and polymerase chain reaction (PCR) buffer II.

The real-time PCR was performed using a DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The PCR mixture contained 20 pmol of forward and reverse primers and 2 μl of cDNA. Amplification was performed using a DNA Engine Opticon 1 (MJ Research, San Francisco, CA, USA), with preheating at 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. The amplicons were detected directly by measuring the increase in fluorescence caused by the binding of SYBR Green I dye to gene-specific, amplified, double-stranded DNA (SYBR fluorescence systems in real-time PCR can be used to semiquantitatively analyze mRNA expression with assistance by on-line monitoring). Following the completion of PCR amplification, the temperature was raised from the annealing temperature to 95°C for melting curve analysis.

The initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold (C_T) in the exponential phase of the PCR. The number of transcripts was determined based on the threshold cycles of chemokines and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). ΔC_T (C_T-chemokine − C_T-GAPDH) indicated the relative amount of the chemokine transcript. $\Delta\Delta C_T$ (ΔC_T-treated − ΔC_T-control) represented the relative *n*-value compared with the control. The quantity 2^{-n} represented the difference in chemokine expression between TNF-α-treated cells and controls.

The following chemokine sense and antisense primers were used: IL-8, 5′-ATCACTTCCAAGCTGGCCGTGGCT-3′ and 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′; GRO-α, 5′-TGCAGGGAATTCACCCCAAG-3′ and 5′-CAGGGCCTCCTTCAGGAACA-3′; MCP-1, 5′-CCAATTCTCAAACTGAAGCTCGCAC-3′ and

5′-GTTAGCTGCCAGATTCTTGGGTTGTG-3′; RANTES, 5′-TACACCAGTGGCAAGTGCTC-3′ and 5′-GAAGCCTCCCAAGCTAGGAC-3′; GAPDH, 5′-ATCACCATCTTCCAGGAG-3′ and 5′-ATGGACTGTGGTCATGAG-3′.

Statistical analysis

Data are expressed as the mean ± SD. Significant differences between groups of control (without TNF-α) and TNF-α treatment in chemokine production were analyzed by Student's *t*-test. Statistical significance for multiple comparisons were assessed by two-way ANOVA or one-way ANOVA.

Results

Protein levels of chemokines

In experiments, we examined production of IL-8, GRO-α, MCP-1, RANTES, SDF-1α, MIP-1α, and MIP-1β by synovial fibroblasts treated with 10 ng/ml of TNF-α for 24 h (Table 1). Significant increases in IL-8, GRO-α, MCP-1, and RANTES were observed in the conditioned medium from synovial fibroblasts treated with TNF-α. Untreated synovial fibroblasts constitutively produced a large amount of MCP-1 and a small amount of GRO-α. In contrast, the levels of SDF-1α, MIP-1α, and MIP-1β were below the limit of detection in conditioned medium from synovial fibroblasts incubated with or without TNF-α.

We examined the kinetics of production of IL-8, GRO-α, MCP-1, and RANTES by synovial fibroblasts incubated with or without 10 ng/ml of TNF-α for 4, 8, 24 or 48 h. Production of IL-8, GRO-α, MCP-1, and RANTES increased from 4 to 48 h after the addition of TNF-α, compared with the untreated controls (Fig. 1a, c, e, g). In the next experiment, synovial fibroblasts were incubated for 24 h with concentrations of TNF-α ranging from 0.1 to 100.0 ng/ml. Production of IL-8, MCP-1, RANTES, and GRO-α increased in a dose-dependent manner, although there was no significance in production of RANTES between 0.1 ng/ml of TNF-α and the control. Production of GRO-α was significantly increased by all concentrations of TNF-α tested (0.1–100.0 ng/ml), and a plateau was reached approximately at a dose of 10.0 ng/ml (Fig. 1b, d, f, h).

Table 1 Effect of TNF-α on chemokine production in synovial fibroblasts from human TMJ

	Control	*TNF-α*
IL-8	0.02 ± 0.07	32.32 ± 10.80*
GRO-α	1.80 ± 0.13	12.23 ± 1.85*
MCP-1	14.49 ± 0.96	58.85 ± 7.02*
RANTES	0.03 ± 0.01	2.27 ± 0.34*
MIP-1α	< 0.025	< 0.025
MIP-1β	< 0.009	< 0.009
SDF-1α	< 0.156	< 0.156

Results are expressed as mean ± SD ($n = 4$).
TNF, tumor necrosis factor-α; TMJ, temporomandibular joint; IL, interleukin; GRO, growth-related oncogene; MCP, monocyte chemoattractant protein; RANTES, regulated upon activation normal T-cell expressed and secreted protein; MIP, macrophage inflammatory protein; SDF, stromal cell-derived factor.
*$P < 0.005$, compared to the control (without TNF-α).

