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Identification of genomic targets downstream of p38 mitogen-activated protein kinase pathway mediating tumor necrosis factor- α signaling

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Abstract

Inhibition of p38 MAPK suppresses the expression of proinflammatory cytokines such as TNF- α and IL-1 β in macrophages and fibroblast-like synoviocytes (FLS). However, there have been no genomewide studies on the gene targets of p38 MAPK signaling in synoviocytes. Microarray technology was applied to generate a comprehensive analysis of all genes regulated by the p38 MAPK signaling pathway in FLS. Gene expression levels were measured with Agilent oligonucleotide microarrays. Four independent sets of mRNA modulated by TNF- α and vehicle were used to measure the change of gene expression due to TNF- α , and three experiments were done to ascertain the effect of SB-203580, a p38 MAPK inhibitor, on TNF- α -induced genes. Microarray data were validated by RT-quantitative polymerase chain reaction. One hundred forty-one significantly expressed genes were more than twofold upregulated by TNF- α . Thirty percent of these genes were downregulated by the p38 inhibitor SB-203580, whereas 67% of these genes were not significantly changed. The SB-203580-inhibited genes include proinflammatory cytokines such as interleukins and chemokines, proteases including matrix metalloproteinases, metabolism-related genes such as cyclooxygenases and phosphodiesterase, genes involved in signal transduction, and genes encoding for transcription factors, receptors, and transporters. Approximately one-third of the TNF- α -induced genes in FLS are regulated by the p38 MAPK signal pathway, showing that p38 MAPK is a possible target for suppressing proinflammatory gene expressions in rheumatoid arthritis.

Keywords

microarray; synoviocytes; SB-203580; rheumatoid arthritis

RHEUMATOID ARTHRITIS (RA) is a chronic inflammatory disease that affects the synovial joints. RA synovial tissue is characterized by hyperplasia of the synovial lining cells, infiltration of inflammatory cells, and transformation of synoviocytes into destructive pannus tissue, leading to progressive degradation of cartilage and erosion of bones. The etiology of RA is not clear, although it is well established that overproduction of two major proinflammatory cytokines, tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , potentiates and propagates the inflammation.

The synovial lining is composed of two major cell types, the macrophage-like synoviocytes (MLS) and the fibroblast-like synoviocytes (FLS). FLS are a major target of the proinflammatory cytokines (41). Stimulation of proinflammatory cytokines like IL-1 β and TNF- α results in FLS proliferation (6) along with release of degradative enzymes such as collagenases and matrix metalloproteinases (MMPs). Since the FLS are the dominant cell type at the edge of invasion by the tumorlike pannus, they contribute significantly to the destruction of the adjacent cartilage and bone (27,33,44). Cytokine stimulation of the FLS also induces the FLS themselves to produce cytokines, thereby amplifying and perpetuating the inflammatory response. Because the FLS have such a prominent role in the pathology of RA, the effect of TNF- α on FLS provides a useful model to study the bioactivity of TNF- α in RA (11,43,56).

TNF- α activates a variety of signaling cascades that lead to the induction of inflammatory response. The principal cascades are driven by the nuclear factor (NF)- κ B (48,51) and mitogen-activated protein kinase (MAPK) pathways that include p38 MAPK, c-Jun NH₂-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). p38 MAPK and JNK respond to proinflammatory cytokines and cellular stresses and activate AP-1 transcription factors, while ERK mediates effects of mitogens and growth factors.

Blockade of NF- κ B nuclear translocation in TNF- α -stimulated primary RA synovial fibroblasts results in downregulation of antiapoptosis genes, cytokines, and adhesion molecule genes (56). Inhibition of NF- κ B as a treatment for RA has been supported by the efficacy of corticosteroids and sulfasalazine, both of which inhibit NF- κ B (7,50).

Inhibition of the MAPKs is another promising therapeutic strategy. Among these, inhibition of p38 MAPK is of particular interest, because this kinase is prominently expressed in both endothelial cells and the synovial lining (35). Many p38 MAPK inhibitors have been developed (26). One class of such inhibitors has a pyridinylimidazole core structure, exemplified by SB-203580, which inhibits the catalytic activity of p38 MAPK by competitive binding in the ATP pocket (13,22). This class of inhibitor suppressed the release of TNF- α , IL-1 β , IL-6, IL-8, MMP-1, and MMP-3 (5,14,25,26,53). Generally, p38 kinase inhibitors have shown efficacies in both in vitro and in vivo models in downregulating inflammation. However, the specific downstream gene targets of p38 MAPK in FLS as a result of TNF- α signaling have not been comprehensively studied.

In the present experiments, we treated FLS with TNF- α , blocked the activation of p38 MAPK by SB-203580, and conducted a genomewide analysis of all genes regulated by the p38 MAPK signaling pathway in FLS to ascertain the importance of this signaling pathway in mediating the effect of TNF- α that may be relevant to the pathology of RA. We found that p38 could affect the expression of about one-third of the genes inducible by TNF- α . Many of these genes are inflammatory genes relevant to RA, such as metalloproteinases, chemokines, and interleukins. We also found potential novel gene targets of p38 that may be relevant to RA pathogenesis. Our results extend our knowledge of the biological process regulated by p38 MAPK and reaffirm the concept that blockade of the p38 pathway could be a good drug target for the treatment of RA.

MATERIALS AND METHODS

FLS isolation and culture

The animal study was approved by the Animal Care and Use Committee of the Department of Veterans Affairs Greater Los Angeles Healthcare System and fulfilled National Institutes of Health guidelines for use of animal subjects. Male Sprague-Dawley rats were used. Rat knee synovial tissues were excised, rinsed in Dulbecco's modified Eagle's medium (DMEM)

supplemented with penicillin, streptomycin, and amphotericin B, and digested in 0.2% collagenase/dispase, 0.05% pronase for 2 h at 37°C with gentle shaking. The cell suspension was then filtered through a 100- μ m nylon mesh and washed twice with DMEM supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, and amphotericin B. Afterwards, the cells were resuspended, placed in 100-mm tissue culture dishes, and allowed to attach overnight. The culture medium was changed the next day to remove nonadherent cells. The cell culture was split 1:4 once confluence was reached. After third passage of the cell culture, >98% of cells were FLS, which were CD14 negative (macrophage marker) and prolyl-4-hydroxylase positive (FLS marker) as measured by immunofluorescence. FLS cells of fourth to eighth passage were used in the subsequent experiments. Every experiment used a different FLS cell culture prepared from a different animal group.

