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Microarray analysis of IL-1β-stimulated chemokine genes in synovial fibroblasts from human TMJ

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BACKGROUND: Interleukin (IL)-1β is thought to play a key role in several pathologic conditions of the temporomandibular joint (TMJ). Gene expression profile of synovial fibroblasts stimulated with IL-1β was studied by oligonucleotide microarray analysis to elucidate candidate genes associated with intracapsular pathologic conditions of TMJ.

METHODS: RNA was isolated from synovial fibroblasts from five patients after IL-1β treatment. Gene expression profiling was performed with a GeneChip. Changes in gene expression were determined by comparing IL-1β-treated cells with untreated cells.

RESULTS: A total of 121 genes showed a greater than threefold difference in average intensity between untreated and IL-1β-treated synovial fibroblasts in five experiments. Five chemokines were among the 10 most upregulated genes, and the most upregulated gene was CCL20. The 121 IL-1β-responsive genes included 12 chemokines whose mRNA levels were confirmed by real-time PCR.

CONCLUSION: These data should provided useful information about the pathologic conditions of TMJ, especially in support of diagnosis and therapeutic approaches to TMJ.

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Keywords: chemokine; interleukin-1β; oligonucleotide microarray; synovial fibroblasts; temporomandibular joint

Introduction

Synovitis, which often accompanies intracapsular pathologic conditions such as disk displacement (DD)/internal derangement (ID) and osteoarthritis (OA) of the temporomandibular joint (TMJ), is characterized by chronic inflammatory changes, including hyperplasia of the synovial lining (1, 2), growth of small new blood vessels (1), and infiltration of inflammatory cells (1, 3–5). In addition, synovial cells produce a number of putative mediators of inflammation (6, 7).

Interleukin (IL)-1β is a proinflammatory cytokine that affects cell proliferation (8), inflammatory responses (9), and matrix remodeling (10). Elevated expression of IL-1β 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; 8, 11). Under pathologic conditions of the TMJ, levels of IL-1β are elevated in synovial fluids obtained from patients with OA and RA (12). Studies have also indicated that IL-1β in synovial fluid is associated with pain in the TMJ (13, 14). However, the involvement of synovial cells under pathologic conditions in the TMJ is poorly understood, as synovial cells have been difficult to cultivate in quantities sufficient for study.

Our group has isolated synovial cells from the human diseased TMJ and examined how regulation of IL-1β affects the underlying inflammatory status (15–17). We hypothesized that identification of IL-1β-responsive genes in synovial fibroblasts could help develop models for the inflammatory condition of the TMJ. However, obtaining an overview of the molecular and cellular responses of synovial fibroblasts to IL-1β has been difficult, in part because classic approaches for studying inflammatory factors have focused on identification and analysis of specific postulated factors on an individual basis. DNA microarray techniques have recently been developed to facilitate comprehensive expression analysis of large numbers of defined genes (18–20). Microarray analysis is an efficient method for exploring the functions of uncharacterized genes, as the expression pattern of uncharacterized genes can be compared with the expression pattern of characterized genes.

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Microarray analysis can also be used to search for differentially expressed genes, which may include diagnostic and therapeutic markers.

In order to understand how IL-1β can induced the intracapsular pathologic condition of the TMJ, we examined the gene expression profiles of non-induced and IL-1β-induced synovial fibroblasts from the human TMJ using an oligonucleotide microarray approach.

Materials and methods

Cell culture

Human synovial tissue was obtained from five patients with ID who underwent arthroscopy of the TMJ (three females and two males; age range: 17–27 years). Patients gave complete informed consent for the surgery and for 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 as previously described (15). For the experiments, we used synovial fibroblasts from the sixth to eighth passages.

Total RNA extraction

Synovial fibroblasts were incubated with or without 1 unit/ml IL-1β for 4 h. Total cellular RNA from synovial fibroblasts (TMJ 1–5) was extracted using RNeasy kits (Qiagen, Valencia, CA, USA), and was then stored at −80°C until used.

