Microarray Analysis Detection of Signaling Pathway Responsive to IL-1 β in Synovial Fibroblasts from TMJ

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Abstract

Interleukin-1 β (IL-1 β) has important roles in the inflammation and connective tissue destruction observed in joint diseases such as rheumatoid arthritis. IL-1 β is also a key mediator of intracapsular pathologic conditions of the temporomandibular joint (TMJ), including disk displacement/internal derangement and osteoarthritis. To identify putative IL-1 β -responsive genes from arthritic disease tissues, gene expression of IL-1 β treated and untreated synovial fibroblasts was measured using DNA microarray, and IL-1 β -responsive genes were analyzed with GeneSpring. To measure the expression of 8,793 genes in synovial fibroblasts from five TMJ patients, significant changes between controls and IL-1 β -treated cells ($p < 0.05$) were detected in 170 genes; 139 up-regulated genes, and 31 down-regulated genes. In addition, the biological interactions of IL-1 β -regulated genes were investigated using Ingenuity Pathway Analysis, and it was found that IL-1 β affects the expression of several genes in the NF κ B signaling pathway. NF κ B1 gene expression increased in synovial fibroblasts by IL-1 β treatment. Gene expression of I κ B α , TNFAIP3 and TNIP1, which are negative-feedback regulators, were also increased after IL-1 β treatment in synovial fibroblasts. The increase in the expression of these genes was confirmed by real time-PCR analysis. The results suggest that IL-1 β -responsive genes play important roles in the progression of inflammation and destruction of joint components.

Introduction

Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine that has numerous effects, including cell proliferation (1), inflammatory responses (2) and induction of matrix remodeling (3). It has been well documented that elevated expression of IL-1 β in the joints can result in inflammatory and degradative responses in synovial fibroblasts, responses that contribute to the progression of osteoarthritis (OA) and rheumatoid arthritis (RA) (1, 4).

Intracapsular pathologic conditions of the temporomandibular joint (TMJ) are characterized by noises associated with jaw motion, limited jaw motion and joint pain. Patients can also experience disk displacement/internal derangement (DD/ID) and OA of the TMJ. In patients with OA and RA in the TMJ, the level of IL-1 β in synovial fluids is

elevated (5). Several studies have also indicated that an increased level of IL-1 β in the synovial fluid is associated with pain and hyperalgesia in the TMJ (6, 7). The condition (known as synovitis) often accompanies DD/ID and OA of the TMJ and is characterized by chronic inflammatory changes, including hyperplasia of the synovial lining (8, 9), growth of small new blood vessels (9) and infiltration of inflammatory cells (9-11). Synovial fibroblasts are a major source of the mediators of joint inflammation. Propagation of synovial fibroblasts in the presence of IL-1 β provides a useful *in vitro* model with which to dissect the molecular components of IL-1 β bioactivity in joint diseases (12).

The DNA microarray technique provides a means to perform comprehensive expression analysis of a large number of pre-defined genes (12-14). In this study, we performed DNA microarray analysis to search for differentially expressed genes under the notion that they could provide diagnostic and therapeutic markers for pathogene-

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sis and lead to new treatments for IL-1 β -related diseases. In addition, we performed a pathway analysis of microarray data to investigate the key functions and signaling pathways involved in inflammation and joint destruction.

Materials and Methods

Cell culture

Human synovial tissue was obtained from patients with internal derangement who had undergone arthrotomy or arthroscopic synovectomy of the TMJ. There were five patients (three female and two male) aged between 17 and 27 years included in the study set. Patients gave complete informed consent for the surgery and for the use of their tissue in research. Isolation of cells 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 (EC07-004 and EC10-037). Synovial fibroblast cultures were isolated from fresh TMJ synovial tissue biopsy samples as reported previously (15). Experiments were performed with synovial fibroblasts from the 6th to 8th doubling passages.