Inhibition of rat FLS MAPKs by SB-203580

p38 MAPK and JNK activity assays were carried out according to the manufacturer's protocol (Cell Signaling Technology, Danvers, MA) with slight modifications. p38 MAPK activity was assayed as follows. Cultured FLS (0.75×10^6 cells of 4th passage) were seeded on 100-mm culture dishes and synchronized in 1% FCS-containing DMEM for 24 h. The cells were then stimulated with 10 ng/ml TNF- α or sterile water for 15 min. The plates were washed twice with ice-cold PBS, and total cell lysate was collected with 1 ml of cell lysis buffer per plate. The cell lysate was cleared with a 14,000 rpm microcentrifugation for 10 min at 4°C. Eight hundred microliters of the cleared, TNF- α -treated cell lysate was incubated with 80 μ l of immobilized phospho-p38 MAPK (Thr180/Tyr182) MAb primary antibody (Cell Signaling Technology) with gentle shaking at 4°C overnight. The cell lysate/immobilized antibody was microcentrifuged at 14,000 g for 30 s. The pellet was washed three times with 1 ml of 1 $\times$ cell lysis buffer. On the third wash, the resuspended pellet was divided into five equal aliquots. The aliquots were microcentrifuged and washed twice with 500 μ l of kinase buffer containing 0, 0.1, 0.3, 1.0, or 10.0 μ M SB-203580. The pellets were then resuspended in 50 μ l of kinase buffer supplemented with 1 μ g of ATF-2 fusion protein for p38 kinase assay, 200 μ M ATP, and 0, 0.1, 0.3, 1.0, or 10.0 μ M SB-203580. The suspensions were vortexed gently and incubated at 30°C for 30 min. The reactions were terminated by adding 25 μ l of 3 $\times$ SDS buffer. Thirty microliters from each reaction was analyzed by Western immunoblotting.

JNK activity was assayed similarly as described above with a stress-activated protein kinase (SAPK)/JNK assay kit (Cell Signaling Technology), with the addition of an untreated control (a pull-down with 20 μ l of immobilized c-Jun fusion protein bead slurry added to 200 μ l of control cell lysate). The kinase buffer was supplemented with 200 μ M ATP and 0, 0.01, 0.1, or 1.0 μ M SB-203580.

p38 MAPK assay after TNF- α treatment

Nine 60-mm plates were seeded with 0.36×10^6 cells of fourth-passage rat FLS and synchronized in 1% FCS-containing DMEM for 24 h. Four plates were preincubated with 0.3 μ M SB-203580-containing 1% FCS medium for 2 h and then changed to 10 ng/ml TNF- α - and 0.3 μ M SB-203580-containing 1% FCS medium. Cells were collected at 15 min, 1 h, 6 h, and 24 h after TNF- α addition. Cells were collected by two washes with ice-cold PBS and 0.5 ml of cell lysis buffer. Concurrently, the other five plates were preincubated with vehicle containing 1% FCS medium for 2 h, and then four plates were changed to 10 ng/ml TNF- α -containing 1% FCS medium. Cells were collected at 15 min, 1 h, 6 h, and 24 h after TNF- α addition. Cells were collected by two washes with ice-cold PBS and 0.5 ml of cell lysis buffer. The last plate served as the non-TNF- α treatment control and was harvested in the same way as the others. The cell lysates were cleared by a 14,000 rpm microcentrifugation for 10 min at 4°C. p38 MAPK activity was assayed according to the manufacturer's protocol.

TNF- α and SB-203580 treatment for microarray analysis

Cultured FLS (0.5×10^6 cells) were seeded onto 100-mm culture dishes, allowed to attach overnight in DMEM supplemented with 10% FCS, antibiotics, and fungicide, and then synchronized in DMEM containing 1% FCS for 24 h. The cells were placed in culture medium containing 1% FCS and either 0.3 μ M SB-203580 or the equivalent volume of DMSO for 2 h. After the 2-h preincubation, the cells were placed in medium containing TNF- α at a 10 ng/ml final concentration or the equivalent volume of sterile water along with 0.3 μ M SB-203580 or DMSO. The cells were harvested after 24 h of incubation by scraping. Trypan blue uptake showed that the exposure of the cells in this experiment to 0.3 μ M SB-203580 had no effect on cell viability (data not shown), which was the same result as previously reported (15,28).

Microarray procedures

Total RNA was extracted with the Ambion RNAqueous Kit (Ambion, Austin, TX) according to the manufacturer's protocol. One microgram of total RNA was used for fluorescently labeled cRNA synthesis with the Agilent Low RNA Input Fluor Linear Amp Kit and protocol (Agilent, Santa Clara, CA). For hybridization, 3.5 μ g of Cy3- or Cy5-labeled cRNA from treated and vehicle-treated cells was combined and hybridized to an Agilent 44K Whole Rat Genome Oligo Microarray (Agilent) according to the manufacturer's protocol. The oligonucleotide microarray slides were scanned with an Agilent microarray scanner, and the gene expression profiles were analyzed with Agilent Whole Rat Genome Oligo Microarray software.

Four separate experiments were used for calculating changes in gene expression due to TNF- α alone. Fold changes in gene expression were calculated by dividing the signal intensity of the target gene from the TNF- α -treated FLS by the signal intensity of the target gene from the corresponding vehicle-treated FLS. The final fold change for each gene was the average value of the fold changes of the four individual experiments.

