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Effects of IL-1β on gene expression in human rheumatoid synovial fibroblasts

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Abstract

IL-1 is one of the key mediators involved in the pathogenesis of rheumatoid arthritis (RA) and is known to affect the level of gene expression in various settings. We investigated the effects of IL-1β on the expression of 240 genes in rheumatoid synovial fibroblasts (RSFs) using a cDNA microarray. Total RNAs were prepared from RSFs stimulated with IL-1β and hybridized to the microarray. The fluorescence intensity of each gene was compared between the control and IL-1β-treated cells. To confirm the data obtained from the microarray analysis, the level of gene expression was also examined by ELISA, Northern blot, or Western blot depending on the genes to be analyzed. The genes whose levels were significantly changed by IL-1β in the microarray analysis could be divided into three categories; inflammatory mediators, matrix-modifying enzymes, and apoptosis-associated molecules. The increase in the mRNA levels of IL-6, IL-8, MCP-1, and GRO-1 was confirmed by determining their protein levels from the cell culture supernatant using ELISA. The increase in the level of two matrix-degrading enzymes, MMP-1 and MMP-3, was reproducibly observed by an ELISA method, while the decrease in the level of TIMP-3, an inhibitor of MMPs, was confirmed by Northern blot analysis. The fluorescence intensity of two apoptosis-related genes, caspase-3 and Bcl-xL, was significantly lowered. The decreased protein level of caspase-3 was also found. Our data suggested that IL-1β could provoke a series of responses in RSFs leading to the pathologic status of RA, including enhancement of inflammatory cytokines, imbalanced production of MMPs and TIMPs, and dysregulation of apoptosis.

Keywords: cDNA microarray; IL-1β; Rheumatoid synovial fibroblasts

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown cause, which is characterized by synovial hyperplasia, inflammation, and joint destruction. The excessive growth of rheumatoid synovial fibroblasts (RSFs) is thought to be responsible for the formation of pannus, which invades adjacent tissues including cartilage and bone [1,2]. It is not yet clear whether RSFs are permanently changed in association with genetic alterations or are passively changed by virtue of environmental factors such as cytokines [2].

Proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) play major roles in the pathogenesis of RA [3]. In contrast with TNF-α, which is predominantly detected during the early stages of disease, IL-1 is detected long after the onset of RA at high levels in the local area. IL-1 binds to the cellular receptor and increases the release of other inflammatory cytokines and various metalloproteinases (MMPs) [3]. The cartilage destruction observed in RA is mostly caused by the activity of MMPs [4].

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Indeed, administration of IL-1 provoked the synovitis and the loss of proteoglycan from cartilage in animal models [5].

We studied the expression of selective human genes in IL-1β-stimulated RSFs using a cDNA microarray. The expression level of each gene was compared between RSFs treated and untreated with IL-1β. The results obtained from the microarray were confirmed by other methods including ELISA, Northern blot, and Western blot. Our data clearly suggested pleiotropic roles of IL-1β in the pathogenesis of RA.

Materials and methods

Isolation and culture of RSFs. Synovial tissue was recovered from the joint of a male patient undergoing surgery for the management of his RA. Cells were isolated by sequential digestion of synovium with trypsin and collagenase, and cultured in Dulbelcco's modified essential medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 20% (v/v) fetal bovine serum (Gibco-BRL), 120 μg/ml penicillin G (Sigma, St. Louis, MO, USA), and 200 μg/ml streptomycin sulfate (Sigma).

Preparation of total cellular RNAs. RSFs were seeded in 10-cm tissue culture dishes at density of 5×10^5 cells/dish. After 24 h, the cells were treated with IL-1β (R&D Systems, Minniapolis, MN, USA) at a concentration of 0.1 μg/ml. After 40 h, total cellular RNAs were prepared by the guanidine thiocyanate–cesium chloride method.

