Regulation of Interleukin-1β-induced Interleukin-6 Gene Expression in Human Fibroblast-like Synoviocytes by p38 Mitogen-activated Protein Kinase*

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Involvement of p38 mitogen-activated protein (MAP) kinase in interleukin (IL)-6 gene expression of human fibroblast-like synoviocytes (FLSs) was assessed. p38 MAP kinase was constitutively expressed in human FLSs and activated in response to IL-1β. A pyridinylimidazol compound, SB203580, inhibited p38 MAP kinase activity in vitro, since the activity of MAPKAP kinase-2 (a substrate of p38 MAP kinase) in IL-1β-stimulated FLSs was totally suppressed by it. SB203580 concentration-dependently inhibited protein production and gene expression of IL-6 by human FLSs. The effect of SB203580 was dependent on de novo protein synthesis. SB203580 significantly reduced the stability of IL-6 mRNA without affecting the rate of IL-6 gene transcription. Here, we provide evidence that p38 MAP kinase is activated in response to IL-1β in human FLSs and is involved in IL-6 synthesis by stabilizing IL-6 mRNA.

Human fibroblast-like synoviocytes (FLSs) respond to several cytokines and growth factors including interleukin (IL)-1, tumor necrosis factor (TNF), and platelet-derived growth factor and exhibit characteristics of inflammatory cells critically involved in several aspects of rheumatoid pathophysiology (1, 2). They secrete matrix metalloproteinases such as stromelysin-1 (matrix metalloproteinase-3) and collagenase (matrix metalloproteinase-1), enzymes essentially involved in the degradation of cartilage (3, 4). In addition, FLSs are involved in the infiltration and activation of other immune cells in the synovial tissue by secreting IL-6 or by expressing intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (5, 6). Human FLSs, therefore, seem to be the crucial regulator of joint inflammation and destruction featured in rheumatoid arthritis (RA).

IL-6 is a 21-kDa glycoprotein containing 184 amino acid residues derived from a precursor peptide of 212 amino acid and has a variety of biological activities including B- and T-cell activation, stimulation of fever, and release of acute phase response proteins (7–9). This cytokine in co-operation with soluble IL-6 receptor (IL-6R) is involved in the proliferation of synoviocytes of RA patients (10). Immunological disorders often associated with RA include polyclonal plasmacytosis, production of autoantibodies, increased levels of acute phase proteins, and an increased number of peripheral blood platelets, all of which are related to the biological actions of IL-6 (11). In fact, IL-6 levels are greatly increased in thesynovial fluid and serum obtained from RA patients (12, 13). Human FLSs produce biologically active IL-6 in vitro (5) and are considered as a main source of IL-6 in the synovium (14). We have recently reported that human rheumatoid FLSs produce by far the larger amount of IL-6 compared with osteoarthritis (OA) FLSs (15) and that IL-6 gene regulation by the IL6-α receptor is altered in rheumatoid FLSs (16). Inhibition of IL-6 production by FLSs seems to represent an effective target for RA treatment.

The molecular mode of action of pyridinylimidazoles has recently been elucidated (17). The representative compound SB203580 is a highly specific inhibitor of p38 mitogen-activated protein (MAP) kinase (18), which is also termed cytokine-suppressive anti-inflammatory drug-binding protein (19), or RK (20). p38 MAP kinase is activated by the treatment of cells with lipopolysaccharide, cytokines, and stress (21, 22). MAPKAP kinase-2 was first identified as a p38 MAP kinase substrate, which in turn phosphorylated HSP-25/27 (20, 23). Subsequently, several transcription factors including ATF-2 (24), CHOP/GADD153 (25), MAX (26), myocyte enhancer factor 2C (27), and ternary complex factor (28–30) have been found to be activated by p38 MAP kinase. In addition to the original p38 (also termed p38α, cytokine-suppressive anti-inflammatory drug-binding protein 2, or SAPK2A), the p38 subgroup of MAP kinase now consists of cytokine-suppressive anti-inflammatory drug-binding protein 1 (17), Mxi2 (26), p38β (also known as SAPK2B), p38-2 (also known as p38β2) (31, 32), p38γ (also known as ERK6 or SAPK3) (33, 34), and p38δ (also known as SAPK4) (32, 35–37). Pyridinylimidazoles block the kinase activity of p38α, p38β, and p38-2 (18, 31). They inhibit production of TNF-α and IL-1 by LPS-induced human monocytes at the transcriptional level (19, 38–40) and IL-6 production by TNF-α-induced murine fibrosarcoma L929 cells at the transcriptional level (41, 42). It was also reported that the compound has beneficial effects in animal models such as arthritis, bone resorption, and endotoxin shock (43).

