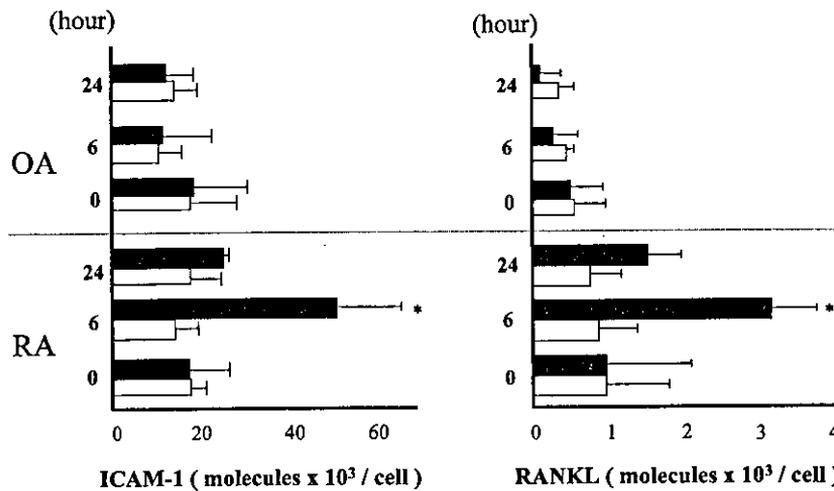**Figure 1.** Effects of fibroblast growth factor 2 (FGF-2) on the expression of intercellular adhesion molecule 1 (ICAM-1) and RANKL on synovial fibroblasts from 10 patients with rheumatoid arthritis (RA). RA synovial fibroblasts (RASFs) were incubated with FGF-2 (1–50 ng/ml) for 6 hours, and the levels of ICAM-1 and RANKL expression were analyzed by FACScan. The number of cell surface antigens on a single cell was calculated by QIFIKIT. Values are the mean and SD. $*$ = $P < 0.05$ and $**$ = $P < 0.01$ versus control (0 ng/ml of FGF-2), by unpaired t-test.

## RESULTS

We initially assessed the effects of FGF-2 on the expression of ICAM-1 and RANKL on RASFs using a flow cytometer. The indicated amounts of FGF-2 (1–50 ng/ml) were added to the cells, and after 6 hours of incubation, the cells were harvested. The cells were then stained using mAb, analyzed by flow cytometry, and the cell surface antigens on 1 cell were quantified with the use of standard beads. Approximately 25,000 molecules of ICAM-1 and 1,000 molecules of RANKL were spon-

Figure 2. Effects of fibroblast growth factor 2 (FGF-2) on the expression of intercellular adhesion molecule 1 (ICAM-1) and RANKL on synovial fibroblasts from 10 patients with RA and 7 patients with osteoarthritis (OA). OA and RA synovial fibroblasts were incubated with FGF-2 (10 ng/ml) in the presence (open bars) and absence (solid bars) of anti–FGF-2 antibody (10 μg/ml) for the indicated durations, and the levels of ICAM-1 and RANKL expression were analyzed by FACScan. The number of cell surface antigens on a single cell was calculated by QIFIKIT. Values are the mean and SD. $*$ = $P < 0.05$ versus 0 hours of incubation, by unpaired t-test.

A

B
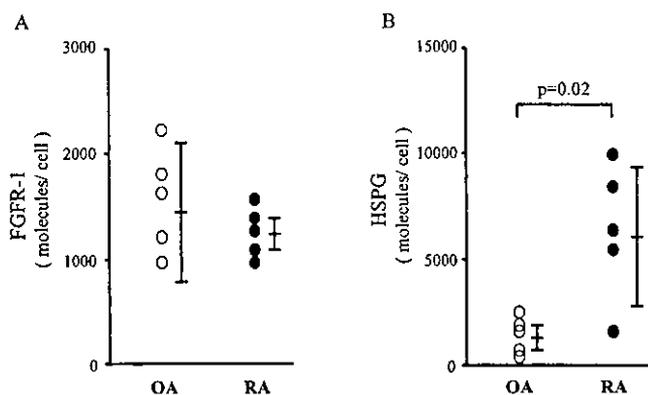
Figure 3. Expression of fibroblast growth factor receptor 1 (FGFR-1) and heparan sulfate proteoglycans (HSPGs) on synovial fibroblasts from 5 patients with rheumatoid arthritis (RA) and 5 patients with osteoarthritis (OA). Staining and flow cytometric analyses of RA and OA synovial fibroblasts were performed with A, anti–FGFR-1 antibody or B, anti–heparan sulfate monoclonal antibody 10E4, with subsequent staining with fluorescein isothiocyanate–conjugated goat anti-mouse IgG and analysis by FACScan. Values are the mean ± SD. P values were determined by unpaired t-test.

taneously expressed on RASFs (Figure 1). However, treatment with FGF-2 increased the numbers of both ICAM-1 and RANKL on RASFs in a dose-dependent manner with 6 hours of incubation. In particular, 10 ng/ml of FGF-2 induced 3 times as many RANKL molecules on the cells compared with spontaneous expression. These results indicate that certain concentrations of FGF-2 induce the expression of both RANKL and ICAM-1 on RASFs.

The effects of FGF-2 on the expression of ICAM-1 and RANKL on RASFs from 10 RA patients were compared with the effects on OASFs from 7 OA patients. We found that 10 ng/ml of FGF-2 failed to enhance ICAM-1 and RANKL expression on OASFs after 6 hours or 24 hours of incubation (Figure 2). In contrast, FGF-2 efficiently induced ICAM-1 and RANKL on RASFs, reaching maximum levels within 6 hours. Furthermore, up-regulation of ICAM-1 and RANKL by FGF-2 on RASFs was completely abrogated by the addition of anti–FGF-2 antibody, indicating that FGF-2 plays a pivotal role in the induction of ICAM-1 and RANKL on RASFs.

We next sought to determine why RASFs, but not OASFs, responded to FGF-2 by up-regulating the expression of ICAM-1 and RANKL in an effort to shed light on the receptors for FGF-2. We found that FGFR-1 was similarly expressed on RASFs and OASFs, with ~1,500 sites on 1 cell in these fibroblasts of different origins (Figure 3A). It is well known that HSPGs are coreceptors for FGF-2, promoting both the binding affinity of FGF for FGFR and the subsequent signaling. HSPGs were highly expressed on RASFs, but were only marginally expressed on OASFs (Figure 3B). Furthermore, removal of heparan sulfate from the surface of RASFs by pretreatment with heparitinase or heparinase markedly reduced the up-regulation of RANKL on these cells by FGF-2 (Figure 4). These results imply that the differential responsiveness of RASFs and OASFs to FGF-2 appears to depend on the significantly higher expression of HSPGs on

RANKL (molecules/ cell)
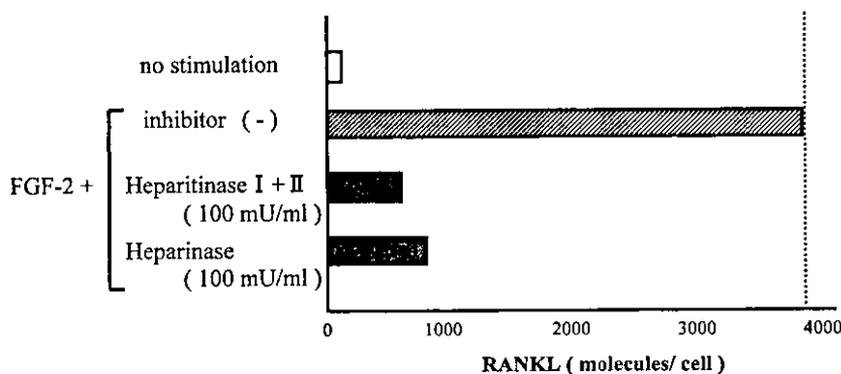
Figure 4. Inhibitory effects of heparitinase and heparinase on fibroblast growth factor 2 (FGF-2)–mediated RANKL expression on synovial fibroblasts from patients with rheumatoid arthritis (RA). RA synovial fibroblasts (RASFs) were treated with a mixture of 100 mU/ml of heparitinase I and II or with heparinase (100 mU/ml) at 37°C for 2 hours. After removal of the added reagents by washing, the RASFs were incubated in the presence or absence of FGF-2 (10 ng/ml) for 6 hours. RANKL expression on RASFs was determined by FACScan. Values are from a representative experiment of cells from 3 RA patients.
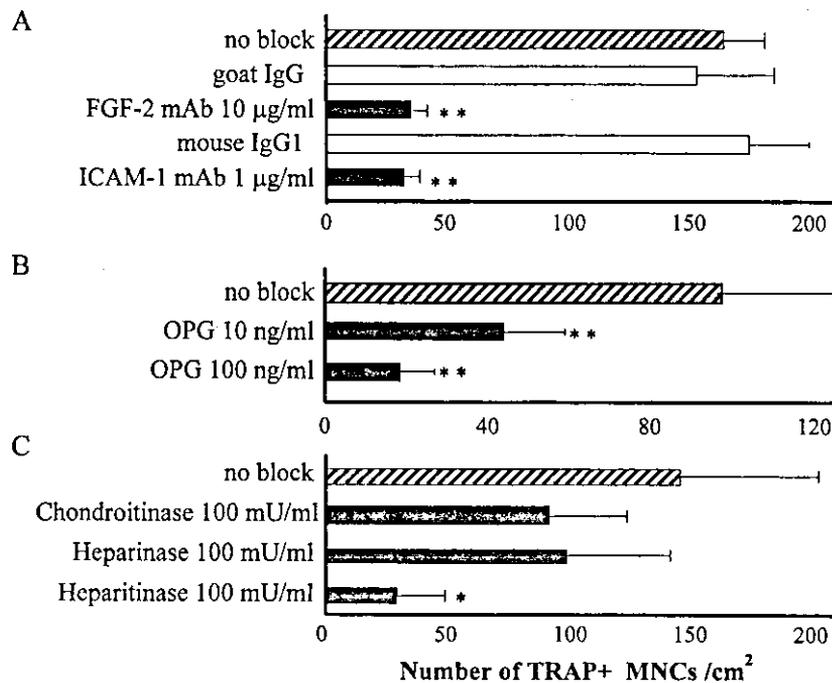
A