Microarray analysis. The cDNA microarray analysis was performed using the IntelliGene Human Cytokine CHIP (TaKaRa Bio, Kyoto, Japan) containing cDNA fragments of 240 human genes. The cDNA probe synthesized by reverse transcription of 20 μg of total RNAs prepared from RSFs treated or untreated with IL-1β. The reverse transcription was carried out using AMV reverse transcriptase (TaKaRa Bio) in the presence of Cy3-dUTP or Cy5-dUTP (Amersham–Pharmacia, Little Chalfont, UK). Probes were hybridized to the cDNA microarray for 16 h at 65 °C in a custom-built hybridization chamber. The cDNA microarray was washed for 5 min at 55 °C with 2 × SSC/0.2% SDS and at 65 °C with 2 × SSC/0.2% SDS. Microarray was scanned using GMS 418 Array Scanner (Genetic MicroSystems, Woburn, MA, USA) and the scanned images were analyzed using the ImaGene software (BioDiscovery, Marina del Rey, CA, USA) for quantification of each spot. The relative expression level of each gene was standardized based on the average expression levels of β-actin.

Enzyme linked immunosorbent assay. Cytokine concentrations in supernatants of the control and IL-1β-treated RSFs were measured using commercial enzyme linked immunosorbent assay (ELISA) kits for IL-6, IL-8 (Pierce Chemical, Rockford, IL, USA), MCP-1, GRO-1, MMP-1, and MMP-3 (R&D Systems), according to the manufacturer's recommendations.

Northern blot analysis. Total cellular RNAs were prepared by the guanidine thiocyanate–cesium chloride method. The DNA probes for TIMP-2, TIMP-3, and β-actin were synthesized in the presence of [^{32}P]dCTP (Amersham–Pharmacia). Ten micrograms of total RNAs was resolved in 1% formaldehyde–agarose gels and blotted onto nitrocellulose membranes. The membranes were hybridized with the probes at 68 °C for 1 h using ExpressHyb hybridization solution (Clontech laboratories, Palo Alto, CA, USA) and then washed twice with 2× SSC/1% SDS at room temperature for 20 min, followed by three times with 0.1× SSC/1% SDS at 65 °C for 20 min. The membranes were exposed to autoradiography film with an intensifying screen at −80 °C.

Western blot analysis. RSFs were harvested and centrifuged at 1200 rpm for 5 min. The pellets were then lysed in a lysis buffer containing 500 mM NaCl, 1 mM EDTA, 10 mM Tris, pH7.4, and 0.5% (v/v) Triton X-100. The solubilized cell homogenate was harvested and centrifuged at 12,000 rpm for 5 min. The resultant supernatant was used for further analysis. Total protein concentration was determined with the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (10 μg) of protein were loaded on 12% SDS–polyacrylamide gel. Proteins were separated electrophoretically and transferred to nitrocellulose membrane. The protein-blotted membranes were blocked with 3% bovine serum albumin (Sigma, Saint Louis, MO, USA) in Tris-buffered saline (TBS; 10 mM Tris, pH 7.5, 0.15 M NaCl) with 0.05% Tween 20 (TBST) for 1 h at room temperature. They were incubated with anti-caspase-3 antibody (NeoMarkers, Fremont, CA, USA) for 1 h at room temperature at 1:1000 dilution in TBST. After washing three times for 5 min with TBST blots were further incubated with for 1 h at room temperature with anti-mouse IgG antibody coupled to horseradish peroxidase (Pierce Chemical) at 1:100,000 dilutions in TBST. Blots were washed three times for 5 min with TBST, and visualized by enhanced chemiluminescence using SuperSignal Substrate Western Blotting (Pierce Chemical).

Results

Microarray analysis of RSFs treated with IL-1β

Human RSFs were cultured for 40 h in serum-containing medium in the presence or absence of human IL-1β. Total RNAs were prepared and used for hybridization with cDNA microarray containing 240 genes. Genes of our particular interest were those involved in inflammation, destruction of cartilage, apoptosis, and angiogenesis. Only the genes producing a hybridization signal 3-fold higher than the background level (non-specific signal of the hybridized microarray) were considered to be worth further analyzed. The fluorescence intensity of each spot was compared between the control and IL-1β-stimulated RSFs.