IL-1β is a potent activator of IL-6 synthesis by human FLSs (5). Involvement of p38 MAP kinase in IL-6 production by TNF-α-induced human SaOS2 osteoblastic cells and IL-1β-induced human MRC-5 lung fibroblasts was demonstrated by the use of SK&F86002 (44) and SB203580 (45), respectively. As
the inhibitor effect was described only at the level of protein production, the mechanism of the inhibition of IL-1β-induced IL-6 production by SB203580 still remains unclear. In addition, regulation of p38 MAP kinase in human FLSs by IL-1β has not been elucidated. This is the first study demonstrating that the inhibition of p38 MAP kinase in human FLSs resulted in the reduced stability of IL-6 mRNA, in a protein synthesis-dependent manner, without affecting the transcription of the gene. Elucidation of the molecular mechanism of IL-6 production by human FLSs may lead to the future development of a effective therapy for RA.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human IL-1β and IL-6 were obtained from Genzyme Corp. (Cambridge, MA). The compound SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-5-(4-pyridylimidazolide) was synthesized by the Central Research Laboratories, Kissei Pharmaceutical Co., Ltd. (Nagano, Japan) according to the procedure reported by Adams et al. (46). The preparation was determined to be >95% pure on the basis of high performance liquid chromatography and NMR analysis (data not shown). PhosphoPlus p38 MAP kinase (Tyr182) antibody kit (catalog no. 9210) was purchased from New England Biolabs (Daiichi Pure Chemicals, Tokyo, Japan). Blotting polyclonal antibody against p38 MAP kinase (C-20; catalog no. sc-535) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MPRKAP kinase-2 immunoprecipitation kinase assay kit (catalog no. 17-162) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cells—Synovial tissue samples were obtained from patients with RA and osteoarthritis (OA) undergoing total joint replacement, as described previously (16). Written informed consent was obtained from each patient. The data obtained using rheumatoid FLSs are presented in this manuscript, since the same results were obtained qualitatively from OA cells. The culture medium was RPMI 1640 without phenol red (Nikken, Kyoto, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Inc.,), and penicillin-streptomycin (20 units/ml; R & D Systems, Minneapolis, MN). Northern blotting was performed as described previously (16). Written informed consent was obtained from each patient. The data obtained using rheumatoid FLSs are presented in this manuscript, since the same results were obtained qualitatively from OA cells.

Quantification of IL-6—IL-6 was measured by a sandwich enzyme-linked immunosorbent assay, as described previously (16), using anti-human-IL-6 monoclonal antibody (2 µg/ml; R & D Systems, Minneapolis, MN) as the capture antibody and biotinylated purified anti-human-IL-6 monoclonal antibody (2 µg/ml; R & D Systems) as the second antibody. The absorbance at 450 nm was measured by a microplate reader (Bio-Rad). The minimum detection limit of the assay was 6.25 pg/ml.