Three experiments were performed to ascertain the effect of SB-203580 on TNF- α -induced genes and to calculate the average ratio of gene downregulation. Fold changes in gene expression were calculated by dividing the signal intensity from the SB-203580-treated FLS by the signal intensity from the corresponding vehicle-treated FLS. The final fold change for each gene was the average value of the fold changes of the three individual experiments. One control experiment to determine the effect of SB-203580 treatment alone in the absence of TNF- α was also carried out. Fold changes in gene expression were calculated by dividing the signal intensity from the individual SB-203580-treated FLS by the signal intensity from the corresponding vehicle-treated FLS.

Reverse transcription-quantitative polymerase chain reaction

To validate the microarray data, RT-quantitative polymerase chain reaction (qPCR) was performed on 11 selected genes. Oligo(dT) 12-18mers were from Invitrogen (Carlsbad, CA). Two micrograms of total RNA and 1.5 μ g of oligo(dT) 12-18mer was used for each reverse transcription with Qiagen's Omniscript RT kit (Qiagen, Chatsworth, CA). Fifty nanograms of the RT product was mixed with 10 μ l of SYBR Premix Ex Taq (Clontech, Mountain View, CA), sense and antisense primers (1 μ M final concentration), and water to a final volume of 20 μ l. qPCR was performed in a DNA Engine Opticon 2 unit (MJ Research). The threshold cycle (C_T) was determined as the cycle number at which the PCR amplification entered the linear exponential phase. The relative expression (E) of the target gene was determined by the Pfaffl method (31):

$$\text{Ratio} = \left(E_{\text{target gene}} \right)^{\Delta C_{T, \text{target}}(\text{control-sample})} / \left(E_{\text{reference gene}} \right)^{\Delta C_{T, \text{reference}}(\text{control-sample})}$$

The reference gene used was Hsp90, because its expression level was constant in both experimental conditions (i.e., with TNF- α and/or SB-203580) and control conditions (i.e., without TNF- α and/or SB-203580) according to our microarray data. Amplification efficiency (E) for each primer pair was determined by a standard curve of C_T against a 10-fold dilution series between 0.1 pg and 0.1 ng of PCR-4-TOPO vectors (Invitrogen) cloned with the amplicon of the gene amplified from the primer pair:

$$E=10^{-1/\text{slope of standard curve}}$$

The amplification efficiencies of all the primer pairs were between 1.9 and 2.1. The primer pair sequences for each gene were as follows: Hsp90: sense (S): 5'-AGGCACTGCGGGACAACCTCG-3', antisense (AS): 5'-ATCTCATCGGAACAGCAGCACT-3'; Cxcl2: S: 5'-AGCTTGACGGTGACCCCTCCAG-3', AS: 5'-AGCCTTGCCTTTGTTTCAGTATCTT-3'; Bmp2: S: 5'-AAGCGTCAAGCCAAACACAAACAG-3', AS: 5'-GATCAGCCAGGGGAAAAGGACATT-3'; Tgm2: S: 5'-TCTGCGGCGCTGGAAGGA-3', AS: 5'-TGGGCGGAGTTGTAGTTGGTCAC-3'; Mmp10: S: 5'-CCGCCCCCTCTCTGATGC-3', AS: 5'-CTTTGGGTAACTGCTTGGACTTC-3'; IL-1 β : S: 5'-GTGGGATGATGACGACCTGCTA-3', AS: 5'-GTCCCGACCATTTGCTGTTTCCTA-3'; Ptg2: S: 5'-GCTGCCGGACACCTTCAACAT-3', AS: 5'-CCAGCAGGGCGGGATACAGT-3'; Il6: S: 5'-AGCCACTGCCTTCCCTACTT-3', AS: 5'-GCCATTGCACAACCTCTTTTCTC-3'; Tlr2: S: 5'-GATGCCCGGCCCTCAGTCT-3', AS: 5'-AGCCACGCCCACATCATTCTC-3'; MMP3: S: 5'-GGACCCTGAGACCTTACCAATGTG-3', AS: 5'-AAGAGACGGCCAAAATGAAGAGAT-3'; Fst: S: 5'-CTGTGGCCCCGGGAAAAAGT-3', AS: 5'-ACACAAGTGGAGCTGCCTGGACA-3'; Adora2a: S: 5'-GAGGGCGAAGGGCATCATTG-3', AS: 5'-TCGCCGCAGGTCTTCGTGGAGT-3'.

Determination of IC₅₀ of SB-203580 on gene expression

FLS were treated with different concentrations of SB-203580 (0, 0.01, 0.03, 0.1, 0.3, 0.5, 1.0, 5.0, 10, and 20 μ M) in the presence or absence of TNF- α as described above. mRNA was prepared and analyzed by RT-qPCR. The relative amounts of target mRNA were calculated by a reciprocal of $2^{\Delta CT}$ of RT-qPCR among the samples of different SB-203580 treatments and normalized to a reference gene, Hsp90. The normalized amount of mRNA prepared in the presence of TNF- α but the absence of SB-203580 was taken as 100%, and the amount of mRNA prepared in the absence of TNF- α and SB-203580 was set at 0%. The relative percentage of mRNA of the sample was calculated from the relative amounts of mRNA prepared at different concentrations of SB-203580 in the presence of TNF- α , and then the IC₅₀ was calculated by a nonlinear regression method with GraphPad Prism version 4.02 (GraphPad Software, San Diego, CA).

Quantification of chemokine Cxcl2 protein product

Cell cultures were treated with SB-203580 in the presence and absence of TNF- α with the corresponding vehicles as mentioned above. Culture supernatants were collected. The cells from each plate were isolated and counted with a Cellometer (Nexcelcom Bioscience). The concentration of Cinc2 α and Cinc2 β , the protein products of Cxcl2 and GM1960 mRNAs, from each cell culture supernatant was quantified by Quantikine Rat CINC-2 α/β Immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The experiment was done in duplicate.