GeneChip expression analysis

For gene expression profiling, we used the Affymetrix GeneChip Human Genome Focus Array (Affymetrix, Santa Clara, CA, USA) according to Affymetrix protocols. Briefly, double-stranded cDNA was generated from 7 μg of total RNA using a T7-oligo (dT) primer and the Superscript Choice System kit (Invitrogen, Carlsbad, CA, USA). cDNA was then purified with a GeneChip Sample Clean-up Module (Affymetrix). Biotin-labeled cRNA was synthesized by *in vitro* transcription using the BioArray high-yield RNA transcript labeling system (ENZO, Farmingdale, NY, USA). cRNA was then purified using a GeneChip Sample Clean-up Module. Biotin-labeled cRNA was added to the fragmentation buffer (Affymetrix), and was heated for 35 min at 95°C. Fragmented cRNA (10 μg) was hybridized to the Human Genome Focus Array (8793 genes) for 16 h at 45°C. Arrays were subjected to washing and staining with R-phycoerythrin streptavidin (SAPE, Molecular Probes, Eugene, OR, USA) using GeneChip Fluidics Station 400 (Affymetrix).

In order to amplify the signals, the arrays were further stained with goat biotinylated antistreptavidin antibody (Vector Laboratories, Burlingame, CA, USA), followed by SAPE. After scanning (scanner from Affymetrix), the oligonucleotide hybridization data were exported for gene expression value analysis using AFFYMETRIX MICROARRAY SUITE (version 5.0). The hybridization intensity for each gene was calculated with a probe set containing 24 specific probes for perfect matches and mismatches. The expression of each gene was also categorized as 'present', 'absent', or 'marginal'. For quality control, cRNA samples were hybridized to the test microarray chip (Test 3; Affymetrix) in order to ensure equal hybridization to 5′- and 3′-oligonucleotides of the genes chosen for standardization (GAPDH and β-actin) before hybridization with Focus Array.

Data analysis

Raw data from 10 GeneChips were loaded into GENESPRING software (version 6.2; Agilent Technologies, Waldbronn, Germany). Data were normalized using the median raw data from each array as a reference. Changes in gene expression were determined by comparing controls (average normalized intensity of untreated cells) with IL-1β treatment (average normalized intensity of IL-1β-treated cells).

Real-time PCR

cDNA was synthesized using a GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT, USA). 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 pre-heating at 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 30 s. 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 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 PCR. The number of transcripts was determined based on the threshold cycles of chemokines and GAPDH. ΔC_T (C_T-chemokine minus C_T-GAPDH) indicates the relative amount of chemokine transcripts. $\Delta\Delta C_T$ (ΔC_T-treated minus ΔC_T-control) represents the relative n-value compared with the control. The quantity 2^{-n} represents the difference in chemokine expression between IL-1β-treated cells and controls. Chemokine primer sequences are given in Table 1.

Results

The expression of 8793 genes on the Human Genome Focus Array was compared between controls (average values of five non-treated cells from five patients) and IL-1β-treated cells (average values of IL-1β-treated cells from five patients). A total of 121 genes showed a greater than threefold difference in intensity between control and IL-1β-treated synovial fibroblasts; 111 were upregulated and 10 were downregulated.

