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Interleukin-1β increases RANTES gene expression and production in synovial fibroblasts from human temporomandibular joint

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BACKGROUND: Synovial fibroblasts of temporomandibular joint (TMJ) are poorly characterized, although they have important roles in progression of temporomandibular disorders (TMD). In this study, we investigated responses of synovial fibroblasts to interleukin (IL)-1β.

METHODS: We examined gene expression profiles of synovial fibroblasts in response to IL-1β, using Affymetrix GeneChip. Regulated upon activation normal T-cell expressed and secreted (RANTES) gene expression was confirmed by polymerase chain reaction (PCR) and real-time PCR. RANTES protein levels were measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS: The RANTES was preferentially up-regulated in synovial fibroblasts by IL-1β. The increase in RANTES gene expression in response to IL-1β was confirmed by PCR and real-time PCR. Protein level of RANTES in synovial fibroblasts was also increased by IL-1β.

CONCLUSIONS: The RANTES, a cc-type chemokine, has chemotactic effects on lymphocytes and monocytes. Increased gene expression and protein production of RANTES in synovial fibroblasts, in response to IL-1β, may play an important role in recruitment of inflammatory cells into synovium and progression of synovitis in TMD.
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Introduction

Synovitis, which is often accompanied by internal derangement (ID) and osteoarthritis (OA) in the temporomandibular joint (TMJ), is characterized by chronic inflammatory changes as hyperplasia of the cells lining the synovium (1, 2) and growth of small new blood vessels (2). Synovitis is frequently associated with infiltration by inflammatory cells (2–5), which are believed to play pathologic roles in the development and continuation of inflammation via their release of degradative enzymes and various products of oxidative metabolism. The mechanism by which inflammatory cells are recruited into the synovial membrane is not completely understood.

Synovial cells have important roles in progression of synovitis. However, synovial cells of temporomandibular disorders (TMD) are poorly characterized, because it is difficult to obtain sufficient quantities of these cells. Also, no existing animal models are universally accepted for investigation of this disease. In orthopedic studies, cultured fibroblast-like cells from the knee have been extensively investigated to elucidate mechanisms of inflammatory response (6–8) and tissue degradation (9, 10). Our group has isolated and characterized synovial cells from human TMJ (11, 12).

Rheumatoid arthritis (RA) and OA patients have elevated levels of the cytokine interleukin (IL)-1β in synovial fluid (13). IL-1β mediates a variety of host defense processes, including inflammation and cellular response to injury involved in joint destruction (14). In several recent studies, levels of IL-1β were elevated in synovial fluid obtained from TMD patients with ID or OA (15–17). Also, studies indicate that IL-1β in synovial fluid is associated with pain and hyperalgesia in the TMJ (18).

Various approaches have been used to assay for differences in gene expression profile between cell types and identify genes preferentially expressed in certain types of cells. Recently, a DNA microarray technique has been developed for differential expression analysis of a large number of defined genes. Many types of DNA microarrays have been proposed and applied to expression profiling. The Affymetrix GeneChip array carries 40 distinct oligonucleotide sequences for each gene examined: 20 oligonucleotide perfect matches,

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and 20 single-base mismatch controls. The hybridization intensity, calculated from averages of perfect matches and mismatch controls, reflects gene expression levels.

In this study, we used the Affymetrix GeneChip array to investigate global gene expression in synovial fibroblasts in response to IL-1β. One of the genes we found preferentially expressed in synovial fibroblasts, regulated upon activation normal T-cell expressed and secreted (RANTES), a member of the chemokine superfamily, was up-regulated in synovial fibroblasts by treatment with IL-1β. We thus confirmed that treatment with IL-1β can alter gene expression and protein production of RANTES in synovial fibroblasts.

Materials and methods

Cell culture

Human synovial tissue was obtained from three patients undergoing TMJ arthrotomy (18-year-old female, 27-year-old female and 20-year-old female). All patients gave complete informed consent for the surgery and use of 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 TMJ were prepared using the method of Ogura et al. (11). For the experiments, we used synovial fibroblasts from the 6th to 8th doubling passages. Synovial fibroblasts from two patients were used for analysis with the Affymetrix GeneChip array, and cells from one patient were used for PCR, real-time PCR and ELISA.