B   **C**
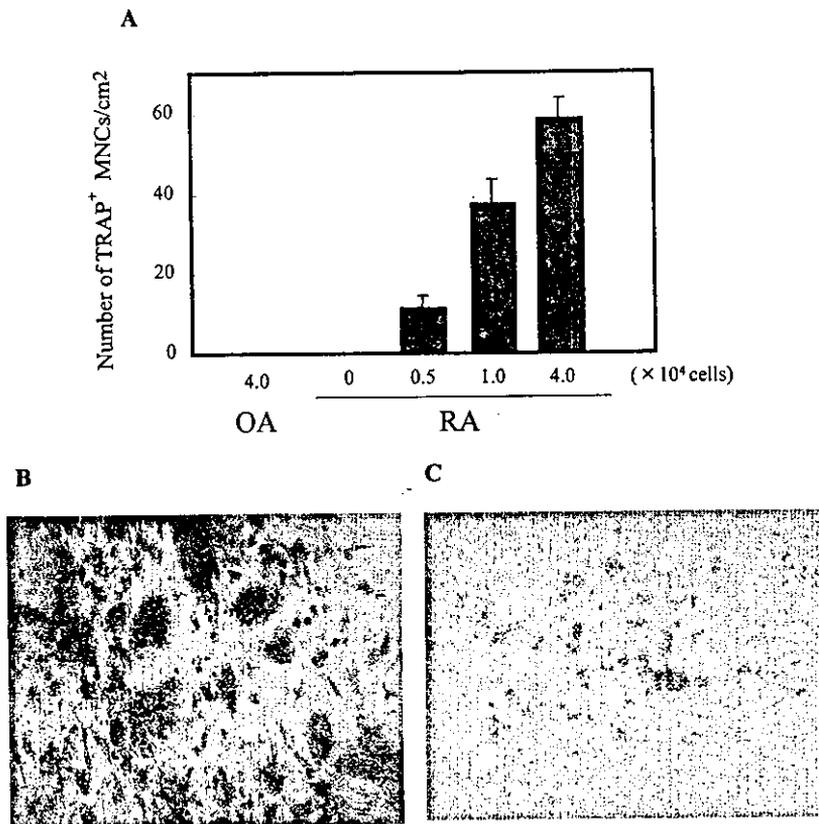
Figure 5. Formation of tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells (MNCs) from coculture of rheumatoid arthritis synovial fibroblasts (RASFs) and peripheral blood mononuclear cells (PBMCs). Coculture of RASFs and adherent PBMCs was maintained in the presence of 50 ng/ml of macrophage colony-stimulating factor and $10^{-7}M$ 1,25-dihydroxyvitamin D_3 for 9 days, and then the dishes were stained for TRAP. On day 11, dentin slices were placed in NH_4OH ($1N$) for 30 minutes and then cleaned by ultrasonication to remove adherent cells. After washing, the dentin slices were stained with hematoxylin and eosin. TRAP-positive multinucleated cells that contained >3 nuclei were identified as osteoclasts, and these were counted by light microscopy. A, Ability of RASFs to support osteoclastogenesis in cocultures with PBMCs. Values are the mean and SD of triplicate measurements. OA = osteoarthritis. B, TRAP-positive multinucleated cells in the coculture system. C, Resorption pits on dentin slices.

RASFs than that on OASFs and that heparan sulfate present on RASFs might hold FGF-2 as a coreceptor for FGFR-1.

The formation of TRAP-positive multinucleated cells from coculture of RASFs or OASFs (3–5 passages) and PBMCs in α-MEM containing 10% FCS and 50 ng/ml of M-CSF and $10^{-7}M$ 1,25(OH)$_2$D$_3$ was then assessed. After 9 days of culture, there was a marked increase in the number of TRAP-positive multinucleated cells in proportion to the number of RASFs in culture, whereas TRAP-positive multinucleated cells were not induced in cocultures of OASFs and PBMCs

(Figure 5A). Furthermore, when the pit-formation assay was performed using the TRAP-positive multinucleated cells induced from the RASF and PBMC coculture, multiple resorption pits were seen on dentin slices, indicating that the TRAP-positive multinucleated cells possess the bone-resorbing function (Figures 5B and C). These results indicate that RASFs, but not OASFs, are involved in osteoclastogenesis and subsequent bone resorption.

The formation of TRAP-positive multinucleated cells by coculture of RASFs and PBMCs was completely inhibited by the addition of anti–FGF-2 antibody, sug-

Figure 6. Inhibitory effects of anti–fibroblast growth factor 2 (anti–FGF-2) monoclonal antibody (mAb), anti–intercellular adhesion molecule 1 (anti–ICAM-1) mAb, osteoprotegerin (OPG), or heparitinase on FGF-2–induced tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells (MNCs) from coculture of rheumatoid arthritis synovial fibroblasts (RASFs) and peripheral blood mononuclear cells (PBMCs). On day 4, anti–FGF-2 antibody (10 μg/ml), goat IgG (control), ICAM-1 mAb 84H10 (1 μg/ml), or mouse IgG1 (control) (A), OPG (10 ng/ml or 100 ng/ml) (B), or RASFs pretreated with a heparitinase mixture (100 mU/ml), heparinase (100 mU/ml), or chondroitinase (100 mU/ml) (C) were added to the coculture system. Values are the mean and SD. * = $P < 0.05$ and ** = $P < 0.01$ versus no block, by unpaired t-test.

gesting that FGF-2 plays a pivotal role in osteoclastogenesis through the up-regulation of ICAM-1 and RANKL on RASFs (Figure 6A). The formation of TRAP-positive multinucleated cells from PBMCs in the presence of RASFs was also abrogated by the addition of anti–ICAM-1 mAb. The addition of 10 ng/ml of OPG to the cocultures on the fourth day also reduced the number of TRAP-positive multinucleated cells in a dose-dependent manner, reaching basal levels with the addition of 100 ng/ml of OPG (Figure 6B). These results imply that the high-affinity adhesion of PBMCs and RASFs is a prerequisite for the efficient signaling of RANKL on RASFs during osteoclast maturation. Furthermore, pretreatment of RASFs with 100 mU/ml of heparitinase, but not the same concentration of heparinase or chondroitinase, significantly reduced the formation of TRAP-positive multinucleated cells from the RASF and PBMC coculture (Figure 6C), suggesting that the heparan sulfate on RASFs, which holds FGF-2 as a

coreceptor and sends it to the FGFR-1 signaling receptor, plays a pivotal role in the osteoclastogenesis involving FGF-2/RANKL on RASFs.