Level of chemokine mRNA

The mRNA expression of IL-8, GRO-α, MCP-1, and RANTES, which were detectable in the conditioned medium from synovial fibroblasts, was examined by real-time PCR. When synovial fibroblasts were treated with 10 ng/ml of TNF-α for 4 h, mRNA levels increased as follows, compared with controls: IL-8, approximately 12-fold; GRO-α, 2.5-fold; MCP-1 and RANTES, five-fold (Table 2).

Discussion

Inflammatory arthropathies are characterized histologically by infiltration of inflammatory cells and enlargement of the synovial lining layer. Accumulation of neutrophils, activated T cells and macrophages at inflamed synovial tissue may lead to significant structural damage to joints with RA (8–10, 25–27). Inflammatory cells have also been detected in synovial tissue and fluid from patients with intracapsular pathologic conditions of the TMJ (2–4). The mechanisms leading to cell infiltration of the synovium and joint degeneration have been somewhat elucidated, but little information is available about synovial fibroblasts from the TMJ.

Chemokines are considered key players in the process of leukocyte diapedesis from the vasculature into tissues in inflammatory diseases (20, 22). Studies of the chemotactic activity of several of these proteins *in vitro* indicate relatively rigid patterns of target cell selectivity. For example, IL-8 and GRO-α (members of the CXC subfamily) are predominantly chemotactic for neutrophils (14, 15). In contrast, MCP-1 (a member of the CC subfamily) is highly specific for monocytes (14, 16), whereas RANTES, MIP-1α, and -1β (members of the CC subfamily) are chemotactic factors for monocytes and T-lymphocytes (14, 17, 18). SDF-1 (a member of the CXC subfamily) is considered a homing factor for hematopoietic stem cells (28). Levels of these chemokines have been found to be elevated in synovial fibroblasts in RA (14, 16, 17, 20–22).

The purpose of the present study was to analyze chemokine production by synovial fibroblasts from the TMJ in response to TNF-α, which is one of the cytokines that has been detected in synovial fluid obtained from patients suffering from intercapsular pathologic conditions with DD or OA (23). Studies suggest that the presence of TNF-α in synovial fluid is associated with pain in the TMJ, and elevated levels of TNF-α have been found in synovium exhibiting chronic inflammation (24). In the present study, production of IL-8, GRO-α, MCP-1, RANTES, SDF-1α, MIP-1α, and -1β was examined in synovial fibroblasts treated with TNF-α. Production of IL-8, GRO-α, MCP-1, and RANTES by synovial fibroblasts significantly increased in response to TNF-α. In contrast, production of SDF-1α, MIP-1α, and -1β by synovial fibroblasts, with or without TNF-α treatment, was below the detection limit of the ELISA kit we used. Gene expression of IL-8, GRO-α, MCP-1, and RANTES