Ten genes showed intensity greater than 3-fold difference between the control and treated RSFs (Table 1). The genes whose levels were significantly altered by IL-1β could be classified into three categories based on their biological roles. IL-1β increased the expression of the genes known to mediate the inflammatory response. They were IL-1β itself, IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), and growth-related oncogene-1 (GRO-1). The expression of the two matrix-degrading enzymes, matrix metalloproteinase-1 (MMP-1) and MMP-3, was significantly increased by IL-1β, while the level of tissue inhibitor of metalloproteinases-3 (TIMP-3) was decreased by almost 7-fold. The expression of two apoptosis-associated genes, caspase-3 and Bcl-xL, was down-regulated by IL-1β. These results showed that IL-1β could modulate the expression levels of various genes in RSFs.

Inflammatory mediators

The dysregulated expression of inflammatory cytokines or chemokines is one of the hallmarks in the

Table 1
Microarray analysis of gene expression in RSFs treated with IL-1β

Gene	Fold[a]	GenBank No.
Inflammatory mediators		
IL-1α	2.9	M28983
IL-1β	22.9	M15330
IL-6	71.0	X04430
IL-8	3.8	M26383
GRO-1	45.0	X54489
MCP-1	14.8	M26683
TNF-α	ND	X02910
Extracellular matrix modifying enzymes		
MMP-1	3.1	AK024818
MMP-3	46.1	X05232
MMP-9	ND	AL162458
TIMP-2	−1.9	AL110197
TIMP-3	−6.9	NM_000362
Apoptosis-associated molecules		
Bcl-xL	−4.7	Z23115
Caspase-3	−5.2	U13737
Caspase-9	ND	U60521
Fas	−2.1	X63717
Angiogenesis-regulating factors		
Angiogenin	−1.1	M11567
Angiopoietin-2	ND	AF004327
FGF-1	ND	X59065
FGF-2	−2.6	NM_002006
HGF	ND	X16323
SDF-1	−1.5	U16752
VEGF	−1.9	AF022375
VEGF-B	−1.4	U43368

[a] Fold indicates the up-regulation or down-regulation (−) of the level of each gene expression in IL-1β-treated RSFs. The mean value of fluorescence intensities of background signals was subtracted from the intensity of each spot. This was then normalized by the mean levels of β-actin. The fold variation was obtained by dividing each normalized value in IL-1β-treated cells by that in untreated cells. ND; the hybridization signal lower than the background level.

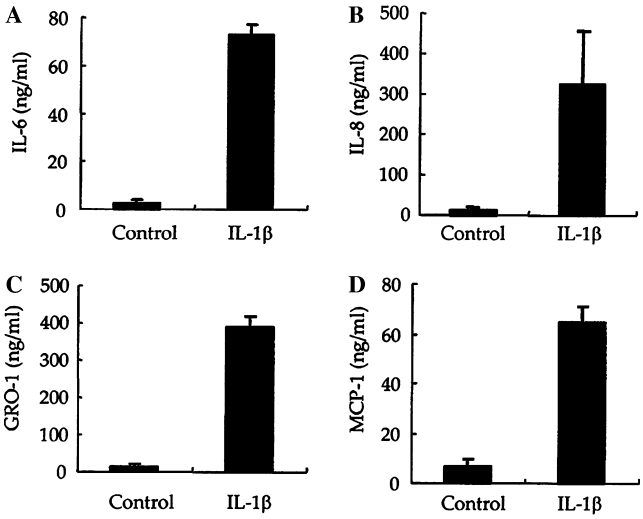

Fig. 1. Effects of IL-1β on production of IL-6 (A), IL-8 (B), GRO-1 (C), and MCP-1 (D). RSFs were treated with IL-1β for 40 h and the levels of representative cytokines were determined by ELISA. Data are means ± standard deviation (SD) of relative values obtained in three experiments.

chronic synovitis, which is one of the major pathologic findings in RA [6]. All of these molecules are secreted proteins whose level can be determined from the culture supernatant by ELISA. We compared the protein concentrations of cytokines in culture supernatants between the control and IL-1β-stimulated RSFs using ELISA. In the microarray analysis, the expression of IL-1β, IL-6, IL-8, MCP-1, and GRO-1 was elevated about 20-, 70-, 4-, 15-, and 45-fold, respectively, by IL-1β (Table 1). Consistent with these data, protein levels of IL-6, IL-8, GRO-1, and MCP-1 were also highly increased in culture supernatants (Fig. 1). These results suggested that IL-1β might contribute to the synovitis by up-regulating the expression of IL-6, IL-8, GRO-1, and MCP-1 in RSFs.