In fibroblast proliferation, activation of MAPKs followed by autophosphorylation of FGFR is known to be involved in FGF-2 signaling. To study the association between MAPK activation and FGF-2-mediated RANKL expression on RASFs, we pretreated RASFs with PD 98059 (a specific inhibitor of ERK) and SB 202190 (a specific inhibitor of p38 MAPK). PD 98059, but not SB 202190, inhibited the up-regulation of RANKL on RASFs in a dose-dependent manner (Figure 7A). These results suggest that the FGF-2–mediated RANKL expression on RASFs requires activation of the ERK cascade. Furthermore, when we added RASFs pretreated with PD 98059 or SB 202190 to cocultures, we found that the formation of TRAP-positive multinucleated cells was also inhibited by PD 98059 in a dose-dependent manner (Figure 7B).
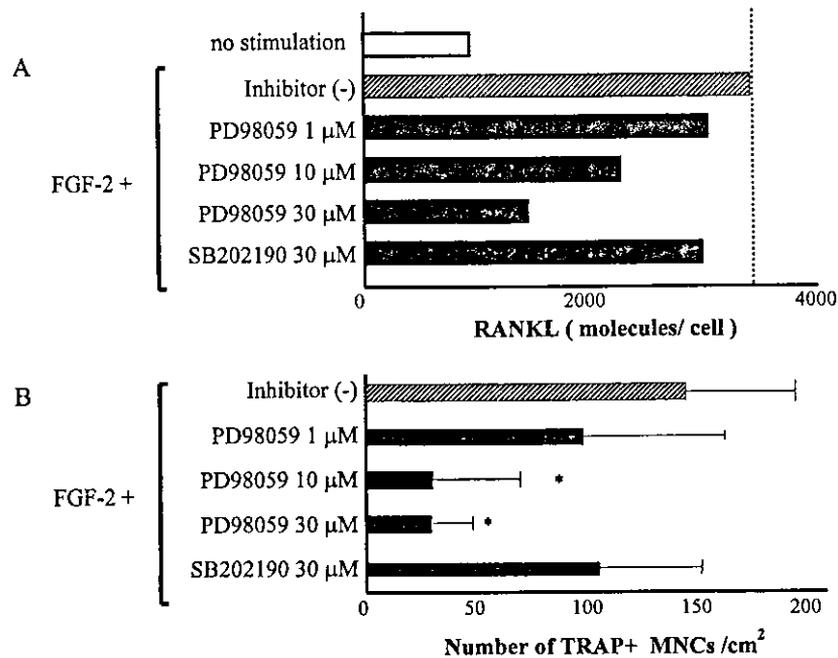
Figure 7. Involvement of MAPK activation in fibroblast growth factor 2 (FGF-2)–mediated RANKL expression on rheumatoid arthritis synovial fibroblasts (RASFs) and on osteoclast formation in a coculture system. A, RASFs were treated with PD 98059 (1, 10, or 30 μM) or SB 202190 (30 μM) at 37°C for 30 minutes. After washing, RASFs were incubated with or without FGF-2 (10 ng/ml) for 6 hours. RANKL expression was determined by FACScan. Values are from a representative experiment of cells from 3 rheumatoid arthritis patients. Dotted vertical line indicates without treatment with MAPK inhibitor. B, For coculture of RASFs and peripheral blood mononuclear cells (PBMCs) for the generation of osteoclasts, RASFs were treated with PD 98059 or SB 202190 at 37°C for 30 minutes. After washing, RASFs were added to adherent PBMCs. Cocultures were maintained in the presence of 50 ng/ml of macrophage colony-stimulating factor and $10^{-7}M$ 1,25-dihydroxyvitamin D_3 for 9 days, and then the dishes were stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells (MNCs) that contained >3 nuclei were identified as osteoclasts, and these were counted by light microscopy. Values are the mean and SD of triplicate measurements. * = $P < 0.05$ versus no inhibitor, by unpaired t-test.

DISCUSSION

Prevention of bone and cartilage destruction is the most important issue in the treatment of RA, but it has never been completely achieved by conventional drug therapy, which mainly targets the suppression of inflammation. Recently, some centers have used bisphosphonates to inhibit osteoclast activity, but this is not a specific therapy for the pathologic process of RA. In this regard, the mechanisms underlying osteoclast differentiation within the synovium, away from osteoblasts or bone marrow stromal cells, which are thought to be indispensable for osteoclastogenesis, have not yet been delineated. However, it has been reported that RASFs in coculture with PBMCs induce osteoclast formation and that this system requires cell-to-cell interaction between RASFs and PBMCs (17), but the underlying mechanism(s) of this interaction is not yet clear. In the present study, we showed that FGF-2, which is produced by RA synovial tissue, induced ICAM-1 and RANKL expression on RASFs.

We and other investigators have reported that osteoblasts, which express ICAM-1 (14) and RANKL (18), promote osteoclast formation by adhesion to PBMCs. It was suggested that in RA synovial tissue, RASFs direct osteoclast formation under the same mechanism as osteoblasts. Furthermore, we found that differences in RANKL and ICAM-1 expression levels on RASFs and OASFs by FGF-2 were not due to differ-

ences in levels of FGFR-1 expression, but rather, differences in levels of HSPG expression, which is a coreceptor of FGFR-1. These results suggest that control of the expression of HSPG is linked to the specific control of bone resorption in RA.

It has been reported that the actions of FGF-2 save RASFs from apoptosis (19), that RASFs express the HOXD9 gene, and FGF-2 promotes the autonomous production of FGF-2 (20), and that the synovial fluid concentration of FGF-2 correlates with the degree of bone destruction more so than do the synovial fluid concentrations of inflammatory cytokines, such as tumor necrosis factor α, interleukin-1α, and interleukin-6 (6). According to these reports, FGF-2 plays a leading role in the pathologic changes of RA, such as cell proliferation and bone and cartilage destruction, and is thought to be an attractive target in any disease-specific treatment.

Since Yayon et al (8) first demonstrated the importance of HSPG for high-affinity binding of FGF-2 to FGFR-1, extensive biochemical and biologic data have been reported. These data indicate that HSPG is essential for FGF/FGFR signaling (9–11,21,22). Although several models have been proposed from binding studies, functional analyses, and crystal structure analyses, the mechanism(s) through which HSPG/heparin assists FGF/FGFR signaling remains poorly understood.

Preincubation of RASFs with heparinase and heparitinase strongly inhibited FGF-2–stimulated expression of RANKL on RASFs. Furthermore, preincubation of RASFs with heparitinase strongly controlled osteoclast formation, whereas preincubation of RASFs with heparinase failed to control osteoclastogenesis.

Extrapolating from the current structural models, we suggest that FGFR dimerization and autophosphorylation is supported by cooperative "heparin-like end structures," and that cell surface association and concentration of these end structures compensate for the relative scarcity of such end structures in native HSPG (23,24). Heparitinase catalyzes the eliminative cleavage of α-N-acetyl-D-glucosaminidic linkage in heparan sulfate, whereas heparinase mainly acts on heparin. In this osteoclast formation assay, we found that heparitinase resolved the heparan sulfate chains of cell surface HSPG most effectively and inhibited FGF receptor dimerization, activation, and signaling.

FGF-2/FGFR signaling enhanced cell proliferation to fibroblasts through ERK (25,26). In studies of the osteoclastogenesis of the murine monocytic cell line RAW264.7, it was reported that the ERK pathway negatively regulated RANKL-induced osteoclast maturation, whereas the p38 MAPK pathway positively reg-

ulated it (27,28). Our results showed that activation of ERK was necessary for RANKL expression on RASFs. Osteoclast formation was suppressed, a finding similar to that when RASFs were preincubated with heparitinase under the control of ERK activation. Previous studies indicated that FGF-2 acts on osteoblasts through activation of FGFR-1 and ERK, causing the expression of RANKL and the stimulation of bone resorption at physiologic or pathologic concentrations (29). It is possible that the same mechanism also exists in RASFs.

In conclusion, the present study demonstrated that FGF-2 binds to FGFR-1 through HSPGs, which are characteristically expressed on RASFs, and results in RANKL- and ICAM-1–mediated maturation of osteoclasts via ERK activation. Thus, FGF-2 is involved in osteoclast maturation, which leads to bone destruction and osteoporosis in RA. These results suggest that FGF-2– and HSPG-mediated signaling could be a suitable target for the treatment of RA.

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Autocrine Induction of the Human Pro-IL-1β Gene Promoter by IL-1β in Monocytes

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IL-1β is produced primarily by activated monocytes/macrophages. We report in this study that IL-1β induces the human pro-IL-1β (*IL1B*) gene promoter in human THP-1 monocytic cells. The −131 to +12 minimal IL1B promoter was induced by IL-1β in a dose-dependent manner. The promoter possesses two important transcription factor binding motifs, one for an ETS family transcription factor Spi-1 (PU.1), and the other a binding site for NF-IL6 (CCAAT/enhancer binding protein β). Autocrine promoter activity was completely inhibited by mutation of the Spi-1 site. Mutation of the NF-IL6 binding motif caused partial loss of activity. EMSAs using THP-1 cell nuclear extracts indicated that IL-1β significantly induced Spi-1 binding to its target site within the IL1B promoter that was maximal at 1 h after stimulation, correlating with the kinetics of IL-1β induction. The importance of Spi-1 was supported by our observation that Spi-1-deficient EL4 thymocytes exhibited IL-1β-induced activity only after transfection with a Spi-1 expression vector. Moreover, TNFR-associated factor 6 also required Spi-1 to activate the promoter. Transfection studies using Spi-1 mutant constructs showed that the TATA-binding protein binding and glutamine-rich domains of Spi-1 were important for IL-1β induction, whereas LPS induction required the proline, glutamic acid, serine, and threonine-rich domain containing serine 148 as well as the TATA-binding protein and glutamine-rich domains. We conclude that the *IL1B* promoter is an IL-1β-responsive sequence as a result of its ability to bind Spi-1 in response to IL-1β. *The Journal of Immunology*, 2002, 168: 1984–1991.