Table 1 Primers for chemokine

Chemokine	*Primers*	*bp*
CCL20 (MIP-3)	F: 5′-GCA AGC AAC TTT GAC TGC TG-3′ R: 5′-CAA GTC CAG TGA GGC ACA AA-3′	342
CXCL3 (GRO-γ)	F: 5′-TAA ATG ACA GGG TGG GGA AC-3′ R: 5′-GCA TTA TGC CCT ACA AGC AA-3′	225
CCL7 (MCP-3)	F: 5′-CCT GGA CAA GAA AAC CCA AA-3′ R: 5′-TTC AAA ACC CAC CAA AAT CC-3′	245
CXCL2 (GRO-β)	F: 5′-CCC TGC CTT ACA GGA ACA GAA-3′ R: 5′-TCC CTG CCG TCA CAT TGA TCT-3′	403
CXC10 (IP-10)	F: 5′-TGC AAG CCA ATT TTG TCC ACG TGT TG-3′ R: 5′-GCA GCT GAT TTG GTG ACC ATC ATT GG-3′	302
CXCL1 (GRO-α)	F: 5′-TGC AGG GAA TTC ACC CCA AG-3′ R: 5′-CAG GGC CTC CTT CAG GAA CA-3′	229
CXCL8 (IL-8)	F: 5′-ATC ACT TCC AAG CTG GCC GTG GCT-3′ R: 5′-TCT CAG CCC TCT TCA AAA ACT TCT C-3′	289
CCL8 (MCP-2)	F: 5′-TGC AAA ATC CTG GTG ATG TG-3′ R: 5′-AGG AGC ACT GAT TGC CAA AG-3′	166
CX3CL1 (fractalkine)	F: 5′-GAG TGG GTC CAA TGC ACT TT-3′ R: 5′-CAC AGA CGT TGG TGA TGA GG-3′	241
CXCL6 (GCP-2)	F: 5′-CAA TGA TCT GTG CTC TGC AA-3′ R: 5′-CCA ACA TGA CAC ACA GGA AAA-3′	218
CCL5 (RANTES)	F: 5′-TAC ACC AGT GGC AAG TGC TC-3′ R: 5′-GAA GCC TCC CAA GCT AGG AC-3′	199
CCL2 (MCP-1)	F: 5′-CCA ATT CTC AAA CTG AAG CTC GCA-C3′ R: 5′-GTT AGC TGC CAG ATT CTT GGG TTG TG-3′	372
GAPDH	F: 5′-ATC ACC ATC TTC CAG GAG-3′ R: 5′-ATC GAC TGT GGT CAT GAG-3′	315

CCL, CC motif chemokine ligand; CXCL, CXC motif chemokine ligand; CX3CL, CX3C motif chemokin ligand; MIP, macrophage inflammatory protein; GRO, growth-regulated oncogene; MCP, monocyte chemoattractant protein; IP, interferon-inducible protein; IL, interleukin; GDP, granulocyte chemotactic protein; RNATES, regulated upon activation, normal T expressed and secreted; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 2 Upregulated genes by treatment with IL-lβ

Rank[a]	*Gene*	*GenBank*	*Fold change*[b] *(IL-lβ/control)*	*Gene title Affy*
1	CCL20	NM_004591	413.6	Chemokine (C–C motif) ligand 20
2	CXCL3	NM_002090	60.8	Chemokine (C–X–C motif) ligand 3
3	BCL2A1	NM_004049	41.0	BCL2-related protein A1
4	PTGS2	NM_000963	38.9	Prostaglandin-endoperoxide synthase 2
5	CXCL2	M57731	36.3	Chemokine (C–X–C motif) ligand 2
6	IL-8	AF043337	35.6	Interleukin 8
7	CSF3	NM_000759	29.4	Colony-stimulating factor 3 (granulocyte)
8	CCL7	NM_006273	21.0	Chemokine (C–C motif) ligand 7
9	IL-1β	M15330	20.5	Interleukin 1, β
10	IL-6	NM_000600	19.9	Interleukin 6

[a]Ranking of upregulated gene by IL-1β.
[b]Average normalized intensitiy of IL-lβ-sitmulated cell in TMJ 1–5/average normalized intensity of control cell in TMJ 1–5.