Matrix-modifying enzymes

In addition to synovial inflammation, IL-1 is known to be a key mediator of bone resorption and cartilage destruction [3]. The microarray analysis showed that the expression of MMP-1 and MMP-3 was increased by 3- and 46-fold, respectively, when RSFs were stimulated with IL-1β. On the contrary, the mRNA level of TIMP-3, one of the inhibitors of MMPs, was decreased by 7-fold under the same condition (Table 1). Other MMPs contained in the microarray include MMP-9, -10, -12, -13, and -15, but their fluorescence intensities were around the background level both in the control and stimulated cells. As expected from the microarray analysis, the protein level of MMP-1 was moderately increased by 2.6-fold in IL-1β-treated RSFs, while the magnitude of increase in the level of MMP-3 was approximately 25-fold (Figs. 2A and B). Because the ELISA kit was not available for TIMP-3, Northern blot was used to confirm the result. It was found that the mRNA level of TIMP-3 was decreased by about 3-fold, while that of TIMP-2, used as a control, remained virtually unchanged (Fig. 2C). The level of TIMP-1 expression was not altered, either (data not shown). These results indicated that IL-1β might be involved in cartilage destruction by inducing the imbalance in the production of MMPs and TIMPs in RSFs.

Apoptosis-associated molecules

The hyperplasia of synovial tissue is ascribed to both an increased cellular activation [7] and an impaired apoptosis of synovial cells [8]. The microarray analysis revealed that the expression of both caspase-3 and Bcl-xL was down-regulated by approximately 5-fold in IL-1β-treated cells as compared with that in the control. The change in the level of Fas expression was less than 3-fold. The hybridization signal of caspase-9 both in

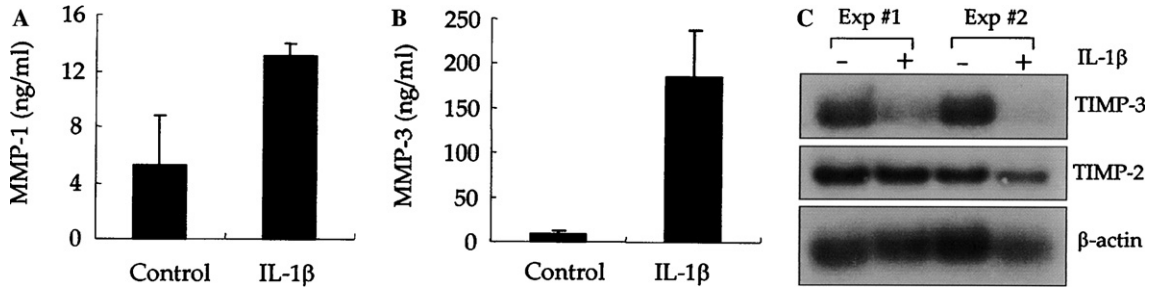

Fig. 2. Effects of IL-1β on expression of matrix-modifying enzymes. Forty hours after RSFs were treated with IL-1β, the levels of MMP-1 (A) and MMP-3 (B) were measured by ELISA. Data represent means ± SD of relative values obtained in three experiments. (C) Northern blot analysis of TIMP-3. Ten micrograms of total RNA extracted from RSFs was subjected to denaturing-gel electrophoresis and blotted onto a nitrocellulose membrane. This blot was probed with the cDNA fragment of TIMP-3 and TIMP-2 (as a control). As a loading control, the blot was probed with the cDNA fragment from the human β-actin gene.