Northern Blotting—Cells were grown to confluence in 75-cm² culture flasks and then harvested. Total RNA was prepared by use of ISOGEN. The pBluescript II KS (+) vector alone, pBluescript vector containing human GAPDH cDNA insert, and pBluescript vector containing human IL-6 cDNA insert, and pBluescript vector carrying human IL-6 cDNA were linearized and immobilized on Hybrid B N nylon membranes (Amerham Pharmacia Biotech) by use of a slot- and polyacrylamide gel loading buffer, boiled for 5 min, and electrophoresed on a 12% SDS-polyacrylamide gel. Proteins were then transferred onto polyvinylidine difluoride membranes by electrobólting (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad) in 25 mM Tris, 192 mM glycine, and 5% methanol at 15 V for 30 min. Membranes were probed with the following antibodies overnight at 4 °C: rabbit polyclonal anti-p38 MAP kinase (C-20; catalog no. sc-535) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MPRKAP kinase-2 immunoprecipitation kinase assay kit (catalog no. 17-162) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Transient Transfection and Luciferase Assay—Cells were grown to confluence in 75-cm² culture flasks containing RPMI 1640 medium supplemented with 10% fetal bovine serum, harvested, and resuspended in RPMI 1640 medium without phenol red to give a cell concentration of 2 × 10⁶ cells/ml. A 500-µl volume of cell suspension, 10 µg of each luciferase reporter plasmid, and 1 µg of pCMV-β-gal were placed on a Bio-Rad 4-mm cuvette, and electroporation (Bio-Rad Gene Pulser) at 0.29 kV, 960 microfarad was performed. The transfected cells were cultured for the designated time periods in RPMI 1640 medium without phenol red. All assays were carried out in 96-well culture plates (Culture Plate™, Packard Instrument Company, Meriden, CT). The luminescence was measured with a Top Count at the conditions of 0.15 min counting time, a 15-min dark adaptation period at 0 °C, and a single photon counting mode (Top Count, Packard). The β-galactosidase activity was measured as described previously (26).

Nuclear Run-on Assay—Cells were treated with appropriate agents, washed twice with ice-cold phosphate-buffered saline, and then collected. Their nuclei were isolated by incubating the cells on ice with the lysing solution containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40. The nuclei were then centrifuged at 14,000 g, 4 °C, for 10 min, resuspended in cell lysing solution containing 10 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.3 M KCl, 5 mM dithiothreitol, 1 mM magnesium chloride, 10 mM dithiothreitol, and 5 mM dithiothreitol, and the mixture was incubated with a human IL-6 cDNA insert, and pBluescript vector containing human GAPDH cDNA insert were linearized and immobilized on Hybrid B N nylon membranes (Amerham Pharmacia Biotech) by use of a slot- and polyacrylamide gel loading buffer, boiled for 5 min, and electrophoresed on a 12% SDS-polyacrylamide gel. Proteins were then transferred onto polyvinylidine difluoride membranes by electrobólting (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad) in 25 mM Tris, 192 mM glycine, and 5% methanol at 15 V for 30 min. Membranes were probed with the following antibodies overnight at 4 °C: rabbit polyclonal anti-p38 MAP kinase antibody (C-20; catalog no. sc-535) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). MPRKAP kinase-2 immunoprecipitation kinase assay kit (catalog no. 17-162) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Analysis of in Vivo p38 MAP Kinase Activity—Cells were lysed in 1 ml of cold lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM NaEDTA, 0.5% Nonidet P-40, 50 µM NaF, 0.5 mM NaVO₃, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble nuclear material was pelleted by centrifugation at 14,000 × g for 10 min at 4 °C, and the supernatant (30 µg protein) was used for immunoprecipitation with anti-p38 MAP kinase antibody. After incubation with anti-rabbit secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature, specific bands were revealed by incubation with the ECL Western blotting detection reagents (Amerham Pharmacia Biotech). Blots were exposed to high performance chemiluminescence film (Hyperfilm ECL; Amerham Pharmacia Biotech) for 0.5–10 min for detection.

Expression of in Vivo p38 MAP Kinase Activity—Cells were lysed in 1 ml of cold lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM NaEDTA, 0.5% Nonidet P-40, 50 µM NaF, 0.5 mM NaVO₃, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Insoluble nuclear material was pelleted by centrifugation at 14,000 × g for 10 min at 4 °C, and the supernatant (50 µg of total protein) was preincubated with 30 µl of protein A-Sepharose beads (Amerham Pharmacia Biotech) for 1 h at 4 °C. Two microliters of rabbit polyclonal anti-p38 MAP kinase antibody (Santa Cruz Biotechnology) was added to the precleared lysates, and each mixture was incubated for 2 h at 4 °C. The mixtures were further incubated for 1 h at 4 °C after the addition of 15 µl of protein A-Sepharose beads. The immunoprecipitates were then washed twice with the lysis buffer and then twice with the kinase buffer (25 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 10 mM MgCl₂, 2 mM dithiothreitol, and 0.1 mM NaVO₃) and resuspended in 30 µl of kinase assay buffer containing 1 µg of GST-ATP-2 fusion protein (Santa Cruz Biotechnology), 50 µM ATP, and 10 µCi of [γ-32P]ATP. The reaction mixtures were incubated at 30 °C for 30 min, and after the reaction had been terminated by the addition of 30 µl of SDS-sample buffer containing 20 µg dithiothreitol, the mixture
was boiled for 5 min. The phosphorylation of the substrate proteins was examined by SDS-polyacrylamide gel electrophoresis (12% gels) followed by autoradiography.