Total RNA extraction

Synovial fibroblasts were incubated with or without 100 pg/ml of IL-1 β for 4 hours (h). Total cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) and was then stored at -80°C until use.

DNA microarray analysis

For gene expression profiling, we used the Affymetrix GeneChip Human Genome Focus Array (Affymetrix, Santa Clara, CA) 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). Biotin-labeled cRNA, which was synthesized by *in vitro* transcription using the BioArray high-yield RNA transcript labeling system (ENZO, Farmingdale, NY), was broken down in the fragmentation buffer (Affymetrix) for 35 min at 95°C . Fragmented cRNA (10 μg) was hybridized to the Human Genome Focus Array (8,793 genes) for 16 μh at 45°C . Arrays were subjected to washing and staining with R-phycoerythrin streptavidin (SAPE, Molecular Probes) using GeneChip Fluidics Station 400 (Affymetrix). To amplify the signals, the arrays were further stained with goat biotinylated anti-streptavidin antibody (Vector

Laboratories), followed by staining with SAPE. After scanning (scanner from Affymetrix), hybridization data were exported for gene expression value analysis using Affymetrix Microarray Suite (version 5.0).

Data analysis

Analysis of oligonucleotide hybridization data was performed using GeneSpring software (Agilent Technologies, Santa Clara, CA). We normalized the expression intensity of each array using the mean value of expression intensity in order to examine the consistency of gene regulation by IL-1 β . Next, Student's t-test was used to detect significant differences ($p < 0.05$) in gene expression between control (non-treated) and IL-1 β -treated fibroblasts from five patients using GeneSpring software.

Pathway analysis

IL-1 β -responsive genes were analyzed using Ingenuity Pathway Analysis (IPA; Ingenuity, Redwood City, CA). Gene accession numbers and ratios of gene expression of IL-1 β -responsive genes were uploaded into the Ingenuity Pathway Knowledge Base (www.ingenuity.com). To build networks, IPA queries the Ingenuity Pathway Knowledge Base for interactions between the uploaded genes ("focus genes") and all other gene objects stored in the Knowledge Base. The IPA then generates a set of networks with a maximum network size of 35 genes/gene products.

Real time-PCR

cDNA was synthesized using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using 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 (BioRad, Hercules, CA), 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. Amplicons were directly detected by measuring the increase in fluorescence caused by binding of SYBR Green using a DNA Engine Opticon 1. Gene expression levels were calculated using the $\Delta\Delta\text{Ct}$ method with normalization against GAPDH (16). Each assay was normalized against GAPDH mRNA levels. Primers were as follows: 5'-tat-gagggtctctctgggggtaca-3' (forward primer for NFkB1), 5'-agt-tagcagtggaggcaccact-3' (reverse primer for NFkB1), (product size; 267 bp), 5'-tcaatgctcaggagccctgta-3' (forward

primer for IKBa), 5'-tctctggcagcatctgaaggt-3' (reverse primer for IKBa), (product size; 222bp), 5'-tgttgaaacggggc-tttgct-3' (forward primer for TNFAIP3), 5'-cggatttcaggcc-cactgtc-3' (reverse primer for TNFAIP3), (product size; 357bp), 5'-ttctgcagaccctgtgtgag-3' (forward primer for TNIP1), 5'-gctccagcatcttcaccttc-3' (reverse primer for TNIP1), (product size; 303bp), 5'-atcaccatvtccaggag-3' (forward primer for GAPDH), 5'-atcgactgtggcatgag-3' (reverse primer for GAPDH), (product size; 318bp).

Results

IL-1 β -responsive genes in synovial fibroblasts

In order to investigate which genes respond to IL-1 β in synovial fibroblasts from patients with ID and/or OA of TMJ, 8793 genes were compared between untreated control and IL-1 β -treated synovial fibroblasts from five patients. By applying Student's *t*-test, we detected a significant difference between control and IL-1 β -treated cells ($p < 0.05$) in 170 genes; 139 up-regulated genes and 31 down-regulated genes.