Table 2 lists the top 10 upregulated genes. The gene most strongly upregulated by IL-1β (rank 1) was CCL20 (MIP-3α), a member of the chemokine superfamily. In fact, five chemokines were among the top 10 upregulated genes; CXCL3 (GRO-γ; rank 2), CXCL2 (GRO-β; rank 5), IL-8 (CXCL8; rank 6), and CCL7 (MCP-3; rank 8). The remaining five genes in the top 10 were BCL2A1 (BCL-related protein A1), PTGS2 (prostaglandin-endoperoxide synthetase 2), CSF3 (granulocyte colony-stimulating factor; G-CSF), IL-1B (IL-1β), and IL-6.

The 121 IL-1β-responsive genes, which showed a greater than threefold difference between control and IL-1β-treated synovial fibroblasts, were categorized based on the ontology of their molecular function (Fig. 1). The category with the most genes was signal transduction (34 genes; 32 upregulated and two downregulated), followed by inflammatory response (20 genes; 20 upregulated), proliferation (15 genes; 13 upregulated and two downregulated), and chemotaxis (12 genes; 12 upregulated). Inflammatory response (20 of 139 genes, 14.4%) and chemotaxis (12 of 88 genes, 13.6%) accounted for the largest percentage of IL-1β-responsive genes among those identified on HG Focus Array. All of the 12 chemotaxis genes were also categorized as inflammatory response genes, such as chemokine superfamily members.

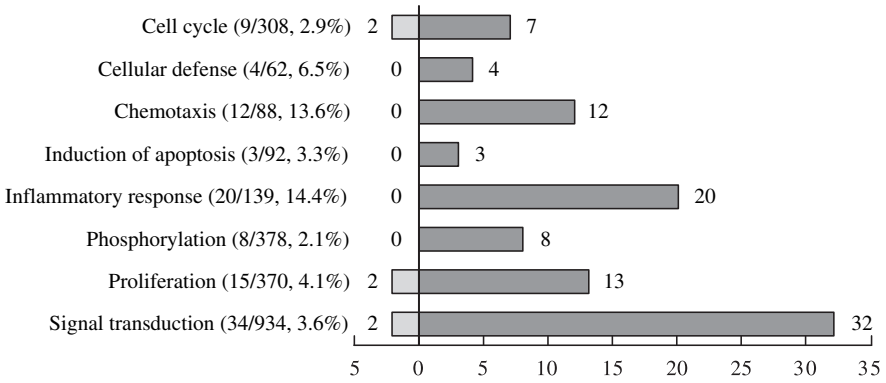

Figure 1 Ontology for IL-1β-responsive genes. The categories of each gene are indicated (number of IL-1β-responsive genes/number of genes identified on HG Focus Array, percentage of IL-1β-responsive genes).

Table 3 Levels of IL-lβ-responsive chemokines mRNA in synovial fibroblasts from TMJ

Chemokine	*GenBank*	*Fold (IL-Iβ/control)*		*Chemoattract target cells*
		GeneChip	*Real-time PCR*	
CCL20 (MIP-3α)	NM_004591	545.2	224.6	Memory T cell, immature dendritic cell
CXCL3(GRO-γ)	NM_002090	61.6	158.3	Neutrophil, endothelial cell
CCL7 (MCP-3)	NM_006273	47.0	86.4	Monocyte, Th1 cell, eosinophil basophil, NK cell, dendritic cell
CXCL2(GRO-β)	NM_002089	34.5	83.7	Neutrophil, melanoma cell
CXC10 (IP-10)	NM_001565	13.8	87.5	Monocyte, Th1 cell, NK cell
CXCL1 (GRO-α)	NM_001511	11.7	72.4	Neutrophil, lymphocyte, monocyte
CXCL-8(IL-8)	NM_000584	11.3	77.4	Neutrophil, $CD8^+$ T cell, NK cells, monocyte
CCL8(MCP-2)	NM_005623	9.9	50.8	$CD4^+$ T cell, $CD8^+$ T cell, monocyte, eosinophil, basophil, NK cell
CX3CL1 (fractalkine)	NM_002996	9.5	8.9	NK cell, intraepithelial lymphocyte, $CD8^+$ T cell, $CD4^+$ T cell, monocytes
CXCL6(GCP-2)	NM_002993	6.4	19.3	Neutrophil
CCLS(RANTES)	NM_002985	4.0	8.3	Eosinophil, T cell, monocyte, basophil
CCL2(MCP-1)	NM_002982	3.5	7.2	Monocyte, T cell, basophil