**Analysis of in Vivo MAPKAP Kinase-2 Activity**—For determination of the effect of SB203580 on the *in vivo* activity of p38 MAP kinase, MAPKAP kinase-2 activity in the human FLS lysates was measured by use of a MAPKAP kinase-2 immunoprecipitation assay kit according to the manufacturer’s protocol. Briefly, cells were incubated with or without SB203580 for the designated time periods and then stimulated. Cells were lysed in cold lysis buffer. After centrifugation and normalization of the protein content, extracts (100 μg of protein) were mixed with 2 μg of sheep anti-MAPKAP kinase-2 antisera (Upstate Biotechnology) for 2 h at 4 °C. The MAPKAP kinase-2 and anti-MAPKAP kinase-2 immune complexes were precipitated with protein G-Sepharose beads (Amersham Pharmacia Biotech). The immunoprecipitates were then washed twice with lysis buffer and then twice with kinase buffer and resuspended in 30 μl of kinase assay buffer containing 100 μM substrate peptide KKLNR1LSVA (20), 50 μM ATP, and 10 μCi of [γ-32P]ATP. The reactions were incubated at 30 °C for 30 min and blotted onto p81 phosphocellulose paper. The papers were washed twice in 1% acetic acid and twice in water and then measured by a scintillation counter.

**Statistics**—Statistical analysis was performed by analysis of variance and Scheffe’s F test on a StatView 4.0 software program (Abacus Concepts, Inc., Berkeley, CA). A *p* value of <0.05 was considered to be significant.

**RESULTS**

**SB203580 Inhibits IL-6 Production Induced by IL-1β**—We examined the effect of a highly specific inhibitor of p38 MAP kinase (18), SB203580, on IL-6 production by IL-1β-induced human FLSs. As shown in Table I, SB203580 (0.01–10 μM) inhibited protein production of IL-6 in a concentration-dependent fashion, clearly indicating that SB203580 directly acts on human FLSs to suppress IL-6 synthesis. Essentially the same results were obtained using FLSs derived from five different donors. The time course of the SB203580 effect on IL-1β-induced IL-6 production was also determined by adding SB203580 (10 μM) at 2 h before and 0, 2, 4, 6, 8, and 12 h following the start of IL-1β stimulation. As shown in Table II, significant inhibition of IL-6 production was observed when SB203580 was added at 2 h before and 0, 2, and 4 h following the start of IL-1β stimulation.

**SB203580 Inhibits IL-6 mRNA Expression Induced by IL-1β**—Northern blot analysis was performed to determine whether SB203580 affected the IL-6 mRNA steady-state level. As shown in Fig. 1, SB203580 (0.1–10 μM) concentration-dependently inhibited IL-6 mRNA expression induced by IL-1β.

**TABLE I**

| Treatment | IL-6 ng/ml |
|-----------|------------|
| Experiment A |          |
| IL-1β (1 ng/ml) | 41.7 ± 0.6 |
| IL-1β + SB203580 (10⁻⁶ M) | 39.2 ± 0.1a |
| IL-1β + SB203580 (10⁻⁵ M) | 36.4 ± 0.6b |
| IL-1β + SB203580 (10⁻⁴ M) | 21.3 ± 0.6a |
| IL-1β + SB203580 (10⁻³ M) | 20.7 ± 0.6b |
| Experiment B |          |
| IL-1β (1 ng/ml) | 50.4 ± 3.8 |
| IL-1β + SB203580 (10⁻⁶ M) | 50.6 ± 8.6 |
| IL-1β + SB203580 (10⁻⁵ M) | 42.3 ± 2.0 |
| IL-1β + SB203580 (10⁻⁴ M) | 25.9 ± 5.4a |
| IL-1β + SB203580 (10⁻³ M) | 19.3 ± 2.6b |

* *p < 0.01 as compared with the values for IL-1β plus vehicle.