I nterleukin-1 is a multifunctional cytokine that mediates a wide spectrum of inflammatory, metabolic, physiologic, hematopoietic, and immunological processes (1–3). A dramatic increase in IL-1 production occurs in response to various stimuli, including LPS, PMA, and cytokines. Some cells, but not exclusively tumor cells, produce cytokines that activate receptors on their own cell surface. It is well known that IL-1 stimulates its own gene expression and synthesis in vascular smooth muscle cells (4), endothelial cells (5), and monocytes (6, 7). Increased IL-1 production has been shown in various human diseases (1–3). These findings suggest that autoinduction of IL-1 may contribute to some pathologic processes via self-amplification of gene expression. IL-1β is a principal mediator in the pathogenesis of rheumatoid arthritis. It is involved in the mechanisms that result in progressive joint destruction in rheumatoid arthritis. Ghivizzani et al. (8) have reported that expression of human IL-1β following gene transfer to rabbit knee joints results in a severe aggressive form of arthritis with elevated levels of rabbit IL-1β and TNF-α in rabbit synovial fluid. Moreover, IL-1β is an autocrine growth factor for human acute myeloid leukemia cells (9, 10). It is noteworthy that in some cases IL-1β production in acute myeloid leukemia cells was partially inhibited by IL-1R antagonist (11).

IL-1β is most abundantly expressed in activated monocytes/macrophages. Production of IL-1β is tightly regulated in monocytes/macrophages. The human pro-IL-1β gene (referred to here by its genomic locus name, *IL1B*) encoding pro-IL-1β, a 31-kDa IL-1β precursor protein, is normally silent but is rapidly transcribed in competent cells upon stimulation (12, 13). The best-characterized stimulus that triggers IL-1β production is LPS. It was previously reported that human blood monocytes possess receptors for IL-1 (14). IL-1β production in monocytes has been demonstrated to be induced in response to IL-1 treatment (6, 7). However, the mechanism by which IL-1β itself activates the *IL1B* gene remains unclear.

Monocyte/macrophage-specific expression of the *IL1B* gene depends upon its promoter located between positions −131 and +12 (15, 16). The *IL1B* promoter contains two important transcription factor binding motifs: one is a binding site for NF-IL6, which is the β form of the CCAAT/enhancer binding protein (C/EBP)[2] of the basic leucine zipper family; and the other for Spi-1 (PU.1), a myeloid and B cell-specific winged helix-turn-helix transcription factor that belongs to the ETS family of proteins.

In the present study, we examined the promoter activity of the *IL1B* gene in transiently transfected human THP-1 monocytic cells and demonstrated that IL-1β induced the *IL1B* promoter in a dose-dependent manner. Mutation of the Spi-1 binding site within the *IL1B* gene promoter completely inhibited IL-1β-induced promoter activity, whereas mutation of the NF-IL6 site resulted in a partial loss of promoter activity. The results of EMSA using nuclear extracts prepared from IL-1β-treated THP-1 monocytes showed that

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[2] Abbreviations used in this paper: C/EBP, CCAAT/enhancer binding protein; Q, glutamine-rich; CAT, chloramphenicol acetyltransferase; TRAF6, TNFR-associated factor 6; PEST, proline, glutamic acid, serine, and threonine-rich; TBP, TATA-binding protein; IRAK, IL-1R-associated kinase.

IL-1β induced binding of Spi-1 to the *IL1B* target site. The importance of Spi-1 was further supported by our finding that IL-1β induction of the *IL1B* promoter was not observed in Spi-1-deficient EL4 cells, but was detected in EL4 cells carrying a Spi-1 expression vector. TNFR-associated factor 6 (TRAF6) also required Spi-1 expression in EL4 cells to activate the promoter. Transfection studies using various activation domain deletions of the Spi-1 cDNA revealed that IL-1 induction of the *IL1B* promoter depends upon the TATA-binding protein (TBP) binding and glutamine-rich (Q) domains of the Spi-1 protein, whereas activation by LPS requires the proline, glutamic acid, serine, and threonine-rich (PEST) domain containing serine 148 as well as the TBP and Q domains. Based on these results, we propose that the *IL1B* promoter is a IL-1β-responsive sequence as a result of its ability to bind Spi-1 in response to IL-1β.

Materials and Methods

Endotoxin tests

Very low concentrations of endotoxin induce *IL1B* gene transcription in THP-1 monocytes, as described previously (17). Therefore, all materials and solutions including RPMI 1640 medium, FBS (Equitech-bio, Ingram, TX), and human rIL-1β were tested for endotoxin by the *Limulus* amebocyte lysate test, as described previously (15). In particular, IL-1β used in the present study contained <0.003 ng/mg endotoxin. Moreover, sterile irrigation water and disposable sterile pipettes and tubes were used to reduce endotoxin levels. Basal *IL1B* gene activation was avoided by culturing cells in endotoxin-free medium.

rIL-1β protein and other reagents

Human rIL-1β protein (1 × 10⁸ U/mg as assayed by a thymocyte proliferation assay) was provided by Otsuka Pharmacia (Fukuoka, Japan). Human rIL-1α protein had a specific activity of 1 × 10⁸ U/mg (Dainippon Pharmacia, Osaka, Japan). Anti-Spi-1 Ab and anti-Oct-1 Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Spi-1 Ab was raised against a synthetic peptide corresponding to amino acids 251–271 mapping at the carboxyl terminus of Spi-1 of mouse origin. Anti-Oct-1 Ab was raised against a synthetic peptide corresponding to amino acids 723–743 mapping at the carboxyl terminus of Oct-1 of human origin. The two Abs did not cross-react with other transcription factors. LPS (*Escherichia coli* O55:B5), PMA, rabbit anti-human IL-1β Ab, and rabbit anti-human IL-1α Ab were purchased from Sigma-Aldrich (St. Louis, MO). One milligram of rabbit anti-human IL-1β Ab neutralizes a minimum of 7,000 U of human IL-1β. One milligram of rabbit anti-human IL-1α Ab neutralizes a minimum of 35,000 U of human IL-1α.

Cells

The human THP-1 monocytic cell line (JCRB0112) was purchased from Health Science Research Resources Bank (Osaka, Japan). The mouse EL4 thymoma cell line was kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Cells were carefully maintained in endotoxin-free complete RPMI 1640 medium supplemented with 10% FBS. Cells were split at a 1/3 dilution every 3 or 4 days to avoid overcrowding and were further split at 1/2 on the day before transfection or preparation for nuclear extracts.

Plasmids

Human *IL1B* genomic DNA fragments were derived from clone BDC454 (18). We used identical sequence numbering to that described previously (15). The construct, 3MEHT, contained the *IL1B* promoter HT sequence (16). The HT sequence (construct 3MEHT) located between positions −131 and +12 was cloned into the chloramphenicol acetyltransferase (CAT) gene plasmid vector pA10CAT3ME (3ME). Mutations of the Spi-1 and NF-IL6 sites were the same as those reported previously (16). These mutations were verified by sequencing. MHC/*fos*CAT contains three tandem repeats of the NF-κB binding site of the *MHC class I* gene enhancer as described previously (19, 20). The series of Spi-1 pECE expression vectors consisting of a wild-type Spi-1 cDNA and deletion constructs were gifts from Dr. R. A. Maki (Burnham Institute, La Jolla, CA) (21). Expression vectors for the full-length NF-IL6 (pcNF-IL6) and a truncated NF-IL6 with a deletion of the internal *SpI/SpII* fragment (amino acid sequence between residues 41 and 205) (pcmNF-IL6(Δ*spI*)) were generated by inserting the respective coding regions into pcDNA1 (Invitrogen, Carlsbad,

CA) (19). A TRAF6 expression vector, pcTRAF6, was constructed by inserting the TRAF6 cDNA into the pcDNA3.1 expression vector (Invitrogen).

Transfection and CAT assay

THP-1 cells and EL4 cells were transfected by the DEAE-dextran method as described previously (15, 22). This technique was used because, unlike electroporation, it did not generate induction of the endogenous *IL1B* gene. Cells (1 × 10⁷ cells per plate) were transfected with 10~18 μg of plasmids. After transfection, the cells were left untreated or were treated with IL-1β (1~10 ng/ml for THP-1 cells and 2 ng/ml for EL4 cells) for 24 h. CAT assays were performed using the liquid scintillation method as described previously (15), except using Pica Gene cell lysis buffer (Toyo Ink, Tokyo, Japan). CAT activities were determined by calculating slopes from plots of time vs cpm within a linear range of the response.

Nuclear extracts and EMSA

Nuclear extracts were prepared from THP-1 monocytes and EL4 thymocytes as reported previously (15). Protein concentrations of extracts were determined using the Bio-Rad protein assay kit (Melville, NY). Oligonucleotides were labeled by filling in 3' recessed ends with the DNA polymerase Klenow fragment and [α-³²P]dNTP (Amersham Life Science, Little Chalfont, Buckinghamshire, U.K.). Binding reactions were conducted as described previously, followed by analysis on a 4% polyacrylamide gel using 0.5× TBE buffer (89 mM Tris-borate and 2.5 mM EDTA) as the running buffer (16).