CCL, CC motif chemokine ligand; CXC, CXC motif chemokine ligand; CX3CL, CX3C motif chemokin ligand; MIP, macrophage inflammatory protein; GRO, growth-related gene product; MCP, monocyte chemoattractant protein; IP, interferon-inducible protein; IL, interleukin; GDP, granulocyte chemotactic protein; RNATES, regulated upon activation, normal T expressed and secreted.

We focused on genes that encode chemokine superfamily members, as this gene family exhibits the most significant responses to IL-1β (Table 2 and Fig. 1). The 12 chemokines included five CC motif chemokines, six CXC motif chemokines, and one CX3C motif chemokines (Table 3). Figure 2 shows scatter plots of the 121 IL-1β-responsive genes and the 12 indicated chemokines. CCL20, CCL2, IL-8, CXCL1, CXCL2, and CXCL3 exhibited high expression in IL-1β-treated cells. Table 3 shows the IL-1β-induced fold-change using microarray and real-time PCR analysis in the TMJ 5 sample. Although the fold-change between the control and IL-1β-treated synovial fibroblasts determined by real-time PCR differed from that found on microarray, the rank order of the IL-1β response was similar between microarray and real-time PCR (Table 3).

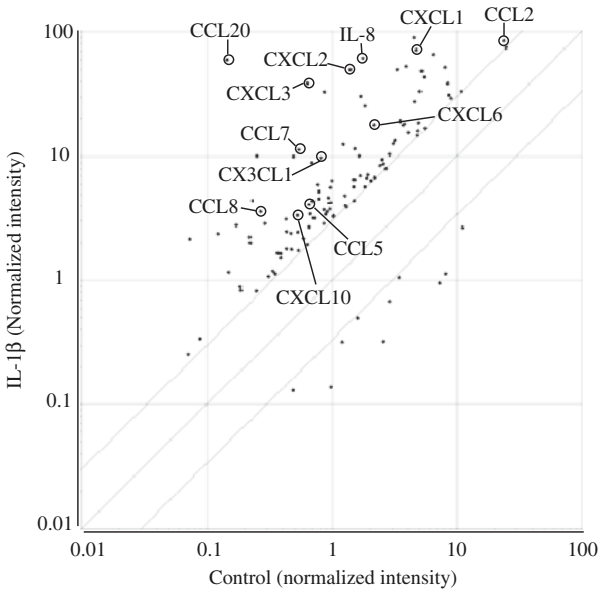

Figure 2 Chemokines in scatter plots of IL-1β-responsive genes. Normalized intensity was calculated from five experiments using samples TMJ 1–5.

Discussion

Changes in the expression and production of inflammatory factors have an impact on both homeostasis and the pathologic condition of the TMJ. We hypothesized that identification of IL-1β-responsive genes in synovial

fibroblasts could help develop models for the inflammatory condition of the TMJ. Cell culture systems provide a well-established means for initial identification of cytokine-responsive genes. Of the cells from patients with intracapsular pathology of the TMJ, we decided to analyze the cells that produced the lowest levels of cytokines under untreated conditions, as we were unable to obtain synovial cells from healthy individuals.

We performed oligonucleotide microarray analysis in order to examine the consistency of gene regulation in synovial fibroblasts treated with IL-1β. Among the 8793 genes tested, a total of 121 exhibited a greater than threefold change in intensity between control and IL-1β-treated synovial fibroblasts, and these genes were categorized based on their molecular functions. Many of the upregulated genes can be categorized as 'signal transduction' or 'inflammatory response' genes. It has previously been suggested that IL-1β contributes to the production of inflammatory factors by modulating the expression of signal transduction.