The 170 IL-1 β -responsive genes were categorized based on the gene ontology of molecular function using GeneSpring software (Fig.1). The category with the most genes was "enzyme" (54 genes; 46 up-regulated and 8 down-regulated), followed by "signal transducer" (46 genes; 41 up-regulated and 5 down-regulated), and "nucleic acid binding" (39 genes; 32 up-regulated and 7 down-regulated). It was speculated that IL-1 β generates a number of signals, and some genes fall into several overlapping ontological categories.

Network analysis of IL-1 β -responsive genes

The 170 IL-1 β -responsive genes were subjected to pathway analysis using IPA to determine the biological interactions. It is immediately evident from looking at Figure 2 that NF κ B (NF κ B; nuclear factor of kappa-light polypeptide gene enhancer in B-cells) complex such as NF κ B1-RelA is a key regulatory molecule for genes in the network. In mammals, the NF κ B family contains five homologous members; RelA (also known as p65), RelB, cRel, NF κ B1 and NF κ B2. These proteins interact in combination to form a family of homodimers and heterodimers that constitute the transcriptionally active proteins(17). NF κ B1 transcription is partially mediated by members of the NF κ B family by binding the κ B element in the NF κ B1 promoter (18, 19).

Fig.1 Gene Ontology of IL-1 β -responsive genes. IL-1 β -responsive genes (170 genes in total) show a significant difference ($p < 0.05$) between control and IL-1 β -treated cells. Note that some genes can be categorized into several ontological groups.

Activation of NF κ B stimulates TNFAIP3 (TNF- α -induced protein 3, also known as A20) (18) and TNIP1 (TNFAIP3-interacting protein 1, also known as ABIN1; A20-binding inhibitor of NF κ B1) (Fig.2) (18). These molecules have been recognized as negative regulators for NF κ B activation signaling(20, 21).

Canonical Pathway of NF κ B activation

The network was linked to a graphical representation of the Canonical Pathway "NF κ B signaling" in IPA. As shown in Fig.3, this illustration exhibits the canonical pathway for "NF κ B signaling" through IL-1 and TNF, which are key cytokines in inflammation and destruction in joint diseases (22, 23). Nodes, which are shown as genes and/or gene products, are associated with NF κ B activation. The red nodes showed average gene expression levels on microarray analysis in synovial fibroblasts from five patients. We divided the nodes into two parts; the left shows relative expression levels for controls and the right shows relative expression levels in IL-1 β -treated samples.

The IL-1 type I receptor and its adaptor molecules were constitutively expressed in synovial fibroblasts (Fig.3). Expression of IL-1 α and IL-1 β was up-regulated after treatment with IL-1 β , an effect that may be mediated by NF κ B. IL-1 β was predominant when compared to IL-1 α in synovial fibroblasts from all five patients.

TNF- α was indicated "absent" in synovial fibroblasts, irrespective of IL-1 β treatment by microarray analysis. Type I TNF receptor and several recruited molecules were constitutively expressed in synovial fibroblasts. Although

Fig.2 Network One Pathways as determined by IPA. Nodes (genes/gene products) of the network are displayed as various shapes that represent the functional classes of the gene products (see key at bottom of figure).

the type II TNF receptor was up-regulated by IL-1 β , expression levels of this receptor in synovial fibroblasts treated with IL-1 β were lower than those observed for type I receptor.

NF κ B1 (NFKB1) and NF κ B2 (NFKB2) were significantly induced by IL-1 β . In contrast, RelA and RelB were constitutively expressed in synovial fibroblasts and only slightly stimulated by IL-1 β (data not shown). Inhibitor κ B (I κ B) has several identified isotypes, including I κ B α , I κ B β and I κ B ϵ (17). I κ B α (IKBa), which was detected in controls, was significantly enhanced in synovial fibroblasts treated with IL-1 β in this study.