RT-PCR

THP-1 cells were harvested after treatment with 10 ng/ml of IL-1β, and total RNA was extracted by ISOGEN RNA extraction kit (Nippon Gene, Tokyo, Japan). After spectrophotometric quantification, 200 ng of total RNA was used along with a reverse transcriptase RNA PCR kit, Access RT-PCR System (Promega, Madison, WI) according to the manufacturer's instructions. An aliquot of the PCR mixture was subjected to electrophoresis in 2% agarose gel. PCR primers were synthesized as follows: human IL-1β sense, 5'-CAGAGAGTCCTGTGCTGAAT-3'; human IL-1β antisense, 5'-GTAGGAGAGGTCAGAGAGGC-3' (23); β-actin sense, 5'-TCATGAAGTGTGACGTTGACATCCGT-3'; and β-actin antisense, 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'.

Results

Autocrine induction of the IL1B promoter by IL-1β

We previously demonstrated that monocyte-specific expression of the human *IL1B* gene depends upon the *IL1B* promoter, located between positions −131 and +12 of the gene (15, 16). IL-1β has been reported to induce production of G-CSF in human THP-1 monocytic cells (24). In the present study, we examined the effect
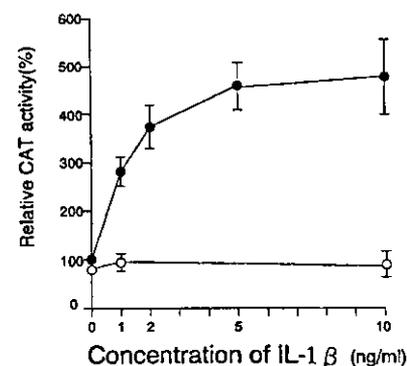
FIGURE 1. IL-1β dose-dependently induces the *IL1B* promoter activity in THP-1 monocytes. Ten micrograms of 3MEHT (●) or 3ME (○) was transfected into THP-1 cells. After transfection, cells were treated with various concentrations of IL-1β or left untreated. The CAT activity was calculated as described in *Materials and Methods*. Data represent the mean ± SD of three experiments. The CAT data were normalized to the average activity elicited by the 3MEHT construct in the absence of IL-1β.

of IL-1β on *IL1B* promoter activity. The −131 to +12 *IL1B* promoter element (HT fragment) was introduced into the 3ME CAT vector and assayed for IL-1β-induced CAT activity following transfection into THP-1 cells. As shown in Fig. 1, IL-1β induced *IL1B* promoter activity in a dose-dependent manner. When 10 ng/ml of IL-1β was used, an ~5-fold increase in activity over control was observed. 3ME vector, in contrast to 3MEHT, did not show any increased CAT activity following IL-1β treatment. These results indicate that IL-1β induces the *IL1B* promoter in THP-1 monocytic cells.

The −50 to −39 Spi-1 binding site is essential for IL-1β induction of the IL1B promoter

The *IL1B* promoter HT fragment contains two important transcription factor binding motifs: one is a binding site for NF-IL6 and the other is a binding site for Spi-1 (Fig. 2*A*). In the present study, two distinct mutated CAT constructs, 3MEHTmSpi-1 and 3MEHT-
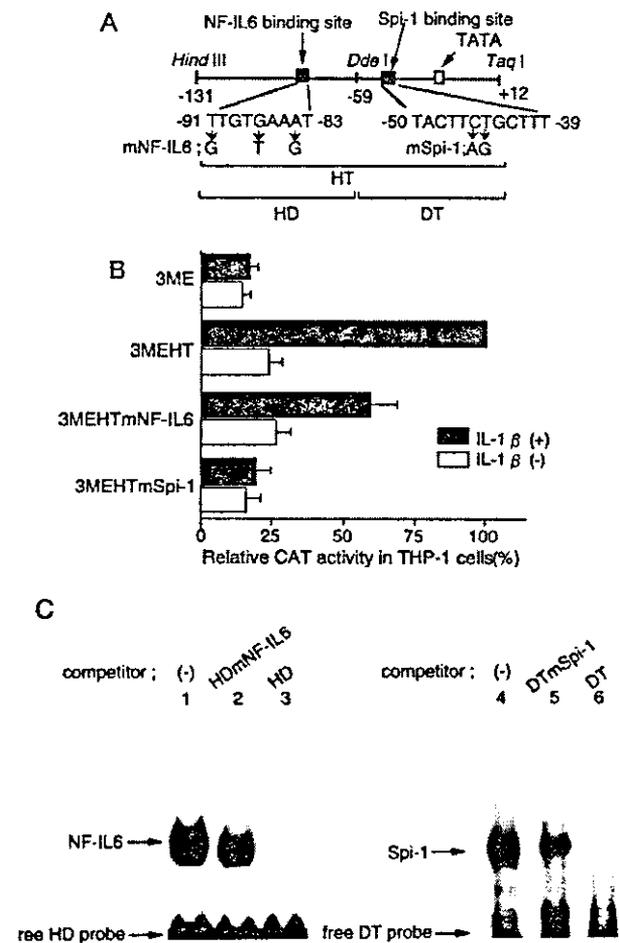

FIGURE 2. Mutation of the −50 to −39 Spi-1-binding site abrogates IL-1β-induced promoter activity. *A*, The schema shows the wild-type *IL1B* promoter HT fragment and two distinct mutated HT (HTmNF-IL6 and HTmSpi-1). As indicated by arrows, these mutations are located at specific sites known to be critical for NF-IL6 and Spi-1 binding, respectively. *B*, CAT reporters were transfected into THP-1 cells and treated with 2 ng/ml IL-1β. The CAT data were normalized to the average activity elicited by IL-1β-induced wild-type 3MEHT. Data represent the mean ± SD of three experiments. *C*, EMSAs were performed with rNF-IL6 and Spi-1 proteins. HD and DT fragments (*A*) were used as radiolabeled probes. Unlabeled competitor DNAs were used at a 30-fold molar excess over the radiolabeled probes.

mNF-IL6, were used to examine specific sequence requirements for IL-1β induction in THP-1 monocytic cells. Specific nucleotide substitutions (Fig. 2*A*) were introduced into either the −50 to −39 Spi-1 binding site (HTmSpi-1) or the −91 to −83 NF-IL6 site (HTmNF-IL6). As shown in Fig. 2*B*, mutation of the Spi-1 site almost completely abolished IL-1β-induced promoter activity. These results reveal that the Spi-1 site is essential for IL-1β induction of the *IL1B* promoter. In contrast, the −91 to −83 NF-IL6 binding motif mutation (3MEHTmNF-IL6) caused only a partial loss of activity (60% of the wild-type 3MEHT), suggesting that the NF-IL6 site is not essential for IL-1β induction but is important for maximal transcriptional activity. This argument is supported by our finding in EMSA experiments that the mutation of the −91 to −83 NF-IL6 binding motif completely abolished binding of rNF-IL6 to the IL1B promoter HD fragment (Fig. 2*C*, lanes 1–3). HDmNF-IL6, which was generated by restriction endonuclease digestion, was identical to the wild-type HD but contained nucleotide substitutions, mNF-IL6, within the −91 to −83 region. Moreover, when the binding affinity of rSpi-1 for the wild-type DT was compared with that for DTmSpi-1, the mutation significantly inhibited binding of rSpi-1 (Fig. 2*C*, lanes 4–6).
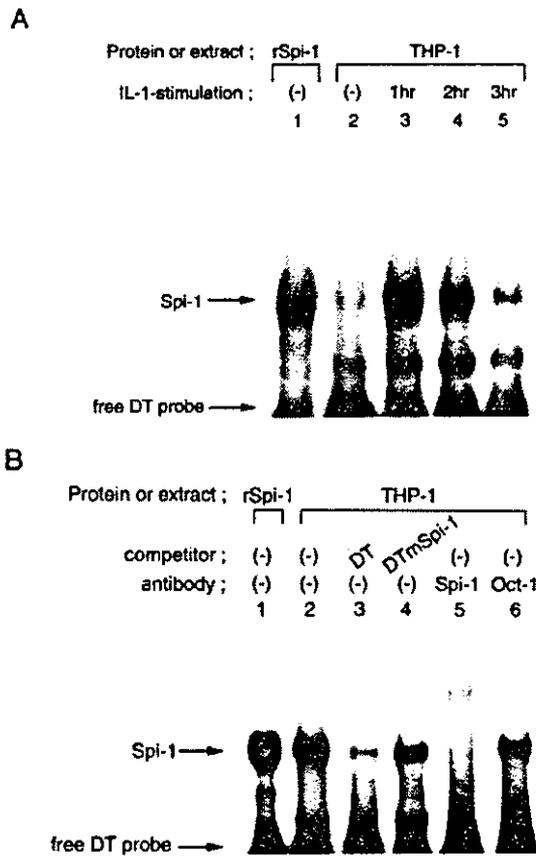
IL-1β induces binding of Spi-1 to the IL1B promoter

Our mutation studies showed that IL-1β-induced transcriptional activation of the *IL1B* promoter requires the −50 to −39 Spi-1 binding motif. To examine binding of specific protein to the Spi-1 site, EMSA studies were performed using a radiolabeled DT probe containing the −50 to −39 Spi-1 binding site (Fig. 2*A*). Nuclear extracts were prepared from THP-1 cells treated with IL-1β. As shown in Fig. 3, *A* and *B*, THP-1 nuclear extracts generated a DNA-protein complex (Fig. 3, arrow), which comigrated with in vitro expressed Spi-1 protein (Fig. 3, lanes 1). Although untreated THP-1 nuclear extract showed a constitutive binding activity (Fig. 3*A*, lane 2), the complex formation was markedly enhanced following treatment with IL-1β. The intensities of the complex reached a maximum at 1 h after stimulation (Fig. 3*A*, lane 3) and returned to a basal level 3 h after treatment (Fig. 3*A*, lane 5). The complex was competed for by a 30-fold molar excess of unlabeled DT fragment itself (Fig. 3*B*, lane 3). In contrast, DT containing site-specific mutation of the Spi-1 binding site (DTmSpi-1) did not compete for the protein-DT complex (Fig. 3*B*, lane 4). Furthermore, the complex was abrogated and supershifted by the addition of anti-Spi-1 Ab (Fig. 3*B*, lane 5), whereas it was not recognized by anti-Oct-1 Ab (Fig. 3*B*, lane 6). These results demonstrate that the IL-1β-induced DT-protein complex contains Spi-1 protein.