Polymorphonuclear cell separation

Polymorphonuclear (PMN) cells were prepared as previously described (10). Pentobarbital (1 ml of 50 mg/ml) was subcutaneously injected into one rat, and blood (9 ml) was collected and combined with 5% sodium citrate (1 ml). Anticoagulated whole blood (5 ml) was layered over 5 ml of Polymorphprep (Axis-Shield USA, Norton, MA) combined with 0.4 ml of 1.5% NaCl in a 12-ml centrifuge tube and centrifuged at 450–500 *g* for 30 min at 18–22°C. Mononuclear cells were on the band at the interface of the sample and Polymorphprep layer and PMN cells in the lower band. PMN cells were collected and diluted by addition of 1 vol of 0.45% NaCl to restore normal osmolarity, and the cells were spun down at 400 *g* for 10 min at 22°C. The cells were resuspended in 1.5 ml of DMEM with 1% FCS. An aliquot of the cell suspension (0.1 ml) was combined with 0.9 ml of DMEM with 1% FCS, and cell number was counted with a Cellometer.

PMN chemotaxis

The supernatants of TNF- α - or vehicle-treated cell cultures (0.6 ml) prepared as described above were placed in a 24-well polycarbonate filter plate. PMN cells (1.8×10^6) in 0.1 ml of DMEM with 1% FCS were placed on a Transwell insert with a 5- μ m filter membrane, and the plate was incubated for 45 min at 37°C in 5% CO₂. The insert was then removed, and the migrated PMN cells on the bottom plate were counted. The experiment was done in triplicate.

Statistical analysis

Differential expression was concluded if the genes met the following criteria: a minimum expression level at least 10 times above background ($>1,000$) and a minimum 2-fold difference in the mean expression values between nontreated and TNF- α -treated groups. Microarray data were statistically analyzed with the HDBStat! software program (University of Alabama at Birmingham, Birmingham, AL) (49,56). Genes with statistically significant difference were selected with a *t*-test *P* value cutoff of 0.05 and with a Chebyshev's CC1 test *P* value of <0.1 . In Tables 1 and 4, fold increases of results are expressed as means $\pm$ SD.

Materials

DMEM was purchased from Cellgro (Herndon, VA). Penicillin, streptomycin, L-glutamine supplement, goat anti-mouse IgG-FITC secondary antibody, and DMSO were from Sigma (St. Louis, MO). Amphotericin B was from Fisher Biotech (Hampton, NH). Collagenase/dispase and pronase were from Roche (Basel, Switzerland). FCS was from Atlanta Biologicals (Norcross, GA). Mouse anti-rat CD14-FITC antibody was from Antigenix America (Huntington Station, NY), and mouse anti-rat prolyl-4-hydroxylase β -subunit monoclonal antibody was from Chemicon (Temecula, CA). SB-203580 was from Calbiochem (La Jolla, CA), and recombinant rat TNF- α was from PeproTech (Rocky Hill, NJ). Cy3-CTP and Cy5-CTP were from PerkinElmer (Wellesley, MA). Polymorphprep was purchased from Axis-Shield USA. Rat Cxcl2 quantification was done with Quantikine for rat CINC-2 α/β (R&D Systems). p38 MAP Kinase assay kit and SAPK/JNK assay kit were purchased from Cell Signaling Technology.

RESULTS

SB-203580 effects on gene expression profiles of TNF- α -treated FLS

FLS obtained from rat knee synovial tissue were cultured and used for the microarray analysis. After the third passage, $>98\%$ of cells were FLS, similar to several human synovial fibroblast cultures described previously (54,57). In this study, 10 ng/ml of recombinant rat TNF- α or vehicle was applied to the fourth to eighth passage of FLS cell cultures to obtain the gene expression profiles. SB-203580 inhibited p38 MAPK of the rat FLS dose-dependently (Fig.

1A). To ensure specific inhibition of p38 MAPK without interfering with the other MAPKs 0.3 μ M SB-203580 was used, since the IC_{50} of SB-203580 for p38 MAPK is known to be $\sim$ 0.3 μ M (5,12,23). At this concentration, SB-203580 does not inhibit JNK (Fig. 1B). It is accepted that low concentrations of SB-203580 below 1 μ M inhibit only p38, not ERK or JNK, in FLS (15). p38 MAPK activity was maximal at 15 min after TNF- α treatment, and then its activity declined rapidly (Fig. 1C).

Gene expression profiles were analyzed with Agilent Whole Rat Genome Oligo Microarray software. The signal intensity of any given spot on the microarray over 1,000, that is, 10-fold higher than the background intensity, was chosen and considered as significant gene expression. This selection provided 17,550 of the 41,000 spots on the microarray. These genes were applied to a *t*-test and a Chebyshev's CC1 test with HDBStat! software (49,56). This selected 950 genes with a *t*-test *P* value of <0.05 and a Chebyshev's CC1 test *P* value of <0.1 . With these criteria, we normalized the gene expression of the TNF- α -treated cells to the control cells and thus identified 141 genes that were greater than twofold upregulated by TNF- α and 64 genes that were more than twofold down-regulated (see Supplemental Data 1 and 2).¹ Although many of the upregulated genes were unknown or hypothetical genes (29 of 141), the majority of genes that were upregulated by TNF- α in our array experiment have been reported to be related to arthritis. These are mostly cytokines, proteases, and genes involved in metabolism and signal transduction (Fig. 2A). Selected genes upregulated by TNF- α are shown in Table 1. All of the microarray data have been submitted as a web supplement at the Gene Expression Omnibus (GEO) repository of the National Center for Biotechnology Information (NCBI) (available at <http://www.ncbi.nlm.nih.gov/geo/GSE7826>).

Among those 141 genes with a signal intensity above 1,000 and with >2 -fold upregulation by TNF- α , 30% were >1.5 -fold downregulated and 3% were >1.5 -fold upregulated by SB-203580; 67% of genes were not significantly changed by treatment with the inhibitor (Fig. 2B).