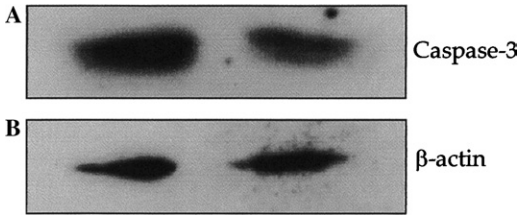

Fig. 3. Effects of IL-1β on the production of caspase-3. RSFs were cultured in the presence of IL-1β for 40 h. Total proteins extracted from RSFs were subjected to SDS–PAGE, and followed by immunoblotting with antibodies specific for caspase-3 (A) and β-actin (B; loading control).

the control and stimulated cells was too low to be quantitated (Table 1). Among these molecules, the protein level of caspase-3, the effector enzyme of apoptosis, was examined. Consistent with the result from the microarray analysis, Western blot analysis showed that the level of the caspase-3 protein was decreased by 3-fold when RSFs were treated with IL-1β (Fig. 3). Taken together, IL-1β might be involved in the suppression of apoptosis in RSFs.

Discussion

IL-1 is readily detected in the synovial fluid of patients with RA, and is widely believed to play major roles in the pathogenesis of RA including inflammation and joint destruction [4]. In this study, we showed that IL-1β could significantly alter the expression of various genes involved in the pathophysiology of RA. These genes included inflammatory mediators (IL-1β, IL-6, IL-8, MCP-1, and GRO-1), matrix-modifying enzymes (MMP-1, MMP-3, and TIMP-3), and apoptosis-regulating molecules (caspase-3 and Bcl-xL).

IL-1 binds to its receptor on the surface of target cells and induces the production of various cytokines that mediate inflammatory reactions [4]. Our observation also showed the elevation in the level of inflammatory cytokines when RSFs were treated with IL-1β. Five of seven genes whose level was increased by more than 3-fold in the microarray analysis are inflammatory mediators. IL-6 that revealed the greatest increase is involved in T cell stimulation, leading to immunological disorders associated with RA [9]. Other cytokines such as IL-8 mainly recruit lymphocytes, monocytes, and neutrophils to inflammatory sites [4].

The level of MMP-3 was also found to be highly increased by IL-1β in the microarray analysis. The level of MMP-1 was also augmented. MMP-3 and MMP-1 are known to play a major role in the destruction of cartilage seen in RA. The activity of these enzymes is regulated by TIMPs [4]. Unlike MMPs, the expression of TIMP-3 was highly decreased in stimulated RSFs. To our knowledge, the down-regulation of TIMP-3 by IL-1β has not previously been reported. In addition to the inhibition of the activities of MMPs, TIMP-3 was reported to be involved also in the induction of apoptosis and the suppression of angiogenesis [10,11].

Other interesting observation made in this study was no significant variation in the level of angiogenic or anti-angiogenic factors including VEGF, FGF, and angiopoietin-2. It is possible that IL-1β might exert an influence on the angiogenesis in an indirect manner. Chemokines such as IL-8 and GRO-1 were reported to play a role in neovascularization [12,13]. MMPs and TIMPs are also involved in the formation of new vessels [11,14].

There are some discrepancies between our results and those from previous reports. The expression of TIMP-3 [15] and VEGF [16] was reported to be enhanced by IL-1, while that of Fas was claimed to be decreased [17]. These differences might result from the variation inherent to the use of primary RSFs or the different culture conditions employed in different studies, such as the concentration of serum in culture medium and the duration of IL-1β treatment. More standardized conditions might have to be employed by investigators to resolve the differences.

Our data showed that IL-1β could invoke the enhancement of expression of inflammatory cytokines, the imbalance between the productions of MMPs and TIMPs, and the dysregulated expression of apoptosis-related genes in the synovial fibroblasts during the pathologic status of RA. The responses of RSFs to IL-1β may be an integral part of the development of various aspects of the pathophysiology of RA including synovitis, cartilage destruction, and synovial hyperplasia.

Acknowledgments

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