Total RNA extraction

Synovial fibroblasts were plated at 1×10^6 cells in 10-cm dish 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 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) and 10% fetal calf serum (FCS). The confluent-stage cells were cultured for 24 h in the medium containing 2% FCS, and were then treated with or without 1 unit/ml of IL-1β (Cistron Biotecnology, Pine Brook, NJ, USA) for 4 h. Cells were homogenized using TRIZOL Reagent (Life Technologies, Gaithersburg, MD, USA). Total RNA was isolated using a Fast RNA kit (BIO 101, Vista, VA, USA) and FastPrep FP120 Instrument (BIO 101), and was stored at −80°C until used.

GeneChip expression analysis

Gene expression profiling was performed using the Affymetrix GeneChip Human Genome Focus Array (Affymetrix, Santa Clara, CA, USA) according to Affymetrix protocols. Analysis of GeneChip data was performed using Gene Springs software (Silicon Genetics, Redwood City, CA, USA).

Endpoint-polymerase chain reaction

cDNA synthesis and amplification by PCR were performed using a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA). Amplification of the PCR mixture was performed with a Gene Amp PCR system 9600 (Perkin-Elmer), beginning with preheating at 94°C for 5 min, followed by 18 or 32 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 2 min. The primers, designed by referring to sequences of RANTES (19) and GAPDH (20) cDNA in previous reports, were as follows: RANTES forward primer, 5′-TAC ACC AGT GGC AAG TGC TC-3′; RANTES reverse primer, 5′-GAA GCC TCC CAA GCT AGG AC-3′; GAPDH forward primer, 5′-ATC ACC ATC TTC CAG GAG-3′; GAPDH reverse primer, 5′-ATC GAC TGT GGT CAT GAG-3′. PCR fragments were electrophoresed on 1.5% agarose gel, followed by staining with ethidium bromide and examination of fragment sizes (RANTES, 199 base pairs; GAPDH, 315 base pairs).

Real-time polymerase chain reaction

Real-time PCR was performed using a DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The PCR mixture, containing 20 pmol of forward and reverse primers and 2 μl of cDNA, was subjected to amplification with a DNA Engine Opticon 1 (MJ Research, San Francisco, CA, USA), with preheating at 95°C for 10 s, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 min. The amplicons were detected directly by measuring the increase in fluorescence caused by binding of SYBR Green I dye to gene-specific, amplified, double-stranded DNA, using a DNA Engine Opticon 1. After the PCR reaction was complete, 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 in the exponential phase of the PCR reaction (C_T-value). The number of transcripts was determined based on the threshold cycles of RANTES and GAPDH. ΔC_T (C_T-RANTES − C_T-GAPDH) designated the relative abundance of the RANTES transcript. $\Delta\Delta C_T$ (ΔC_T-treated − ΔC_T-control) represented the relative n-value compared with the control. The value 2^{-n} represented the difference in RANTES expression between IL-1β treated cells and control.

Enzyme-linked immunosorbent assay

Synovial fibroblasts were plated at 5×10^4 cells per well in 24-well plates with Ham's F12 medium supplemented with 10% FCS and antibiotics. The confluent-stage cells were cultured for 24 h in the medium containing 2% FCS, and were then treated with or without IL-1β (Cistron Biotecnology, Pine Brook, NJ, USA). The culture supernatants were then collected, centrifuged and kept at −80°C until used. RANTES levels in

conditioned media were measured using an ELISA kit (PIERCE Endogen, Rockford, IL, USA).

Statistical analysis
Data are expressed as the mean ± SD Differences between groups were analyzed by Student's *t*-test.

Results

RANTES expression by GeneChip array
The gene expression profiles of synovial fibroblasts from TMJ of two patients were examined using Affymetrix GeneChip probe arrays. Among the genes examined in GeneChip analysis, RANTES was preferentially up-regulated in synovial fibroblasts by treatment with IL-1β (data not shown).

RANTES mRNA levels
Figure 1 shows relative expression of RANTES in synovial fibroblasts by endpoint-PCR. RANTES mRNA levels were elevated in synovial fibroblasts treated with 1 unit/ml of IL-1β for 4 h, compared with untreated cells.