**TABLE II**

| Time of drug addition after IL-1β stimulation | IL-6 ng/ml |
|---------------------------------------------|------------|
| 0 h |          |
| 2 h | 54.8 ± 1.8 |
| 4 h | 55.2 ± 1.5 |
| 6 h | 54.5 ± 1.5 |
| 8 h | 54.9 ± 0.8 |
| 10 h | 55.6 ± 4.9 |
| 12 h | 56.9 ± 1.9 |

* *p < 0.001 as compared with the corresponding value for IL-1β plus vehicle.

* *p < 0.05 as compared with the corresponding value for IL-1β plus vehicle.

**Fig. 1. Dose-response study of SB203580 effect on IL-1β-induced IL-6 mRNA.** Human FLSs were stimulated with IL-1β for 6 h. Various concentrations of SB203580 were added at the start of the designated cultures. Total cellular RNA (20 μg) was then prepared, fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and then hybridized to the appropriate cDNA probes. A, a Northern blot autoradiograph representative of three experiments with similar results. B, relative levels of IL-6 mRNA expression were determined by densitometric scanning of the autoradiographic bands and normalized to the GAPDH signal. Data are expressed as the mean ± S.D. of three experiments. ***, *p < 0.001 as compared with the value for IL-1β alone (control).
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**Fig. 2.** Time course study of SB203580 effect on IL-1β-induced IL-6 mRNA expression. Human FLSs were incubated with IL-1β (1 ng/ml) or not incubated for 4 h, and then the cytokine-stimulated cells were treated with SB203580 (10 μM) for the indicated time periods (0–4 h). Total RNA (20 μg) was fractionated on a 1.2% (w/v) agarose gel, transferred onto Hybond N nylon membranes, and then hybridized to the appropriate cDNA probes. Results shown are representative of two experiments. The percentage values refer to the signal intensity of IL-6 mRNA normalized with respect to GAPDH mRNA as assessed by densitometry of autoradiographs. The 100% value is from cells stimulated with IL-1β (1 ng/ml) for 6 h.

**Effect of Cycloheximide on SB203580 Inhibition of IL-1β-induced IL-6 mRNA Expression.** To analyze whether the SB203580 effect was dependent on *de novo* protein synthesis, we performed experiments using an inhibitor of translation, cycloheximide. A representative result is shown in Fig. 3. Cycloheximide (10 μM) alone slightly increased both the basal (lane 2 versus lane 1) and IL-1β-induced (lane 6 versus lane 5) levels of IL-6 mRNA. SB203580 inhibited IL-1β-stimulated IL-6 mRNA expression (lane 7 versus lane 5). The concurrent presence of cycloheximide abolished the inhibitory effect of SB203580 (lane 8 versus lane 7) but rather slightly enhanced IL-6 mRNA expression, clearly demonstrating that the suppressive effect of SB203580 on IL-1β-stimulated IL-6 mRNA expression depends on *de novo* protein synthesis.

**Effects of IL-1β and SB203580 on IL-6 Gene Transcription.** To investigate whether the effect of SB203580 on IL-6 synthesis was exerted at the transcriptional level, we constructed human IL-6 promoter-luciferase reporter plasmids (Fig. 4A) and transfected human FLSs with them. pIL6–3BLuc contains the −1158 to +11 human IL-6 promoter region. pIL6–κB-tk contains four tandem copies of the IL-6-κB motif (−77 to −59) connected to the thymidine kinase promoter gene. It has been shown that the IL6-κB motif is the NF-κB and CBF1 overlapping binding element and is critical for the induction of the IL-6 promoter (16). A representative result is shown in Fig. 4B. pIL6–3BLuc and pIL6–κB-tk were clearly transcribed in response to IL-1β. SB203580 (10 μM) did not affect the transcription of either reporter construct. In this regard, genistein, an inhibitor of protein-tyrosine kinases, suppressed the transcription of both reporter constructs (data not shown).

The findings were confirmed at the level of endogenous IL-6 gene transcription assessed by the nuclear run-on experiments. As shown in Fig. 5, incubation of the cells with IL-1β (1 ng/ml) for 6 h increased the rate of IL-6 gene transcription. The autoradiogram clearly shows that SB203580 did not affect the rate of IL-6 gene transcription at all, when SB203580 was added either at 0 or 4 h following the start of IL-1β stimulation. No significant change was detected in the rate of GAPDH gene transcription. No hybridization to the vector plasmid was detected either. Genistein, again, suppressed the rate of IL-6 gene transcription (data not shown).