TNFAIP3 and TNIP1, which inhibit NF κ B activation (20, 21), were up-regulated by IL-1 β treatment. cIAP (inhibitor apoptosis protein), which fails to TNF-induced apoptosis,

Fig.3 Canonical Pathway of NF κ B activation through IL-1 or TNF signaling. The shading of the nodes shows the average level of gene expression in synovial fibroblasts from five patients as determined by microarray analysis. The highest levels of expression correspond to the darkest shading. Shading in left part of a molecule shows the relative levels of expression in the control and shading in the right part shows the relative levels of expression in IL-1 β -stimulated samples.

was also up-regulated by IL-1 β , whereas other molecules in the IL-1 β signaling pathway for NF κ B activation were not responsive to treatment with IL-1 β .

Time course of gene expression

Gene expression profiles were investigated in synovial fibroblasts treated for 4h. Next, the time-course of gene expression was examined in synovial fibroblasts after incubation with or without IL-1 β for 2, 4 or 8h using real-time PCR. Microarray data revealed that expression of NFKB1 and IKBa was predominant among NF κ B family

Fig.4 Time course of gene expression. (A) NFKB1, (B) IKBa, (C) TNFAIP3 and (D) TNIP1. Synovial fibroblasts were stimulated by IL-1 β (100pg/ml) for 2, 4 or 8 h. * $P < 0.05$, ** $P < 0.01$; control vs. IL-1 β -treatment at the indicated time point, as analyzed by Student's t -test ($n=3$).

members in synovial fibroblasts, and was induced by 4 h of IL-1 β treatment. Relative gene expression was higher in synovial fibroblasts after 2h of IL-1 β treatment, and this increase persisted at 4h, but the levels decreased after 8h of treatment (Fig.4A, B). Gene expression of TNFAIP3 and TNIP1 was also examined by real-time PCR because these were suppressed in NF κ B activation signaling. The relative gene expression of TNFAIP3 was maximal at 2h, and then decreased toward 8h in synovial fibroblasts treated with IL-1 β (Fig.3C). In contrast, the relative gene expression of TNIP1 peaked at 4h after 8h of treatment (Fig.4D)

Discussion

In this study, we performed DNA microarray analysis in order to examine the consistency of gene regulation in synovial fibroblasts treated with IL-1 β . We hypothesized that identification of IL-1 β -responsive genes in synovial fibroblasts could help develop models for the inflammatory status of the TMJ.

Among the 8,793 genes tested, a total of 170 genes exhibited a significant difference between controls and IL-1 β -treated synovial fibroblasts ($p < 0.05$), and these genes were categorized based on their molecular functions. Many of the up-regulated genes can be categorized as "enzymes", "signal transducers" and "nucleic acid binding"

genes (Fig.1). Thus, it was suggested that IL-1 β contributes to the production of inflammatory factors by modulating the expression of signal transduction.

The 170 IL-1 β -responsive genes were assessed by IPA in order to determine biological interactions, and we found that the NF κ B signaling pathway is a key regulator in IL-1 β -treated synovial fibroblasts (Fig.2). NF κ B was originally described as a nuclear protein in B lymphocytes that bound to κ B target DNA sites (22), controlling transcription of a large number of cellular activation events critical for development, proper control of cell growth and proliferation, the immune response, control of apoptosis and survival, and stress responses to a variety of noxious stimuli (23, 24). The NF κ B network is critical for health, and aberrant NF κ B activity plays a role in a variety of human cancers (25) and chronic inflammatory diseases, such as rheumatoid arthritis (26). An understanding of the underlying molecular mechanisms of the control of signaling by NF κ B may well lead to the design of modulators of chronic inflammation and better chemotherapeutic agents (26, 27).