Using SYBR fluorescence systems and real-time PCR, we were able to quantitatively analyze RANTES and GAPDH expression, with assistance by on-line monitoring. When synovial fibroblasts were treated with 1 unit/ml of IL-1β for 4 h, RANTES expression was up-regulated 10.987-fold (Table 1).

RANTES protein levels
We examined production of RANTES in synovial fibroblasts in response to IL-1β. Figure 2 shows the time course of RANTES levels in conditioned media from synovial fibroblasts treated with or without 1 unit/ml of IL-1β for 4, 8, 24 or 48 h. A significant increase in RANTES production was observed from 4 to 48 h after treatment with IL-1β, compared with untreated controls. In the next experiment, synovial fibroblasts were incubated in the presence of increasing concentrations of IL-1β (0.01–10.0 unit/ml) for 24 h. The stimulatory effect of IL-1β on RANTES production was apparent at a concentration of 0.1 unit/ml, and a plateau was reached at approximately 1.0 unit/ml (Fig. 3).

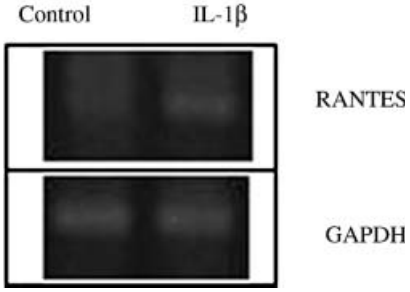

Figure 1 PCR of RANTES in synovial fibroblasts from human TMJ. The cells were treated with or without 1 unit/ml of IL-1β for 4 h. GAPDH was used as an internal control to ensure equal loading between samples.

Table 1 Effect of IL-1β on RENTES gene expression in synovial fibroblasts from human TMJ

Sample	*Gene*	C_T*	ΔC_T**	$\Delta\Delta C_T$*** *(n-value)*	*Fold* (2^{-n})
Control	RANTES	18.804	6.950	–	1.0
	GAPDH	11.885			
IL-1β	RANTES	15.990	3.491	−3.458	10.987
	GAPDH	12.500			

C_T*, the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction; ΔC_T**, C_T-RANTES − C_T-GAPDH; $\Delta\Delta C_T$***, ΔC_T-treated − ΔC_T-control; The 2^{-n} represented the difference in RANTES expression in IL-1β treated cells relative to control.

Characterization of the cells
Cell-specific markers in synovial fibroblasts were examined using immunocytochemistry. The synovial fibroblasts were positive for prolyl 4-hydroxylase and vimentin, which are fibroblast markers. In contrast, they were negative for macrophage marker, HLA class II antigen and dendritic cells marker (data not shown). Results of immunocytostaining for cell-specific markers did not significantly differ between fibroblasts treated with IL-1β and untreated controls (data not shown).

Discussion

In several studies, inflammatory changes were frequently found in synovial tissue and fluid from TMD patients (1–5, 15–18). In another study, inflammatory cells were detected in fluid from patients with TMD but not from healthy individuals (21). Inflammatory cells have also been detected in synovial tissue in histological studies using discectomy specimens from patients with TMD (2–4). Using immunohistochemical staining, Gynther et al. (3) detected cells positive for CD45RO (T cells) and/or CD 68 (macrophage) in synovial biopsy samples from patients with generalized OA or RA. In a study by Kardel et al. (5), about 85% of TMJ specimens from patients with painful clicking or OA stained positive for CD45RO cells. However, the mechanism by which inflammatory cells are recruited into the synovial tissue and fluid is not completely understood.

In this study, we investigated gene expression in synovial fibroblasts from two TMD patients using Affymetrix GeneChip array. When we compared the gene expression of synovial fibroblasts treated with and without 1 unit/ml of IL-1β for 4 h, we found that the level of RANTES mRNA is significantly augmented by treatment with IL-1β in both cases.