Expression of Spi-1 in EL4 cells mediates IL-1β-induced IL1B promoter activation

To clarify the functional involvement of Spi-1 protein in IL-1β induction of the *IL1B* promoter, various amounts of a Spi-1 expression vector, pECE Spi-1, were cotransfected into EL4 thymoma cells along with the *IL1B* promoter CAT reporter, 3MEHT. Following cotransfection, cells were treated with 2 ng/ml IL-1β or left untreated, and assayed for CAT activity. Untransfected EL4 cells do not express Spi-1 protein (Fig. 5*C*, lane 2). As shown in Fig. 4, IL-1β-induced activity in EL4 cells was increased in a Spi-1 dose-dependent manner. In the presence of 4 μg of pECE Spi-1, IL-1β treatment resulted in an ~3-fold increase in activity over control without IL-1β treatment. In contrast, IL-1β failed to augment the *IL1B* promoter activity in Spi-1-deficient EL4 cells. These results demonstrate that Spi-1 functions as a transcriptional activator that is essential for IL-1β induction.

A

Protein or extract : rSpi-1 THP-1

IL-1-stimulation : (-) (-) 1hr 2hr 3hr

1 2 3 4 5

Spi-1 ⟶

free DT probe ⟶

B

Protein or extract : rSpi-1 THP-1

competitor : (-) (-) DT DTmSpi-1 (-) (-)

antibody : (-) (-) (-) (-) Spi-1 Oct-1

1 2 3 4 5 6

Spi-1 ⟶

free DT probe ⟶

FIGURE 3. Spi-1 binds to the *IL1B* promoter through IL-1β stimulation. DT (Fig. 2*A*) oligonucleotide was used as a radiolabeled probe. *Lanes 1,* rSpi-1 protein obtained by in vitro translation (rSpi-1) was used as a control. The arrows locate the mobility of Spi-1. *A,* Nuclear extracts were derived from THP-1 cells untreated (*lane 2*) or treated with IL-1β for the time indicated over lanes (*lanes 3-5*). *B,* Nuclear extract was prepared from THP-1 cells treated with IL-1β for 1 h (*lanes 2-6*). Unlabeled competitor DNAs were used at a 30-fold molar excess over the radiolabeled DT probe (*lanes 3* and *4*). In supershift experiments, nuclear extracts were preincubated with either anti-Spi-1 Ab or anti-Oct-1 Ab at room temperature for 15 min (*lanes 5* and *6*).

IL-1β induction depends on a Q domain and a TBP binding domain of Spi-1 protein

Spi-1 protein contains at least three independent transcriptional activation domains: a TBP binding region, a Q domain, and a PEST region (Fig. 5*A*) (21). In the present study, to determine the domain of Spi-1 protein necessary for IL-1β induction of the *IL1B* promoter, various deletion mutant Spi-1 proteins were assayed in transient transfection studies using EL4 cells. As shown in Fig. 5*B*, removal of the PEST sequence did not reduce IL-1β-dependent activity, indicating that the PEST domain was dispensable for IL-1β induction of the *IL1B* promoter. This is consistent with our observation that mutation of a serine to alanine at codon 148 (S148A) also failed to inhibit the IL-1β-induced activity. However, deletion of either the Q domain (Δ75/100) or the TBP binding region (Δ8/32) markedly inhibited the IL-1β-inducible activity. This argument is further supported by our EMSA data using nuclear extracts prepared from EL4 cells transiently transfected with expression vectors for the mutant Spi-1 proteins. As shown in Fig. 5*C,* the relative ex-
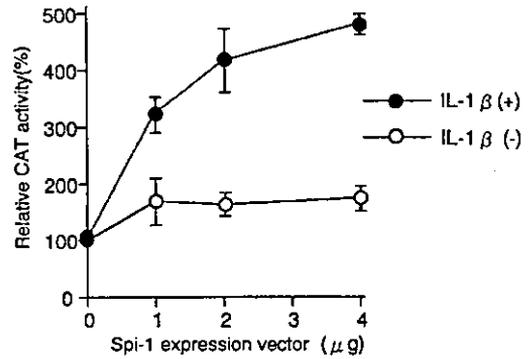
FIGURE 4. IL-1β induces the *IL1B* promoter in Spi-1-expressing EL4 cells. Increasing amounts of a Spi-1 expression vector, pECE Spi-1 were cotransfected into EL4 thymoma cells along with 3MEHT reporter (10 μg). The total amount of transfected DNA was kept constant (14 μg) by addition of control vector. At 24 h after transfection, the cells were treated with 2 ng/ml IL-1β (●) or left untreated (O). The CAT data were normalized to the average activity elicited by the IL-1β-induced 3MEHT construct in the absence of expression vector cotransfection. Data represent the mean ± SD of three experiments.

pression levels of the mutant Spi-1 proteins in EL4 cells were almost the same. Moreover, when the various deletion mutant Spi-1 proteins were expressed in THP-1 monocytes, PMA induction also did not require serine 148. In contrast, S148A markedly inhibited the ability of Spi-1 to transactivate the *IL1B* promoter in the presence of LPS.

We further performed transient transfection studies using neutralizing Abs against IL-1α and IL-1β to elucidate the effect of endogenous IL-1 expression on activation of the *IL1B* promoter by LPS or PMA. Neutralizing activities of the Abs were assessed in transfection studies using EL4 cells and MHC/*fos*CAT vector containing tandem repeats of NF-κB binding site. As shown in Fig. 5*D,* anti-IL-1β Ab almost completely inhibited IL-1β-induced activity for MHC/*fos*CAT. IL-1α-induced CAT activity for MHC/*fos*CAT was also neutralized by the addition of anti-IL-1α Ab. However, neither LPS induction nor PMA induction of the *IL1B* promoter in THP-1 cells was inhibited by the addition of a mixture of anti-IL-1α Ab and anti-IL-1β Ab, showing that endogenous IL-1 expression has no effect on *IL1B* promoter activity induced by either LPS or PMA. Spi-1 protein activation by either LPS or PMA was not also inhibited by the addition of the Abs. In particular, the addition of the Abs failed to affect activation of Spi-1 S148 mutant by PMA.

*TRAF6-mediated activation of the IL1B promoter requires Spi-1*

The IL-1 signal transduction is initiated by the association of IL-1 with the three extracellular Ig domains of IL-1 type I receptor, which, in turn, results in the association of IL-1R accessory protein (2, 25-27). This signaling complex recruits the adapter protein MyD88, which mediates the interaction of the IL-1R-associated kinases (IRAK). IRAK recruits TRAF6 to the activated IL-1 type I receptor. TRAF6 as well as MyD88 and IRAK are critical signal transducers for IL-1 (28, 29). In the present study, a TRAF6 expression vector, pcTRAF6, and a Spi-1 expression vector, pECE Spi-1, alone or in combination was cotransfected into EL4 cells along with the *IL1B* promoter CAT reporter 3MEHT and assayed for TRAF6-induced promoter activity in the presence and absence of Spi-1. As shown in Fig. 6, transfection of pcTRAF6 did not