NF κ B members, NF κ B1, NF κ B2, RelA, RelB and cRel, dimerize to form homo- and hetero-complexes of various transcriptional activities. RelA, RelB and cRel contain active transcriptional activation domains but NF κ B1 and NF κ B2 lack transcriptional activity (17). In this study, NF κ B1 and NF κ B2 were significantly induced by IL-1 β , while RelA and RelB were constitutively expressed in synovial fibroblasts and only slightly stimulated by IL-1 β (data not shown). RelA/NF κ B1 complex may be predominant in synovial fibroblasts, because the expression levels of NF κ B1 and RelA were higher in synovial fibroblasts when compared with NF κ B2, RelB and cRel (data not shown).

NF κ B binds to I κ B in the cytoplasm, and activation of NF κ B requires degradation of I κ B, which exposes the NF κ B nuclear localization sequence, allowing NF κ B to translocate to the nucleus (17, 23). Several I κ B proteins have been identified, including I κ B α , I κ B β and I κ B γ . The inhibitory potential of most I κ B family members is carried by I κ B α , whose synthesis is controlled by a highly NF κ B-responsive promoter generating auto-regulation of NF κ B signaling (28). In this study, I κ B α , which is detected in controls, is significantly enhanced in synovial fibroblasts treated with IL-1 β (Fig.4). I κ B α may be predominant among the I κ B members in synovial fibroblasts, because the expression of other I κ Bs were low or absent in microarray analysis (data not shown).

TNFAIP3 and TNIP1, which inhibit NF κ B activation (20, 21), were up-regulated by IL-1 β treatment (Figs.2 and 3). The zinc finger protein TNFAIP3 has an essential role in limiting the strength and duration of NF κ B signaling (29). TNFAIP3-deficient mice die prematurely from multiorgan inflammation and cachexia, and TNFAIP3-deficient cells exhibit a defect in the termination of tumor necrosis factor- α (TNF- α) and lipopolysaccharide-induced NF κ B signaling (30, 31). TNFAIP3 inhibits the E3 ligase activity of TRAF6 and TRAF2 by antagonizing interactions with the E2 ubiquitin conjugating enzymes (32). TNIP1 is known as a TNFAIP3-associated molecule that inhibits NF κ B activation by interacting with TNFAIP3. It has been reported that TNIP1 controls toll-like receptor-mediated CCAAT/enhancer-binding protein β activation, protecting from inflammatory diseases (33). Combination analysis of microarray and signaling pathways showed that IL-1 β stimulates both activators and inhibitors during NF κ B activation signaling through IL-1 and TNF.

In this study, gene expression of NF κ B1, I κ B α and TNIP1 peaked after 4 h of IL-1 β -treatment in synovial fibroblasts, but the levels decreased significantly after 8 h of treatment (Fig.4). Our previous study reported that gene expression of monocyte attractant protein-1 (MCP-1), which was stimulated by NF κ B, peaked at 4 h in IL-1 β -treated synovial fibroblast (34). Therefore, NF κ B activation may peak in synovial fibroblasts after 4 h of IL-1 β -treatment, and is then reduced by negative-feedback factors, such as I κ B α , TNFAIP3 and TNIP1.

Gene expression of TRAF6 and TAB1 in the IL-1 signaling pathway, and TRAF2 and NIK in the TNF signaling pathway were absent in synovial fibroblasts irrespective of IL-1 β treatment. This suggests that expression of these transcription factors is very weak and/or is enhanced at very early time points (within several minutes).

In conclusion, combination analysis with microarray and IPA provides a powerful tool for analysis of genes that are differentially expressed in synovial fibroblasts. In this study, we examined synovial fibroblasts from five patients and tested the statistical significance of differences in gene expression in order to compare control and IL-1 β -treated cells. Furthermore, many of the IL-1 β -responsive genes in synovial fibroblasts were well-characterized genes known to interact biologically. We believe that the present data will be useful for identification of candidate genes with key roles

in the initiation and progression of intracapsular pathological conditions such as TMJ.

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