The RANTES, a basic 8-kDa polypeptide of the cc chemokine surperfamily initially cloned from an antigen-stimulated T-lymphocyte cell line, is also known as CCL5. RANTES is a potent chemoattractant for monocytes, CD4+/CD45RO+ memory helper T-lymphocytes, eosinophils, basophils and mast cells (22, 23). RANTES is relay expressed in normal adult tissue, but its expression markedly increases in inflammatory sites and some tumors (24). RANTES mRNA has been detected by *in situ* hybridization in synovial

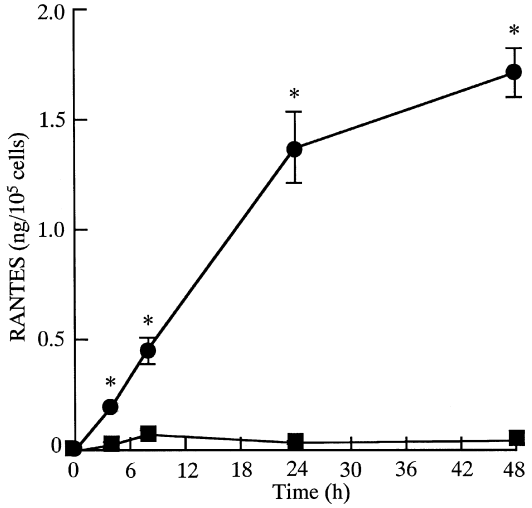

Figure 2 Time course of RANTES levels in conditioned medium of synovial fibroblasts from human TMJ. The cells were treated with (●) or without (■) 1 unit/ml of IL-1β for 4, 8, 24 or 48 h. Results are expressed as mean ± SD ($n = 4$). *$P < 0.005$, compared with the untreated control.

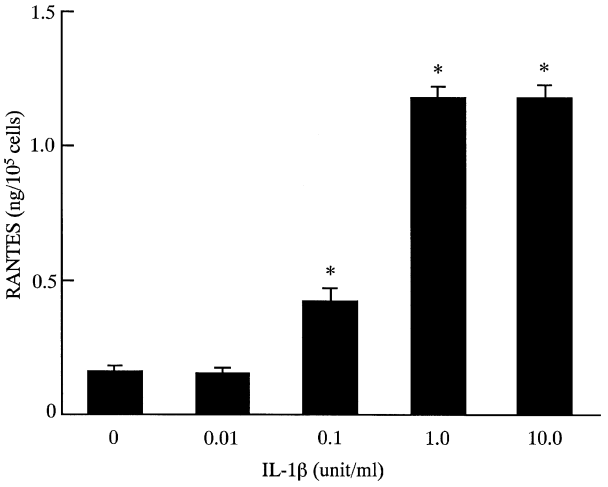

Figure 3 Dose response of RANTES levels in conditioned medium of synovial fibroblasts from human TMJ. The cells were treated with varying concentrations of IL-1β for 24 h. Results are expressed as mean ± SD ($n = 4$). *$P < 0.005$, compared with the untreated control.

lining cells from RA patients. High levels of RANTES have been detected in fluid from RA and OA patients (25, 26). Memory helper T-lymphocytes and monocyte-derived macrophages are also present in arthritic synovium (25, 26). The finding that anti-RANTES antibody can inhibit RA synovial fluid-induced monocyte chemotaxis indicates that RANTES is involved in RA (25).

In the present study, to confirm expression pattern exhibited in the GeneChip array, PCR and real-time PCR were performed with synovial fibroblasts treated with or without IL-1β. In each of these analyses, RANTES gene expression in synovial fibroblasts was augmented by IL-1β treatment. In real-time PCR analysis, the level of RANTES mRNA in synovial fibroblasts was increased approximately 11-fold by treatment with IL-1β, compared with the untreated cells.

We also examined levels of RANTES protein in synovial fibroblasts. When synovial fibroblasts were treated with 1 unit/ml of IL-1β, RANTES production was increased for 48 h in a time-dependent manner. We also incubated synovial fibroblasts for 24 h in the presence of 0.01–10.0 unit/ml (1–1000 pg/ml) of IL-1β, the range of IL-1β concentrations detected in TMJ synovial fluid in previous studies. The stimulatory effect of IL-1β on RANTES production was apparent at a concentration of 0.1 unit/ml, and a plateau was reached at a concentration of approximately 1.0 unit/ml. All cells were immunocytochemically positive for propyl 4-hydroxylase and vimentin, which are fibroblast markers. In contrast, all cells were negative for macrophage marker, dendritic marker and HLA class II antigen. These results suggest that the cells we isolated were synovial fibroblasts.