FIGURE 5. TBP binding and Q domains of Spi-1 are required for IL-1β to induce the *IL1B* promoter. *A*, Schematic diagram of Spi-1 functional domains previously identified and the regions contained within various Spi-1 expression vectors. *B*, Various Spi-1 expression vectors (4 μg) as depicted in *A* were cotransfected into either EL4 thymoma or THP-1 monocyte cells along with 3MEHT CAT reporter (10 μg). Following transfection, EL4 cells were treated with 2 ng/ml IL-1β or left untreated. THP-1 cells were treated with either 100 ng/ml of LPS or 50 ng/ml of PMA. The CAT data were normalized to the average activity elicited by the IL-1β-induced 3MEHT construct in wild-type (WT) Spi-1-expressing EL4 cells. Data represent the mean ± SD of three experiments. *C*, DT was used as a radiolabeled probe. Nuclear extracts were prepared from EL4 thymoma cells carrying various Spi-1 expression vectors as indicated over lanes. At 24 h after transfection, the cells were stimulated by 2 ng/ml IL-1β for 1 h. *D*, The effects of endogenous IL-1 expression on LPS- or PMA-induced *IL1B* promoter activity were investigated. Spi-1 expression vectors (4 μg) were cotransfected into THP-1 cells along with 3MEHT CAT reporter (10 μg). As a control study, MHC/*fos*CAT (10 μg) was transfected into EL4 cells. Following transfection, the cells were treated as indicated. LPS, PMA, IL-1, and Abs were used at the following concentrations: LPS, 100 ng/ml; PMA, 50 ng/ml; IL-1β, 1 ng/ml; IL-1α, 1 ng/ml; anti-IL-1β Ab, 10 μg/ml; and anti-IL-1α Ab, 2 μg/ml. The total amount of added IgG was kept constant by addition of control IgG. The CAT data were normalized to the average activity elicited by the MHC/*fos*CAT in unstimulated EL4 cells or PMA-induced 3MEHT in THP-1 cells. Data represent the mean ± SD of three experiments.
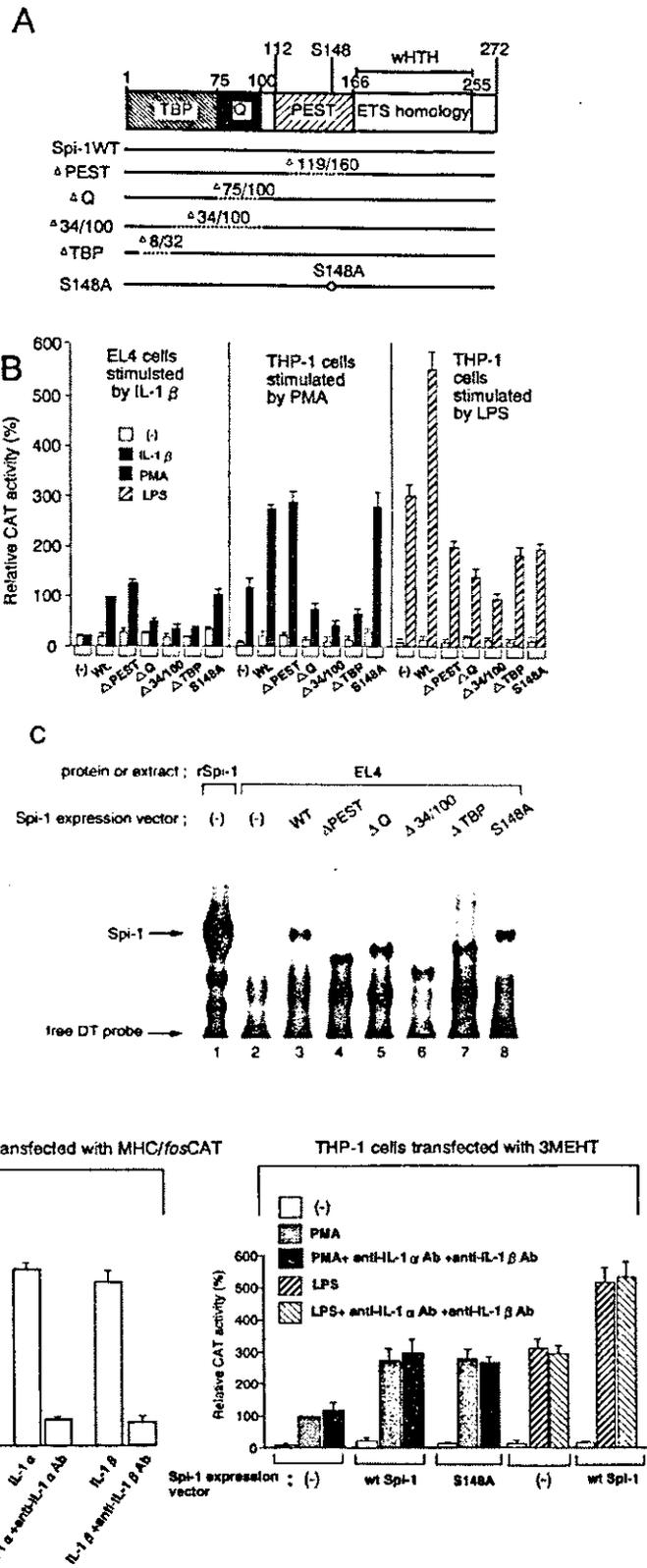
affect *IL1B* promoter activity in the absence of Spi-1. Similar results were observed when the cells were treated with IL-1β. However, when pECE Spi-1 was cotransfected into EL4 cells with pcTRAF6, transfection of pcTRAF6 significantly induced the *IL1B* promoter. Moreover, treatment of Spi-1-expressing EL4 with IL-1β

enhanced the TRAF6-induced promoter activity. In agreement with our data, previous studies reported that overexpression of TRAF6 is sufficient to activate NF-κB without additional exogenous stimuli (28), suggesting that overexpression of TRAF6 may cause their oligomerization, which leads to downstream signal events (30, 31).
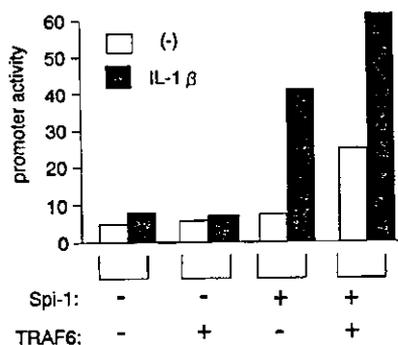
FIGURE 6. TRAF6 requires Spi-1 to activate the *IL1B* promoter. A TRAF6 expression vector, pcTRAF6 (4 μg), and a Spi-1 expression vector, pECE Spi-1 (4 μg), alone or in combination was cotransfected into EL4 cells along with the *IL1B* promoter CAT reporter 3MEHT (10 μg) and assayed for TRAF6-induced promoter activity. The total amount of transfected DNA was kept constant (18 μg) by addition of control vector. At 24 h after transfection, the cells were treated with 2 ng/ml IL-1β or left untreated. The results from a representative experiment are shown.

NF-IL6 lacking the transactivation domain (NF-IL6(Δspl)) inhibits IL-1β-induced promoter activity even in the absence of its cognate binding site (3MEHTmNF-IL6)

The *IL1B* promoter containing an intact Spi-1 site and a mutated NF-IL6 site (HTmNF-IL6) retained significant ability to be activated by IL-1β in THP-1 cells. In the present study, to evaluate functional involvement of NF-IL6 in IL-1β induction of HTmNF-IL6, various amounts of pcmNF-IL6(Δspl), encoding a NF-IL6 mutant lacking the transactivation domain, were cotransfected into EL4 cells with a full-length Spi-1 expression vector, pECE Spi-1, and assayed for IL-1β-induced promoter activity. The truncated NF-IL6 (NF-IL6(Δspl)), which lacks amino acids between residues 41 and 205, has been demonstrated to act as a dominant-negative factor in LPS induction of the *IL1B* enhancer (19). As shown in Fig. 7A, IL-1β induced the promoter containing an intact Spi-1 site and a mutated NF-IL6 site (HTmNF-IL6) in Spi-1-expressing EL4 cells. The IL-1β-induced activity for 3MEHTmNF-IL6 was significantly inhibited by expression of the truncated NF-IL6(Δspl). Cotransfection of 2 μg of pcmNF-IL6(Δspl) resulted in an ~80% loss of IL-1β-inducible activity. In contrast, when a full-length NF-IL6 expression vector, pcNF-IL6, was used instead of pcmNF-IL6(Δspl), cotransfection of pcNF-IL6 enhanced IL-1-induced activity for 3MEHTmNF-IL6 in the presence of Spi-1 (Fig. 7B).

IL-1β induces IL-1β mRNA expression in THP-1 monocytes

RT-PCR analysis for IL-1β mRNA expression was performed on total cellular RNA of THP-1 monocytes treated or untreated with IL-1β. The cells were carefully maintained in endotoxin-free culture medium. In addition, disposable sterile pipettes and tubes were used to reduce endotoxin levels. As shown in Fig. 8, IL-1β mRNA was detected in IL-1β-treated THP-1 cells. In contrast, untreated THP-1 monocytes failed to generate significant signals for IL-1β, indicating that IL-1β induces *IL1B* gene transcription in THP-1 monocytic cells. This result is consistent with our CAT data showing very low levels of background activity in the absence of IL-1β.