Numerous chemokine superfamily members, which were categorized inflammatory response and chemotaxis genes, were also among the top 10 IL-1β-responsive genes identified in this study. Chemokines are considered key players in the diapedesis of leukocytes from the vasculature into tissues in inflammatory diseases (21–23). Inflammatory arthropathies are characterized histologically by infiltration of inflammatory cells and enlargement of the synovial lining layer (24, 25). Accumulation of neutrophils, activated T cells, and macrophages in inflamed synovial tissues may lead to significant structural damage to joints with RA (11, 24, 25). Inflammatory cells have also been detected in synovial tissue and in fluid from patients with intracapsular pathologies of the TMJ (1, 3, 5). The mechanisms leading to cellular infiltration of the synovium and joint degeneration have been elucidated to a degree by studying the release of degradative enzymes (26), various products of oxidative metabolism (27), and inflammatory cytokines (24). 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 IL-1β, matrix degradative enzymes, and various products of oxidative metabolism; (iii) the enzymes and oxidative metabolites cause degradation of the extracellular matrix; (iv) the inflammatory cytokines stimulate synovial fibroblasts to produce more chemokines. Furthermore, the subset of CXC-type chemokines that contain the sequence Glu-Leu-Arg (the 'ELR' motif), CXCL1, 2, 3, 6, 8, are also thought to promote angiogenesis (28). Thus, induction of the ELR-CXCs may lead to both recruitment of inflammatory cells and new small vessels in synovial tissues. Although chemotaxis is a necessary function of homeostasis, inappropriate infiltration of inflammatory cells may cause joint degeneration.

The other genes among the top 10 responsive genes were BCL2A1 (Bfl-1/A1 protein), PTGS2 (COX2), CSF3 (G-CSF), IL-1B, and IL-6. Many reports have demonstrated that prostaglandin E2 synthesized by COX2, G-CSF, IL-1β, and IL-6 is increased in synovial tissues and fluids with severe inflammation, such as in RA or OA (29–31). Bfl-1/A1 protein is a member of the Bcl-2 family, which also includes Bcl-2 and Bcl-x (L), and suppresses apoptosis (32). This factor may be associated with hyperplasia of the synovial lining.

The development and application of genome-scale technologies for studying the IL-1β response may help to understand inflammation and develop new treatments. This study looked at changes in the gene expression of chemokines in response to IL-1β in synovial fibroblasts from the human TMJ using microarray. We also compared the gene expression levels detected by microarray and real-time PCR methods. Although the fold-change between control and IL-1β-treated synovial fibroblasts measured by real-time PCR differed slightly from that on microarray analysis, the rank order of IL-1β-responsive genes was similar, thus suggesting that gene chip analysis is a valid and powerful tool for analyzing the expression of a large number of molecules.

We used microarray analysis to identify differentially expressed genes in order to characterize synovial fibroblasts. As expected, synovial fibroblasts express vimentin, prolyl 4-hydroxylase, and type I collagen, which are fibroblast markers. In contrast, the expression of HLA class II antigens were not detected (data not shown). We observed no significant differences in the gene expression levels of cell markers between control and IL-1β-treated cells. These data were consistent with the notion that synovial fibroblasts are fibroblasts-like cells with no role in antigen presentation.

In conclusion, our comparative gene expression profile analysis has revealed interesting features of synovial fibroblasts from human TMJ. Upon IL-1β stimulation, synovial fibroblasts expressed high levels of several chemokines, suggested that IL-1β promotes the recruitment of leukocytes to the inflammation sites in TMJ synovium. These findings demonstrated that synvoial fibroblasts in TMJ should be considered as important as its surrounding cells or tissue in the initiation or progression of inflammatory TMJ. In addition, we also suggest that the present data will be useful for the identification of candidates for genes with key roles in the initiation and progression of pathologic intracapsular conditions of the TMJ.

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