SB-203580 was very effective in suppressing many proinflammatory cytokines. The interleukins that were upregulated by TNF- α , IL-1 α , IL-1 β , and IL-11, all known participants in RA, were all downregulated by the p38 inhibitor treatment (see Supplemental Data 1). Among the interleukins, IL-1 β is an important proinflammatory cytokine in RA. SB-203580 was able to downregulate many chemokine mRNA levels such as Cx3cl1, Cxcl2, and Gm1960. Among these, Cx3cl1 and Cxcl2 have been implicated in RA pathology (32, 34, 36, 46). SB-203580 was also effective in inhibiting protease-related gene expression. All of the MMPs and plasminogen activators that were upregulated by TNF- α were downregulated by the p38 inhibitor, particularly Mmp10, Mmp13, and Mmp3. Some genes encoding for signaling transduction proteins, such as insulin-like growth factor binding proteins 3 and 5 (Igfbp3 and Igfbp5), Toll-like receptor 2 (Tlr2), and some components of the NF- κ B pathways were all upregulated by TNF- α and inhibited by SB-203580.

We also found genes that have not been previously reported to be regulated by TNF- α and p38 MAPK. Among the genes that are involved in cell adhesion and motility, SB-203580 inhibited the expression of Emp2, Rho β (Arhgdib), and a precursor to protocadherin 16.

Validation of microarray data and determination of IC_{50} by RT-qPCR

The validity of the microarray data was confirmed by RT-qPCR analysis using 11 genes that were highly induced by TNF- α : IL-1 β , IL-6, Mmp3, Mmp10, Cxcl2, Tlr2, Ptgs2, Tgm2, Bmp2, Adora2a, and Fst. Table 2 shows expression of these genes normalized against Hsp90 as a housekeeping gene. Similar data were obtained after normalization against GAPDH expression

¹The online version of this article contains supplemental material.

as an alternative housekeeping gene (data not shown). Generally, the fold increase measured by RT-qPCR was higher than that of the microarray analysis. Overall, qPCR confirmed the significantly increased expression of all 11 genes by TNF- α seen on the microarrays. Cxcl2 and Mmp10 were especially most highly upregulated by TNF- α treatment based on RT-qPCR.

SB-203580 inhibition of the gene expression shown by the microarray was confirmed by RT-qPCR (Table 2). We also measured the IC₅₀ of SB-203580 for some of the typical genes suppressed in our experiments to demonstrate the validity of the inhibition seen in our microarray analysis. Expression of a typical proinflammatory cytokine, IL-1 β , was inhibited by SB-203580, with an IC₅₀ of 0.1 μ M. Gene expression of a chemokine, Cxcl2, that was highly upregulated by TNF- α was also inhibited by SB-203580, with an IC₅₀ of 0.4 μ M. Mmp3 was inhibited by SB-203580, with an IC₅₀ of 0.57 μ M. Ptg2 (COX2) was also inhibited by SB-203580, with an IC₅₀ of 0.6 μ M. These data are consistent with the microarray analysis showing that 0.3 μ M SB-203580 suppressed IL-1 β , Cxcl2, Mmp3, and Ptg2 gene expression by 0.69-, 0.46-, 0.56-, and 0.45-fold, respectively (Table 1).

The microarray analysis also identified a group of genes that were upregulated by TNF- α but not inhibited by 0.3 μ M SB-203580 (see Supplemental Data 1). Among these genes, Adora2 and Fst were chosen and analyzed by qPCR. As expected, the RT-qPCR data confirmed the upregulation of these two genes by TNF- α , but no inhibition by 0.3 μ M of SB-203580 was found corresponding to the microarray data (IC₅₀ > 5 μ M; Table 2).

Correlation of mRNA levels to protein levels in FLS treated with SB-203580 and TNF- α

In our microarray experiments, Cxcl2 showed the most dramatic increase of nearly 60-fold by TNF- α treatment. The alternatively spliced form of the gene, GM1960, also showed an increase of 3.7-fold. Both isoforms were downregulated by more than half by SB-203580. We therefore decided to check whether these changes in mRNA levels reflect actual changes in protein levels of Cinc2 α and Cinc2 β (Cxcl2 encodes for Cinc2 α and GM1960 for Cinc2 β) in the FLS. The culture supernatants of cells that were treated with SB-203580 and/or TNF- α or vehicle were collected, and the levels of Cinc2 α/β were measured with ELISA (Table 3). The changes in the amount of Cinc2 α/β in the culture supernatants resembled the changes in the mRNA level. TNF- α induced a 20-fold increase in Cinc2 α/β , and this amount was reduced nearly by half in the presence of SB-203580.

The Cinc proteins are potent neutrophil chemoattractants (55). We examined whether the observed changes in Cinc2 α/β level could lead to a change in the ability of the FLS to recruit PMN cells. We compared the ability of the supernatants from TNF- α - or mock-treated FLS cultures to induce PMN cell chemotaxis. From the Transwell chemotaxis assay, 68% of the PMN cells had migrated to the bottom well after incubation with the TNF- α -treated cell supernatant, which contains 40.5 ng of Cinc2 α/β per milliliter. Forty-eight percent of the PMN cells had migrated after incubation with the supernatant containing 10 ng of Cinc2 α/β per milliliter, while 36% had migrated with vehicle-treated cell supernatant, which contained 2.75 ng of Cinc2 α/β per milliliter. There was a correlation between the concentration of Cinc2 α/β and the migration of PMN cells, which is consistent with previous reports (37,55).

Genes downregulated by TNF- α

The majority of the 64 genes downregulated by TNF- α were not affected by SB-203580 (~81%). Only six genes whose expressions could be rescued by p38 MAPK inhibition and another six genes were further downregulated by SB-203580 in addition to TNF- α (see Supplemental Data 2). Selected TNF- α -downregulated genes are shown in Table 4.

Among the genes downregulated by TNF- α but upregulated by SB-203580, Cxcl12 and Lgals3 are chemoattractive to Cxcr4-expressing CD4⁺ T cells and monocytes, respectively (3,16). Cxcl12 has been reported as downregulated by TNF- α or IL-1 β in RA FLS in other microarray studies (19,43). However, in vivo Cxcl12 is reported as being elevated in RA synovium and RA patient serum/plasma (16). Some genes such as Rasd1 and Wisp2 were downregulated by both TNF- α and SB-203580.