In recent studies, inflammatory cytokines have been detected in synovial fluids from patients with TMD (15–18, 27, 28). In several studies, IL-1β was detected in synovial fluid obtained from TMD patients with OA (15–18). Alstergren et al. (18) demonstrated that IL-1β in synovial fluid was associated with pain and hyperalgesia in the TMJ, suggesting that it is a warning sign of tissue destruction. However, it is not clear whether IL-1β in synovial fluid in TMJ affects the physiology of the synovial tissue. In future studies, we plan to investigate the network of inflammatory cytokines between cells in the TMJ.

In the present study, in synovial fibroblasts from human TMJ, RANTES production and gene expression were increased by treatment with IL-1β. The present findings suggest the following sequence of events: (i) RANTES produced by synovial fibroblasts stimulates chemotaxis of memory T-lymphocytes and macrophages; (ii) the lymphocytes and macrophages attracted by RANTES probably produce inflammatory cytokines such as IL-1β, matrix degradative enzymes and various products of oxidative metabolism; (iii) these enzymes and products of oxidative metabolism may cause degradation of extracellular matrix; (iv) the inflammatory cytokines may stimulate synovial fibroblasts to produce chomokines such as RANTES. Thus, although chemotaxis is a necessary function of homeostasis, excessive production of chemokines seems to contribute to destruction of joints.

In conclusion, stimulation of RANTES production in synovial fibroblasts by IL-1β may be related to abnormalities associated with TMD. The present findings also suggest that cultured synovial fibroblasts from the TMJ have important advantages for studies of cellular and molecular responses in the TMJ.

References

1. Dijkgraaf LC, Liem RSB, de Bont LGM. Synovial membrane involvement in osteoarthiritic temporomandibular joints. A light microscopic study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1997; **83**: 373–86.