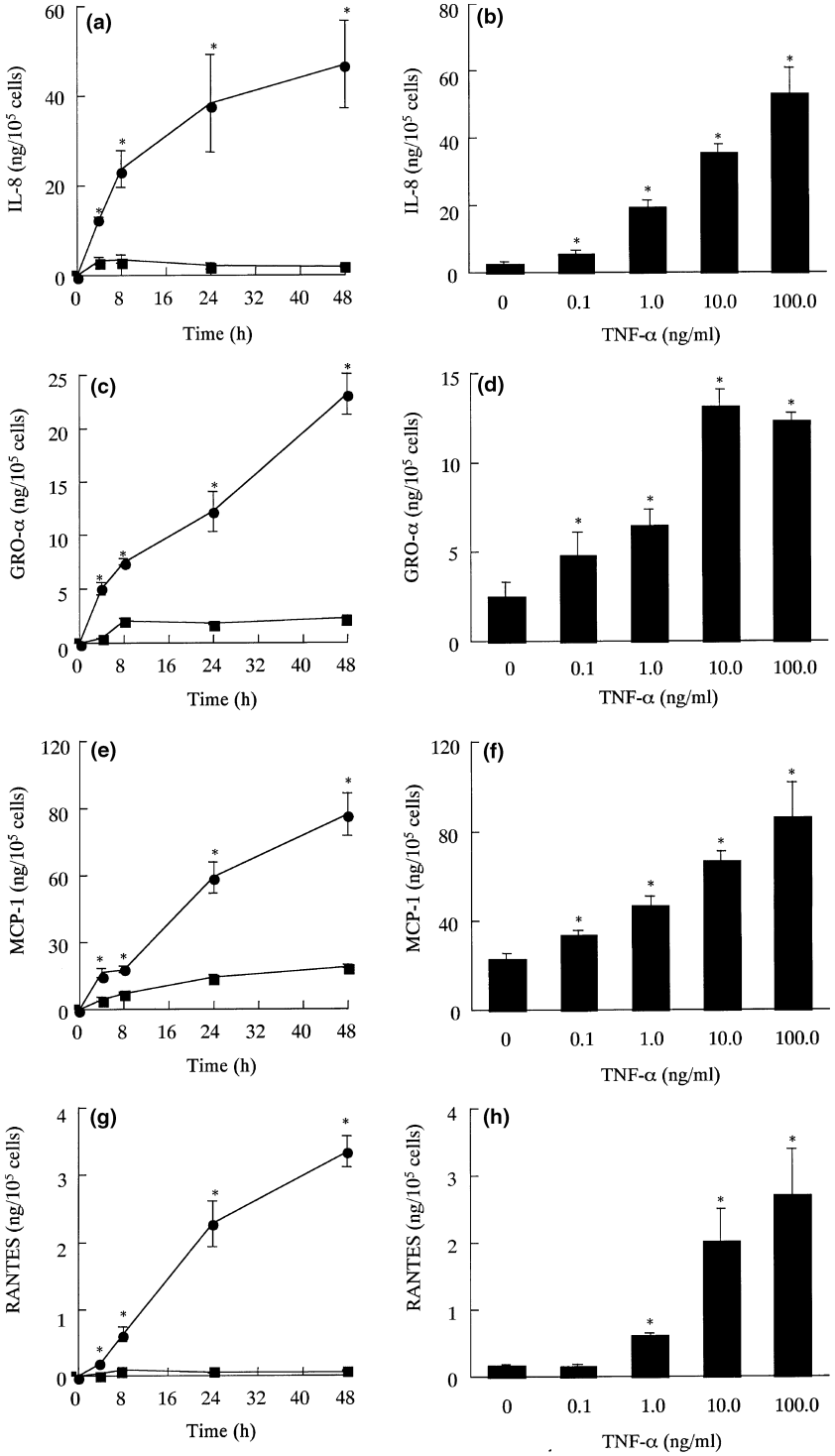

Figure 1 Time courses and dose–responses of chemokine levels in conditioned medium of synovial fibroblasts from human temporomandibular joint (TMJ). In time course experiment, the cells were incubated with (●) or without (■) 10 ng/ml of tumor necrosis factor (TNF)-α for 4, 8, 24 or 48 h. (a) Interleukin (IL)-8; (c) growth-related oncogene (GRO)-α; (e) monocyte chemoattractant protein (MCP)-1; (g) regulated upon activation normal T-cell expressed and secreted (RANTES). In the dose–response experiment, the cells were treated with varying concentrations of TNF-α for 24 h. Results are expressed as mean ± SD ($n = 4$). Asterisk (*) indicated significantly different ($P < 0.005$), for control vs. TNF-α stimulation. Significant difference ($P < 0.001$) for control vs. TNF-α stimulation and during time-course were assessed by two-way ANOVA. Significant difference ($P < 0.001$) during dose dependence in TNF-α were assessed by one-way ANOVA.

by synovial fibroblasts was also increased by treatment with TNF-α, although the degree of stimulation was different for each chemokine.

In the present kinetics experiments, production of IL-8 and GRO-α was increased soon after exposure to TNF-α (4 and 8 h), indicating that neutrophil

Table 2 Effect of TNF-α on chemokine gene expression in synovial fibroblasts from human TMJ

Gene	*Sample*	C_T*	ΔC_T**	$\Delta\Delta C_T$*** (*n-value*)	*Fold* (2^{-n})
IL-8	Control	16.382	3.480	–	1.0
	TNF-α	13.536	−0.150	−3.630	12.38
GRO-α	Control	16.652	2.750	–	1.0
	TNF-α	15.078	1.392	−1.358	2.56
MCP-1	Control	13.050	0.148	–	1.0
	TNF-α	11.353	−2.333	−2.481	5.58
RANTES	Control	23.606	10.704	–	1.0
	TNF-α	22.058	8.372	−2.332	5.03
GAPDH	Control	12.902	–	–	–
	TNF-α	13.686	–	–	–