DISCUSSION

TNF- α modulates a broad range of inflammatory and immunologic processes and has a central role in the pathology of RA. Inhibition of TNF- α is an accepted treatment modality for RA (26,29,51,58). Several genomic studies on the effects of TNF- α on RA FLS have been reported (11,43,56). However, there has been no report of a comprehensive study on TNF- α -responsive genes regulated through the p38 MAPK signaling pathway in FLS. High concentrations of TNF- α have been found in serum and synovial fluid with high disease activity in RA patients (8,17,47), and it was also observed that TNF- α was localized to the synovial lining layer comprised of FLS and MLS (9). Although p38 MAPK signaling is an early event in response to TNF- α stimulation as shown in Fig. 1C, we treated FLS cultures with TNF- α for a relatively long period (24 h) in the presence and absence of SB-203580 to better mimic the in vivo effects of TNF- α and SB-203580 on the FLS.

We found 141 genes upregulated by TNF- α . They could be classified into genes encoding for chemokines, colony stimulating factors, growth factors, interleukins, matrix metalloproteinases, and some genes involved with metabolism such as Ptgs2 (Cox2), Ptges, and Pde4b. These genes were found to be induced in human RA FLS as well by TNF- α (11,43, 56). It is well known that the phenotypes of RA FLS and normal FLS are dramatically different. In vitro, RA FLS cells have a tumorlike growth pattern, and engraftment of RA FLS cells into severe combined immunodeficiency disease (SCID) mice was sufficient to cause cartilage damage (30). Our microarray analysis thus suggested that most of the inflammatory genes and cytokines relevant to RA are intrinsic responses to TNF- α in the FLS, regardless of their activation state. p38 is an intracellular MAPK/SAPK that regulates both the release and the actions of TNF- α and IL-1 β (2). p38 MAPK is highly expressed in the RA synovium (21), and inhibition of p38 is therefore a potential target for developing a novel antirheumatic drug (26). We thus conducted microarray analysis on the effects of SB-203580, a specific p38 MAPK inhibitor (13), on the expression of genes modulated by TNF- α .

Overall, 43 of the 141 genes upregulated by TNF- α were inhibited by SB-203580 treatment (Table 1 and Supplemental Data 1). Most of these genes are thought to play a role in RA. Among them, several genes such as TNF- α , IL-1 β , and MMP-3 have been reported as p38 inhibitor-inhibited proteins (5,14,25,26,53). In this study, we showed that p38 MAPK inhibition suppresses many other genes besides previously reported genes. These inflammatory genes inhibited by SB-203580 are Bmp2, Ptges, Ptgs2 (Cox2), Pde4b, Tlr2, IGF binding protein 5, and IGF binding protein 3. Among the metabolism-related genes inhibited by the p38 inhibitor, Ptgs2 (Cox2) and Pde4b have been drug targets used for RA treatment (18,41,42).

We confirmed the validity of the microarray data with RT-qPCR, and we demonstrated the specificity of the drug inhibition with measurement of the IC₅₀ of the drug for a selected set of genes. We also showed that the mRNA levels that changed translated into comparable changes in protein levels, as in the case for CINC2 α/β , and into observable functional changes to the FLS by measuring the chemotactic ability of PMN cells for culture supernatants of FLS that were treated or not treated with TNF- α . Indeed, SB-203580 can suppress Cxcl2 mRNA, resulting in the inhibition of Cxcl2 protein production, which reduces neutrophil recruitment. SB-203580 inhibition of IL-6 protein production was not measured, since it was previously

reported that SB-203580 inhibits IL-6 protein production and its promoter activity in IL-1 β -treated FLS cells (14).

SB-203580 inhibited the expression of the metalloproteinases Mmp10, Mmp3, and Mmp13 and the chemokines Cx3cl1 and Cxcl2. Metalloproteinases are involved in cartilage destruction (1,33). The expression of Mmp13 mRNA in the synovial tissue was known to be associated with histopathological type II synovitis in RA (52). Suppression of chemokines by SB-203580 suggests that p38 MAPK kinase inhibitors would reduce leukocyte infiltration. This is consistent with the finding that TNF- α blockade reduces inflammatory cell infiltration into the synovium (40).

We also discovered some interesting inhibitory effects that may be of potential therapeutic value. Tgm2 encodes for transglutaminase 2 (TGase 2), which was recently shown to be able to activate NF- κ B through a noncanonical pathway that is independent of IKK activation (24). The inhibition of Tgm2 expression by SB-203580 suggests that p38 may be a potential means for regulation of NF- κ B activation in TNF- α -stimulated inflammatory responses.

We observed 64 genes downregulated by TNF- α (see Supplementary Data 2). Among the genes that responded negatively to TNF- α , Wisp2 belongs to the CCN growth factor family that is regulated by the WNT signaling pathway and estrogen (45), whose expression was found in the fibrotic area of the RA synovium but not in the lining. Our microarray data showed that Wisp2 is negatively regulated by TNF- α and p38 MAPK, which may explain the absence of Wisp2 expression in the synovial lining. We found some genes repressed by TNF- α that are involved in apoptosis and suppression of tumorigenesis (Table 4). For example, Hrasls3 encodes for a class II tumor suppressor (38). Transgelin (Tagln) has been identified as a protein whose expression is lost in virally transformed cell lines and may be a marker for early tumor progression (39), and tumor-associated antigen L6 (Taal6; Tm4sf1 predicted) appeared to be involved in cancer invasion and metastasis (4, 20). The suppressed expression of these genes by TNF- α may help explain the hyperplasia and the activation of normal FLS cells into the aggressive and invasive phenotypes of RA FLS cells.