Discussion

Several studies have previously demonstrated that IL-1 is a potent stimulus for *IL1B* gene expression by using vascular smooth muscle cells (4), endothelial cells (5), and monocytes (6, 7). These
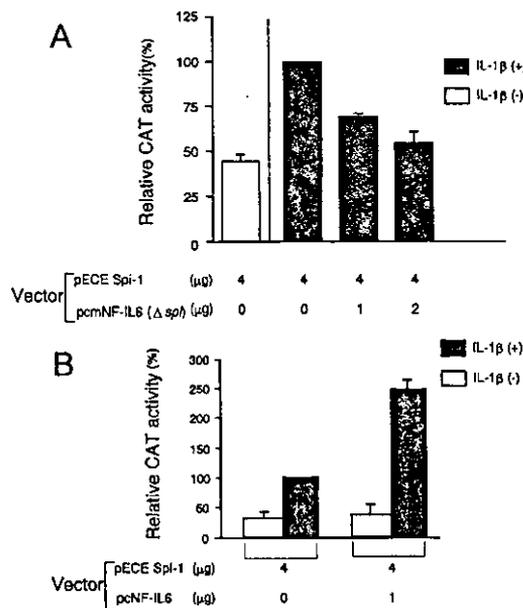
FIGURE 7. Inhibition of IL-1β-induced CAT activity for 3MEHT-mNF-IL6 by expression of a truncated NF-IL6. Increasing amounts of a mutant NF-IL6 expression vector, pcmNF-IL6(Δspl) (*A*), or 1 μg of a full-length NF-IL6 expression vector, pcNF-IL6 (*B*), were cotransfected into EL4 thymoma cells along with 3MEHTmNF-IL6 reporter (10 μg) and pECE Spi-1 (4 μg). The total amount of transfected DNA was kept constant by addition of control vector. At 24 h after transfection, the cells were treated with 2 ng/ml IL-1β or left untreated. The CAT data were normalized to the average activity elicited by the IL-1β-induced 3MEHTmNF-IL6 construct in the presence of Spi-1 expression vector cotransfection. Data represent the mean ± SD of three experiments.

results suggest that IL-1 induced by IL-1 amplifies pathological processes such as local inflammatory responses. In the present study, we demonstrated that IL-1β induces its own gene promoter in monocytes. IL-1β induction of the *IL1B* promoter was not observed in Spi-1-deficient EL4 cells but was detected in EL4 cells carrying a Spi-1 expression vector. Moreover, our transfection data using EL4 cells and a TRAF6 expression vector, pcTRAF6, showed that TRAF6, which is a critical signal transducer for IL-1, also requires Spi-1 to activate the *IL1B* promoter. These results
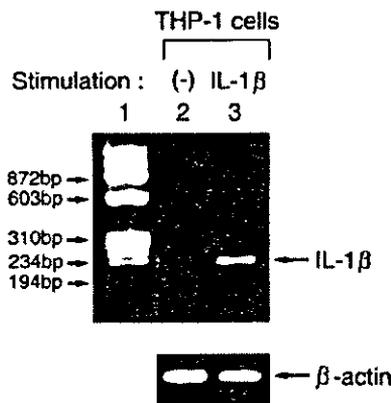
FIGURE 8. IL-1β induces IL-1β mRNA expression in THP-1 monocytic cells. PCR products of IL-1β and β-actin were derived from THP-1 cells treated with 10 ng/ml IL-1β for 1 h or untreated. The size of PCR product for IL-1β was 235 bp. Ten microliters of the reaction mixtures were separated at 50 V in 2% agarose gel. *Lane 1* shows a m.w. marker.

indicate that Spi-1 is a pivotal transcription factor for the IL-1β-*ILIB* induction process in monocytes.

Spi-1, which is an ETS family member protein restricted in expression to monocytes, macrophages, B lymphocytes, mast cells, and erythroid stem cells, has been shown to be a major determinant in cell type-specific expression of genes encoding CD11b (32) and receptors for GM-CSF (33), M-CSF (34), and G-CSF (35). It was reported that LPS and IFN-γ induced binding of Spi-1 to the proximal promoter of the *MHC class II I-Ab* gene in murine tissue macrophages (36). Kim et al. (37) have reported that LPS treatment increases binding of Spi-1 to the *IL-18* gene promoter by using RAW 264.7 macrophages. Moreover, we have recently demonstrated that p40 Tax encoded by human T cell leukemia virus-I genome markedly enhances binding of Spi-1 to the *ILIB* promoter through direct association of Spi-1 with Tax in THP-1 monocytic cells (17). In the present study, EMSA data using IL-1β-treated THP-1 cells further showed that IL-1β-inducible binding of Spi-1 to the *ILIB* promoter plays a crucial role in autocrine induction of the *ILIB* gene in monocytes.

The Spi-1 activation domain comprises at least three functional domains: one that binds TBP, which is necessary for the initiation of transcription; another that contains a Q domain serving as a transactivator; and a third domain that contains the PEST sequence in which phosphorylation of serine 148 is required to bind the lymphoid-specific coactivator NF-EM5/PU.1 interaction partner/IFN regulatory factor-4 (38). In B lymphocytes, Spi-1 promotes binding of NF-EM5/PU.1 interaction partner/IFN regulatory factor-4 to the *Igκ 3'* enhancer (21). The interaction between the two factors contributes to the *Igκ 3'* enhancer activity (38). In contrast, activation of the *Ig J chain* gene depends upon the amino-terminal portion of Spi-1 (39). The GM-CSFR α-chain has been shown to require binding of an uncharacterized factor, PU-SF, to the N-terminal transactivation domain of Spi-1 (33). The Q domain binds CBP/p300 (40). Thus, the functional domains of Spi-1 appear to be differentially used by various genes. In the present study, IL-1β-induced Spi-1 activation required the Q and the TBP binding domains (Fig. 5B). The fact that Spi-1 binds adjacent to the *ILIB* gene TATA box suggests that Spi-1 is involved in the recruitment of TBP to transcription preinitiation complex and in the transcriptional activation through the Q domain.

In addition, it is noteworthy that mutation of a serine to an alanine at codon 148 (S148A) did not affect activation of the *ILIB* promoter by either IL-1 or PMA. Our transfection studies using neutralizing Abs against IL-1α and IL-1β further showed that activation of the Spi-1 S148A mutant by PMA is not mediated by endogenous IL-1 production (Fig. 5D). Lodie et al. (41) have reported that LPS-induced phosphorylation of Spi-1 at serine 148, located within a casein kinase II motif, increases the capacity of Spi-1 to activate transcription. We also observed that S148A inhibited the ability of Spi-1 to transactivate the *ILIB* promoter in LPS-treated THP-1 monocytes. In this regard, the fact that LPS can activate the *ILIB* promoter more strongly than IL-1 and PMA may raise the possibility that, although LPS and IL-1 activate several common genes, LPS-induced phosphorylation of serine 148 recruits an additional transcription factor(s) to activate the *ILIB* promoter. In contrast, in the Spi-1 domain analysis, Spi-1 proteins were probably expressed in excess of the amount required for reporter vector activation. However, in our EMSA studies using nuclear extracts of EL4 cells transfected with various Spi-1 expression vectors, the relative expression levels of the Spi-1 proteins were almost equal (Fig. 5C). Our transfection data that Spi-1 S148A mutant, unlike wild-type Spi-1 protein, functions as a dominant-negative factor in LPS induction (Fig. 5B) further reveal the importance of serine 148 phosphorylation in LPS induction.

The *ILIB* promoter contains NF-IL6 binding site, which is located adjacent to the Spi-1 binding motif. NF-IL6, a member of the C/EBP family of leucine zipper transcription factors (42), is abundant in myeloid cells, including THP-1 monocytes. This factor is activated by IL-1β stimulation (42). However, as shown in Fig. 2B, mutation of the −91 to −83 NF-IL6 site (HTmNF-IL6) resulted in only a partial loss of IL-1β-inducible promoter activity. The IL-1-induced activity for HTmNF-IL6 was further repressed by expression of a NF-IL6 dominant-negative mutant (Fig. 7A). In this regard, a COOH-terminal ETS winged helix-turn-helix domain of Spi-1 has been demonstrated to be involved in both DNA binding and protein-protein interactions with c-Jun (43), C/EBP proteins (44), human CMV immediate early gene products (45), and other proteins. These results may suggest that in IL-1β-stimulated monocytes, NF-IL6 has the capability to activate the ILIB promoter through protein-tethered transactivation mediated by Spi-1. A similar protein-protein interaction between NF-IL6 and Spi-1 was observed in PMA induction (46). In contrast, Shannon et al. (47) have demonstrated that an NF-IL6 binding site in the *G-CSF* gene promoter is essential for IL-1β-induced expression of the *G-CSF* gene in human fibroblasts. They observed that mutation of a Spi-1 binding site within the *G-CSF* promoter does not eliminate promoter function in IL-1β-treated human fibroblasts. In this regard, the fact that fibroblasts lack Spi-1 expression (48, 49) may explain their results.

In conclusion, we have demonstrated that IL-1β activates its own gene promoter. The IL-1β induction process apparently depends upon IL-1β-inducible binding of Spi-1 to its target site within the *ILIB* promoter. Several transcription factors have been implicated in *ILIB* gene induction, including CREB, NF-IL6, LPS/IL-1-inducible-STAT, and NF-κB (19, 22, 50). An understanding of the mechanism of *ILIB* gene autoactivation in response to IL-1β is important for future efforts to modulate IL-1-induced pathological processes in various diseases.

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