**Effect of SB203580 on IL-6 mRNA Stability.** The next experiment was performed to determine the effect of SB203580 on the stability of IL-6 mRNA. Human FLSs were stimulated with IL-1β for 4 h to express IL-6 mRNA. Then either actinomycin D, an inhibitor of overall gene transcription, or actinomycin D plus SB203580 was added. Total cellular RNA was isolated following various time periods after the addition of actinomycin D and examined for the presence of IL-6 mRNA by Northern blot analysis. A representative result is shown in Fig. 6A. For correction for differences in loading, the signal density of each RNA sample hybridized to the IL-6 probe was divided by the density hybridized to the GAPDH probe (Fig. 6B). IL-6...
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mRNA had a calculated half-life of 2 h in human FLSs. SB203580 reduced the half-life of IL-6 mRNA to 0.7 h.

In Vivo Activation of IL-1β-induced p38 MAP Kinase Activity in Human FLSs—p38 MAP kinase is activated by the dual specificity kinases that phosphorylate the threonine and tyrosine residues in its TGY motifs (19). The phosphorylation state of p38 MAP kinase was analyzed by Western blotting using anti-phospho-Tyr182 p38 MAP kinase antibody that selectively targets the fully phosphorylated p38 MAP kinase. As shown in Fig. 7A, p38 MAP kinase was phosphorylated upon stimulation with IL-1β, whereas the protein level of p38 MAP kinase was not significantly affected.

Next, p38 MAP kinase was immunoprecipitated from the cellular extracts with specific anti-p38 MAP kinase antibody, and in vitro kinase assays were performed with GST-ATF-2 fusion protein used as a substrate. As shown in Fig. 7B, treatment of human FLSs with IL-1β increased the p38 MAP kinase activity, which was detectable within 0.5 h and remained elevated for at least 6 h.

In Vivo Activation of MAPKAP Kinase-2 in Human FLSs Stimulated with IL-1β—MAPKAP kinase-2 is a substrate of p38 MAP kinase in several cell types (20). To determine whether MAPKAP kinase-2 is activated in human FLSs stimulated by IL-1β, we immunoprecipitated MAPKAP kinase-2 from cell lysates obtained from unstimulated or stimulated FLSs and then performed in vitro kinase assays using the peptide KKLNRILSVA as a substrate (20). As shown in Fig. 8, IL-1β clearly induced MAPKAP kinase-2 activity, which was significantly inhibited by SB203580 in a concentration-dependent manner. Essentially the same results were obtained using FLSs derived from three different donors.

DISCUSSION

Our present study clearly demonstrated that SB203580, a pyridinylimidazole inhibitor specific for p38 MAP kinase (18), concentration- and time-dependently suppressed protein production and mRNA expression of IL-6 by human FLSs induced by IL-1β (Table I and II; Figs. 1 and 2). p38 MAP kinase and its substrate, MAPKAP kinase-2, were really activated in IL-1β-stimulated human FLSs, and their activity was suppressed by SB203580 (Figs. 7 and 8). The effects of the p38 MAP kinase inhibitor on IL-1β-induced IL-6 mRNA expression were dependent on de novo protein synthesis (Fig. 3) and resulted from the reduction in IL-6 mRNA stability (Fig. 6). The rate of IL-6 gene transcription was not affected by SB203580 (Figs. 4 and 5).
compared with the value for IL-1β that p38 MAP kinase was activated by IL-1β. The mechanism of the inhibition of IL-6 synthesis by p38 MAP kinase has been elucidated. In the present study, we first demonstrated the initial IL-1β stimulation of human FLSs. MAPKAP kinase-2, a substrate of p38 MAP kinase, was also activated upon IL-1β stimulation of human FLSs (16). The results were also confirmed at the level of endogenous gene transcription. Furthermore, the phosphorylated form of p38 MAP kinase (Fig. 7A) and its kinase activity (Fig. 7B) were clearly induced upon IL-1β stimulation of human FLSs. MAPKAP kinase-2, a substrate of p38 MAP kinase, was also activated upon IL-1β stimulation (Fig. 8). At the same time, IL-1β-induced IL-6 gene expression in human FLSs was concentration-dependently suppressed by the p38 MAP kinase inhibitor, SB203580 (Fig. 1). p38 MAP kinase seemed to act directly on IL-6 gene expression but not to affect the initial IL-1β signal transduction pathway, since significant inhibition of IL-6 protein production (Table II) and mRNA expression (Fig. 2) was observed when the p38 MAP kinase inhibitor was added even 4 h after the start of IL-1β stimulation.