In conclusion, our microarray analysis has confirmed that p38 MAPK could mediate a majority of the inflammatory gene expression induced by TNF- α that contributes to different aspects of the pathology of RA such as metalloproteinases in the destruction of bones and cartilages, chemokines for inflammatory cell recruitment, and interleukins for the perpetuation of the inflammatory response (Table 1, Fig. 3). Thus this microarray study demonstrates the potential value of p38 inhibitors as an effective treatment with multiple benefits in TNF- α -mediated inflammatory diseases such as RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A: inhibition of rat fibroblast-like synoviocyte (FLS) p38 mitogen-activated protein kinase (MAPK) by SB-203580. The effect of SB-203580 on the activity of p38 MAPK was measured by an in vitro kinase assay. Phospho-p38 was immunoprecipitated from tumor necrosis factor (TNF)- α -stimulated rat FLS cell lysate and was used to phosphorylate ATF-2 fusion protein in the presence of 0, 0.1, 0.3, 1.0, or 10.0 μ M SB-203580. **B:** SB-203580 effect on rat FLS c-Jun NH₂-terminal kinase (JNK). The effect of SB-203580 on the activity of JNK was measured by an in vitro kinase assay. JNK was immunoprecipitated with immobilized c-Jun fusion protein from either TNF- α - or control-treated rat FLS cell lysates and was used to phosphorylate c-Jun in the presence of 0, 0.01, 0.1, or 1.0 μ M SB-203580. **C:** p38 MAPK kinase assay. The effect of SB-203580 on p38 MAPK in rat FLS in vivo was measured at 15 min, 1 h, 6 h, and 24 h after TNF- α stimulation. The cells were first preincubated with 0.3 μ M SB-203580 or vehicle for 2 h, and then 10 ng/ml TNF- α was added. A negative control without TNF- α stimulation was also included. The cells were harvested at the time points indicated, and the activity of p38 MAPK was measured. SB80, SB-203580.

Fig. 2.

Induced gene expression at 24 h after TNF- α stimulation and SB-203580 inhibition. **A:** 141 genes with signal intensity >1,000 were upregulated >2-fold by TNF- α . Pie chart represents the subsets of functionally assigned genes with the numbers found in each group. **B:** FLS were preincubated with 0.3 μ M SB-203580 for 2 h, followed by 24-h TNF- α stimulation. c upregulated 3% and downregulated 30% of the 141 genes upregulated >2-fold by TNF- α shown in A.

Fig. 3.

Gene targets of p38 MAPK inhibition. Functional categories of TNF- α -induced genes found to be inhibited by SB-203580 in the microarray experiments are shown. They can be categorized into genes encoding for proteases and cytokines, metabolism-related genes, and genes involved in signal transduction, proliferation, and apoptosis (see Supplemental Data 1). Many of these genes, shown in bold, have been associated with rheumatoid arthritis.

Table 1
Gene expression profiles of rat fibroblast-like synoviocytes upregulated by TNF- α and inhibited by SB-203580

Gene Name	Systematic Name	Description	Ratio of TNF- α (+) to TNF- α (-)	Signal Intensity of TNF- α (+)	Ratio of SB80 (+) to SB80(-) (+ TNF- α)
Cytokines					
Cxcl2	NM_053647	Chemokine (C-X-C motif) ligand 2	59.83 $\pm$ 27.40	257,302.8	0.46 $\pm$ 0.02
Gm1960	NM_138522	Cytokine-induced neutrophil chemoattractant-2b	3.73 $\pm$ 1.60	298,841.3	0.44 $\pm$ 0.04
Cx3cl1	NM_134455	Chemokine (C-X3-C motif) ligand 1	2.12 $\pm$ 1.00	260,865.1	0.69 $\pm$ 0.09
Il1b	NM_031512	Interleukin 1 β ; beta;	11.52 $\pm$ 2.85	12,303.8	0.69 $\pm$ 0.39
Bmp2	NM_017178	Bone morphogenetic protein 2	4.50 $\pm$ 1.80	28,567.7	0.75 $\pm$ 0.04
Proteases and protease inhibitors					
Mmp10	NM_133514	Matrix metalloproteinase 10	18.14 $\pm$ 1.91	21,343.8	0.65 $\pm$ 0.14
Mmp3	NM_133523	Matrix metalloproteinase 3	2.53 $\pm$ 1.00	300,288.3	0.56 $\pm$ 0.24
Mmp13	M60616	UMRCase	5.40 $\pm$ 2.12	229,030.9	0.43 $\pm$ 0.05
Serpine2	XM_343604	Serine (or cysteine) proteinase inhibitor, clade E, member 2 (Serpine2)	3.22 $\pm$ 1.20	40,057.2	0.72 $\pm$ 0.12
Metabolism					
Ptges	NM_021583	Prostaglandin E synthase	4.78 $\pm$ 2.37	89,878.9	0.76 $\pm$ 0.12
Ptgs2	NM_017232	Prostaglandin-endoperoxide synthase 2	7.74 $\pm$ 2.78	97,096.8	0.45 $\pm$ 0.05
Pde4b	NM_017031	Phosphodiesterase 4B, cAMP specific	2.01 $\pm$ 0.53	16,247.5	0.67 $\pm$ 0.07
Cell adhesion and motility					
Emp2	NM_001007721	Epithelial membrane protein 2	2.47 $\pm$ 0.23	28,383.0	0.63 $\pm$ 0.16
Athg1b	NM_001009600	Rho, GDP dissociation inhibitor (GDI) β	3.10 $\pm$ 0.63	22,087.4	0.64 $\pm$ 0.10
Pcdh16	XM_219128	Protocadherin 16 precursor; protocadherin 16	2.17 $\pm$ 0.33	23,333.7	0.58 $\pm$ 0.01
Other genes					
Igfbp3	NM_012588	Insulin-like growth factor binding protein 3	2.47 $\pm$ 0.52	12,427.3	0.46 $\pm$ 0.09
Igfbp5	AW917764	Insulin-like growth factor binding protein 5	3.71 $\pm$ 0.47	5,795.1	0.33 $\pm$ 0.02
Ereg	NM_021689	Epiregulin	4.05 $\pm$ 0.79	14,294.2	0.74 $\pm$ 0.43
Tlr2	NM_198769	Toll-like receptor 2	2.59 $\pm$ 0.98	151,051.0	0.61 $\pm$ 0.08
RGD1307189	NM_001008349	Similar to nuclear factor- κ B subunit p100	2.21 $\pm$ 0.19	15,070.3	0.68 $\pm$ 0.13
Slc20a1	NM_031148	Solute carrier family 20 (phosphate transporter)	2.65 $\pm$ 0.91	101,728.4	0.62 $\pm$ 0.07
Pcp4	NM_013002	Purkinje cell protein 4	6.22 $\pm$ 1.57	49,690.0	0.65 $\pm$ 0.03