C_T*, the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR; ΔC_T**, C_T-chemokine − C_T-GAPDH; $\Delta\Delta C_T$***, ΔC_T-treated − ΔC_T-control; 2^{-n}, difference in chemokine expression in TNF-α-treated cells relative to control; TNF, tumor necrosis factor-α; TMJ, temporomandibular joint; IL, interleukin; GRO, growth-related oncogene; MCP, monocyte chemoattractant protein; RANTES, regulated upon activation normal T-cell expressed and secreted protein.

infiltration occurs in the early stage of inflammatory responses. After exposure to TNF-α for 4 h, production of RANTES (a potent chemoattractant for CD4+/CD45RO+ memory helper T-lymphocytes) was increased to a lower degree than the other chemokines. This suggests that T-lymphocytes do not migrate into inflammatory sites as early as neutrophils and monocytes.

It has been reported that IL-8 levels in synovial fluid from patients with RA correlated with the parameters associated with disease activity and neutrophil turn over (28). Neutrophils, which product degradative enzymes by degranulation and reactive oxygen species through the action of the membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, may be trigger in the extracellular matrix degradation. Although neutrophils are predominantly associated with acute phases inflammation, the production of reactive oxygen species has even been proposed as a mechanism leading to persistent synovitis of chronic disease, although a hypoxic-reperfusion event (29). Furthermore, CXC chemokines containing the sequence Glu-Leu-Arg, the so-called ELR motif, including IL-8 and GRO-α, promote angiogenesis (30). Enhance of IL-8 and GRO-α productions from synovial cells may also lead to growth of small new blood vessels in synovium from TMJ.

The synovial fibroblasts constitutively produced MCP-1 at high levels. Cells in synovium are classified as type A or type B based on their ultrastructural characteristics. Type A cells are frequently described as macrophage-like, and probably arise from bone marrow-derived monocytes. Evidence suggests that MCP-1 plays an important role in the emergence of type A cells in synovium.

In a recent study using bone marrow stromal cells, IL-1β increased production of IL-8 and GRO-α (CXC chemokines) to a greater degree than MCP-1 and RNATES (CC chemokines), and TNF-α increased production of MCP-1 and RANTES to a greater degree than IL-8 and GRO-α (21). In a previous study, we found that chemokine production by TMJ synovial fibroblasts was increased by treatment with IL-1β (12, 13). Those previous findings are similar to the present increases in production of IL-8 and MCP-1 by synovial fibroblasts in response to treatment with TNF-α. TNF-α increased production of RANTES to a greater degree than treatment with IL-1β, whereas IL-1β increased production of GRO-α to a greater degree than treatment with TNF-α. Both of IL-1β and TNF-α are known to induced a rapid activation of transcription factors, such as NF-κB and AP-1 (31, 32), and increase in mitogen-activated protein (MAP) kinase phosphorylation, such as p38 MAP kinase, and the subsequent activation of its enzyme (33) in synovial fibroblasts, although IL-1β and TNF-α bind to distinct cellular receptors. Recently, Barchowsky et al. reported that IL-1 is more effective than TNF at inducing MMP-1 gene expression in rabbit primary synovial fibroblasts (RSF). They suggested that this is due in part to the fact that TNF is less effective than IL-1 at activating the MAPK/AP-1 pathway (34). The dichotomy of IL-1 and TNF-elicited signaling may be contribute to different regulation between IL-1β and TNF-α in chemokine production by synovial fibroblasts from TMJ.

The present findings indicate that the protein and gene expression levels of chemokines IL-8, GRO-α, MCP-1, and RANTES by TMJ synovial fibroblasts are increased by treatment with TNF-α, although the levels of basal and TNF-α-stimulated production were different for each chemokine. These results suggest trafficking of various leukocyte populations depending on the inflammatory status of the TMJ. The following sequence of events is consistent with these findings: (i) chemokines produced by synovial fibroblasts stimulate chemotaxis of neutrophils, macrophages and T-lymphocytes; (ii) these inflammatory cells produce inflammatory cytokines such as TNF-α, matrix degradative enzymes, and various products of oxidative metabolisms; (iii) the enzymes and oxidative metabolisms cause degradation of extracellular matrix; and (iv) the inflammatory cytokines stimulate synovial fibroblasts to produce more chemokines. Thus, although chemotaxis is a necessary element of homeostasis, excessive production of chemokines appears to contribute to destruction of joints.

In conclusion, TNF-α-stimulated chemokine production by synovial fibroblasts appears to be related to abnormalities associated with intracapsular pathologic conditions of the TMJ. The present findings also indicate that cultured synovial fibroblasts from TMJ have important advantages for studies of cellular and molecular responses in the TMJ.

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