The most outstanding finding of our present study is that the inhibition of p38 MAP kinase activity significantly reduced the stability of the IL-6 mRNA (Fig. 6). Involvement of p38 MAP kinase in the stabilization of mRNA in a human mesenchymal cell has thus first been demonstrated by our study. The inhibition was dependent on protein synthesis, since the addition of cycloheximide abrogated the effect of SB203580 (Fig. 3). It was reported that a p38 MAP kinase inhibitor suppressed TNF-α synthesis without affecting the mRNA level (19, 39), probably at the translational level through AU-rich elements in its 3′-untranslated regions. Repeated AUUUA sequences in the 3′-untranslated region of many protooncogenes and cytokine mRNAs were not only the target for rapid degradation (59–61) but also suppressed the translation of the corresponding mRNAs (62, 63). IL-6 mRNA contains six copies of the AUUUA sequence in its 3′-untranslated region (64). There is a possibility that p38 MAP kinase or some downstream kinases influence the proteins that bind to the AUUUA sequence of IL-6 mRNA, resulting in mRNA stabilization. Indeed, several molecules such as AUF1 and AUBF bind AU-rich sequences to reduce and increase mRNA stability, respectively (65–67). In addition, the formation of the AUUUA sequence-binding complexes and the accelerated mRNA turnover are dependent on phosphorylation (67). The p38 MAP kinase inhibitor prevented the accumulation of steady-state IL-6 mRNA when added either 0 h or 4 h after the start of IL-1β stimulation (Figs. 2 and 3). Thus, a protein that destabilizes IL-6 mRNA might be produced soon after the addition of IL-1β, the action of which seems to be blocked by the activated p38 MAP kinase.

In our present study, no significant effect of the p38 MAP kinase inhibitor on the IL-6 promoter was demonstrated (Fig. 4). p38 MAP kinase activates several transcription factors such as ATF-2 (24), CHOP/GADD153 (25), MAX (26), myocyte enhancer factor 2C (27), and ternary complex factor (28–30), none of which seem to be involved in the activation of the IL-6 promoter by IL-1β in human FLSs (16). The results were also confirmed at the level of endogenous gene transcription as assessed by the nuclear run-on assay (Fig. 5), consistent with the notion that the effect of SB203580 on IL-6 gene expression results from the down-regulation of IL-6 mRNA stability. However, Beyaert et al. reported that IL-6 mRNA expression and activation of an NF-κB reporter gene by TNF-α-induced murine fibrosarcoma L929a cells were completely inhibited by SB203580 and concluded that p38 MAP kinase modulated IL-6 synthesis at the transcriptional level (41, 42). The differences in species, cell types, and IL-6 inducers may account for the discrepancy between the report by Beyaert et al. and ours.

Our present data collectively indicate that p38 MAP kinase is critically involved in IL-6 synthesis by human FLSs. The mechanisms by which p38 MAP kinase stabilizes cytokine mRNA warrant further investigation. Potent inhibitors of p38 MAP kinase may greatly improve our understanding of the pathophysiology of RA and could prove to be significantly beneficial for RA therapy.

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FIG. 8. In vivo activation of MAPKAP kinase-2 by IL-1β-stimulated human FLS. Human FLSs were treated with IL-1β (1 ng/ml) in the absence or presence of SB203580 for 6 h. Specific polyclonal antibodies were used to immunoprecipitate MAPKAP kinase-2 from cell lysates, and in vitro kinase assays were performed using 10 μCi of [γ-32P]ATP and the substrate peptide KKLRLTSLVA (20). Data are expressed as the mean ± S.D. of triplicate cultures. ***, p < 0.001 as compared with the value for IL-1β alone (control).