Gene Name	Systematic Name	Description	Ratio of TNF- α (+) to TNF- α (-)	Signal Intensity of TNF- α (+)	Ratio of SB80 (+) to SB80(-) (+TNF- α)
Tgm2	NM_019386	Transglutaminase 2, C polypeptide	3.62 $\pm$ 2.69	1,668.2	0.46 $\pm$ 0.05

Values for fold changes in gene expression are means $\pm$ SD. Fold changes in gene expression by tumor necrosis factor (TNF)- α were calculated by dividing the signal intensity of the target gene from TNF- α -treated fibroblast-like synoviocytes (FLS) [TNF- α (+)] by the signal intensity of the target gene from corresponding vehicle-treated FLS [TNF- α (-)]. The final fold change for each gene was the average value of the fold changes of the 4 individual experiments. The signal intensity of TNF- α (+) is the average of the signal intensities obtained from TNF- α -treated samples of 4 independent experiments. Fold changes in gene expression by SB-203580 were calculated by dividing the signal intensity from SB-203580-treated FLS by the signal intensity from corresponding vehicle-treated FLS. The final fold change for each gene was the average value of the fold changes of the 3 individual experiments. SB80(+), FLS cultures preincubated with SB-203580, followed by TNF- α treatment; SB80(-), FLS cultures treated with TNF- α but not with SB-203580.

Table 2RT-qPCR analysis on gene expression and IC₅₀ of SB-203580

Gene Name	Fold Increase	IC ₅₀ of SB-203580 on Gene Expression, μ M
IL-1 β	6.62	0.10
Mmp10	28.44	0.71
Cxcl2	58.83	0.40
Tgm2	4.44	0.47
Bmp2	5.31	0.91
Ptgs2	6.70	0.60
IL-6	3.30	0.11
Tlr2	3.70	0.23
Mmp3	10.8	0.57
Adora2a	9.28	8.01
Fst	9.92	5.62

Table 3Cinc2 α / β level from FLS culture supernatants

	Cinc2 α / β , ng/ml	Cinc2 α / β , ng/10 ⁶ cells
No treatment	2.75	31.8
SB-203580 only	1.48	13.4
TNF- α only	40.5	424.0
TNF- α and SB-203580	23.2	272.0

FLS were preincubated with SB-203580 or vehicle for 2 h and then with TNF- α or vehicle for an additional 24 h. Culture supernatants were collected, and the Cinc2 α / β concentration was determined by ELISA. Cinc2 α / β protein amount was normalized to 1×10^6 cells.

Table 4

Selected gene expression profiles of rat fibroblast-like synoviocytes downregulated by TNF- α

Gene Name	Systematic Name	Description	Ratio of TNF- α (+) to TNF- α (-)	Signal intensity of TNF- α (+)	Ratio of SB80(+) to SB80(-) (+TNF- α)
Upregulated by SB-203580					
Cxcl12	NM_022177	Chemokine (C-X-C motif) ligand 12	0.48 $\pm$ 0.27	73,344.4	1.52 $\pm$ 0.16
Lgals3	NM_031832	Lectin, galactose binding, soluble 3	0.45 $\pm$ 0.17	72,728.1	1.59 $\pm$ 0.31
Downregulated by SB-203580					
Rasd1	XM_340809	RAS, dexamethasone-induced 1	0.14 $\pm$ 0.07	2,486.6	0.44 $\pm$ 0.16
Wisp2	NM_031590	WNT1-inducible signaling pathway protein 2	0.28 $\pm$ 0.05	7,782.1	0.56 $\pm$ 0.12
Others					
Egr1	NM_012551	Early growth response 1	0.49 $\pm$ 0.06	8,512.6	1.15 $\pm$ 0.47
Hrasl3	NM_017060	HRAS-like suppressor	0.47 $\pm$ 0.10	6,960.4	1.10 $\pm$ 0.11
Smpd13a	NM_001005539	Acid sphingomyelinase-like phosphodiesterase	0.47 $\pm$ 0.09	14,173.7	1.21 $\pm$ 0.27
Tagln	NM_031549	Transgelin	0.47 $\pm$ 0.11	3,607.7	0.95 $\pm$ 0.20
Vim	NM_031140	Vimentin	0.44 $\pm$ 0.11	4,3843.9	1.27 $\pm$ 0.42
Empl	NM_012843	Epithelial membrane protein 1	0.43 $\pm$ 0.04	23,627.9	1.48 $\pm$ 0.27
Tgfb3	NM_013174	Transforming growth factor, β 2	0.36 $\pm$ 0.13	2,317.5	0.78 $\pm$ 0.01
Sdc2	NM_013082	Syndecan 2	0.43 $\pm$ 0.04	19,574.2	0.88 $\pm$ 0.03
Spin2c	NM_031531	Serine protease inhibitor	0.44 $\pm$ 0.14	10,337.8	1.00 $\pm$ 0.05
Lxn	NM_031655	Latexin	0.44 $\pm$ 0.08	8,120.0	1.07 $\pm$ 0.25

Values for fold changes in gene expression are means $\pm$ SD. Fold changes in gene expression were calculated by dividing the signal intensity of the target gene from TNF- α -treated FLS by the signal intensity of the target gene from corresponding vehicle-treated FLS. The final fold change for each gene was the average value of the fold changes of the 4 individual experiments. The signal intensity of TNF- α (+) is the average of the signal intensities obtained from TNF- α -treated samples of 4 independent experiments. Fold changes in gene expression were calculated by dividing the signal intensity from SB-203580-treated FLS by the signal intensity from corresponding vehicle-treated FLS. The final fold change for each gene was the average value of the fold changes of the 3 individual experiments. SB80(+), FLS cultures preincubated with SB-203580, followed by TNF- α treatment. SB(-), FLS cultures treated with TNF- α but not with SB-203580.