2. Carls FR, von Hochstetter A, Makek M, Engelke W. Diagnostic accuracy of TMJ arthroscopy in correlation to histological findings. *J Craniomaxillofac Surg* 1995; **23**: 75–80.
3. Gynther GW, Holmlund AB, Reinholt FP, Lindblad S. Temporomandibular joint involvement in generalized osteoarthritis and rheumatoid arthritis: a clinical, arthroscopic, histologic, and immunohistochemical study. *Int J Oral Maxillofac Surg* 1997; **26**: 10–6.
4. Gynther GW, Dijkgraaf LC, Reinholt FP, Holmlund AB, Liem RSB, de Bont LGM. Synovial inflammation in arthroscopically obtained biopsy specimens from the temporomandibular joint: a review of the literature and a proposed histologic grading system. *J Oral Maxillofac Surg* 1998; **56**: 1281–6.
5. Kardel R, Ulfgren A-K, Reinholt FP, Holmlund A. Inflammatory cell and cytokine patterns in patients with painful clicking and osteoarthritis in the temporomandibular joint. *Int J Oral Maxillofac Surg* 2003; **32**: 390–6.
6. Chin JE, Winterrowd GE, Krzesicki RF, Sanders ME. Role of cytokines in inflammatory synovitis. The coordinate regulation of intercellular adhesion molecule 1 and HLA class I and class II antigens in rheumatoid synovial fibroblasts. *Arthritis Rheum* 1990; **33**: 1776–86.
7. Richards CD, Agro A. Interaction between oncostatin M, interleukin 1 and prostaglandin E_2 in induction of IL-6 expression in human fibroblasts. *Cytokine* 1994; **6**: 40–7.
8. Williams RJ, Henderson LM, Naidoo Y, Cassim B, Elson CJ, Bhoola KD. Immunocytochemical analysis of tissue kallikrein and the kinin moiety in rheumatoid synovial fluid neutrophils. *Br J Rheumatol* 1997; **36**: 420–5.
9. Koolwijk P, Miltenburg AMM, van Erck MGM, et al. Activated gelatinase-B (MMP-9) and urokinase-type plasminogen activator in synovial fluids of patients with arthritis. Correlation with clinical and experimental variables of inflammation. *J Rheumatol* 1995; **22**: 385–93.
10. Bläser J, Triebel S, Maasjosthusmann U, et al. Determination of metalloproteinases, plasminogen- activators and their inhibitors in the synovial fluids of patients with rheumatoid arthritis during chemical synoviorthesis. *Clin Chim Acta* 1996; **244**: 17–33.
11. Ogura N, Tobe M, Sakamaki H, et al. Interleukin-1β induces interleukin-6 mRNA expression and protein production in synovial cells from human temporomandibular joint. *J Oral Pathol Med* 2002; **31**: 353–60.
12. Tobe M, Ogura N, Abiko Y, Nagura H. Interleukin-1β stimulates interleukin-8 production and gene expression in synovial cells from human temporomandibular joint. *J Oral Maxillofac Surg* 2002; **60**: 741–7.
13. Horiuchi T, Yoshida T, Koshihara Y, et al. The increase of parathyroid hormone-related peptide and cytokine levels in synovial fluid of elderly rheumatoid arthritis and osteoarthritis. *Endocrine J* 1999; **46**: 643–9.
14. Vincenti MP, Brinckerhoff CE. Early response genes induced in chondrocytes stimulated with the inflammatory cytokine interleukin-1β. *Arthritis Res* 2001; **3**: 381–8.
15. Kubota E, Imamura H, Kubota T, Shibata T, Murakami K. Interleukin-1β and stromelysin (MMP3) activity of synovial fluid as possible markers of osteoarthritis in the temporomandibular joint. *J Oral Maxillofac Surg* 1997; **55**: 20–7.
16. Takahashi T, Kondoh T, Fukuda M, Yamazaki Y, Toyosaki T, Suzuki R. Proinflammatory cytokines detectable in synovial fluids from patients with temporomandibular disorders. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1998; **85**: 135–41.
17. 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* 2002; **40**: 418–23.
18. Alstergren P, Ernberg M, Kvarnström M, Kopp S. Interleukin-1β in synovial fluid from the arthritic temporomandibular joint and its relation to pain, mobility, and anterior open bite. *J Oral Maxillofac Surg* 1998; **56**: 1059–65.
19. Schall TJ, Jongstra J, Dyer BJ, et al. A human T cell-specific molecule is a member of a new gene family. *J Immunol* 1988; **141**: 1018–25.
20. Tokunaga K, Nakamura Y, Sakata, K, et al. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res* 1987; **47**: 5616–9.
21. Kopp S, Wenneberg B, Clemensson E. Clinical, microscopical and biochemical investigation of synovial fluid from temporomandibular joints. *Scand J Dent Res* 1983; **91**: 33–41.
22. Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* 1990; **347**: 669–71.
23. Rot A, Krieger M, Brunner T, Bischoff SC, Schall TJ, Dahinden CA. RANTES and macrophage inflammatory protein 1α induce the migration and activation of normal human eosinophil granulocytes. *J Exp Med* 1992; **176**: 1489–95.
24. von Luettichau I, Nelson PJ, Pattison JM, et al. RANTES chemokine expression in diseased and normal human tissues. *Cytokine* 1996; **8**: 89–98.
25. Volin MV, Shah MR, Tokuhira M, Kenneth Haines G III, Woods JM, Koch AE. RANTES expression and contribution to monocyte chemotaxis in arthritis. *Clin Immunol Immunopathol* 1998; **89**: 44–53.
26. Patterson AM, Schmutz C, Davis S, Gardner L, Ashton BA, Middleton J. Differential binding of chemokines to macrophages and nertrophils in the human inflamed synovium. *Arthritis Res* 2002; **4**: 209–14.
27. Sandler NA, Buckley MJ, Cillo JE, Braun TW. Correlation of inflammatory cytokines with arthroscopic findings in patients with temporomandibular joint internal derangements. *J Oral Maxillofac Surg* 1998; **56**: 534–43.
28. Kaneyama K, Segami N, Nishimura M, Sato J, Suzuki T, Fujimura K, Osteoclastogenesis inhibitory factor/osteoprotegerin in synovial fluid from patients with temporomandibular disorders. *Int J Oral Maxillofac Surg* 2003; **32**: 404–7.

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