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Macrophage Inflammatory Protein-1α Induces Osteoclast Formation by Activation of the MEK/ERK/c-Fos Pathway and Inhibition of the p38MAPK/IRF-3/IFN-β Pathway

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ABSTRACT

Multiple myeloma (MM) is a bone disease that affects many individuals. It was recently reported that macrophage inflammatory protein (MIP)-1α is constitutively secreted by MM cells. MIP-1α causes bone destruction through the formation of osteoclasts (OCs). However, the molecular mechanism underlying MIP-1α-induced OC formation is not well understood. In the present study, we attempted to clarify the mechanism whereby MIP-1α induces OC formation in a mouse macrophage-like cell line comprising C7 cells. We found that MIP-1α augmented OC formation in a concentration-dependent manner; moreover, it inhibited IFN-β and ISGF3γ mRNA expression, and IFN-β secretion. MIP-1α increased the expressions of phosphorylated ERK1/2 and c-Fos and decreased those of phosphorylated p38MAPK and IRF-3. We found that the MEK1/2 inhibitor U0126 inhibited OC formation by suppressing the MEK/ERK/c-Fos pathway. SB203580 induced OC formation by upregulating c-fos mRNA expression, and SB203580 was found to inhibit IFN-β and IRF-3 mRNA expressions. The results indicate that MIP-1α induces OC formation by activating and inhibiting the MEK/ERK/c-Fos and p38MAPK/IRF-3 pathways, respectively, and suppressing IFN-β expression. These findings may be useful in the development of an OC inhibitor that targets intracellular signaling factors. J. Cell. Biochem. 111: 1661–1672, 2010.

KEY WORDS: MIP-1α; OSTEOCLAST; ERK1/2; P38MAPK; IFN-β

In order to achieve normal bone remodeling, a homeostatic balance is required between the activities of bone-forming osteoblasts and bone-resorbing osteoclasts (OCs). Hematopoietic precursors fuse and differentiate to form multinucleated bone-resorbing OCs; this process is induced by an essential tumor necrosis factor (TNF) family-related signal molecule, namely, receptor activator of NF-κB (RANK) ligand (RANKL) in the presence of permissive levels of macrophage colony-stimulating factor (M-CSF) [Hofbauer and Heufelder, 2001; Lerner, 2004]. RANKL is expressed on the surface of osteoblasts or bone marrow stromal cells; it directly interacts with RANK, a membrane receptor, and triggers multiple intracellular signaling cascades, which stimulate gene expression, development, function, and survival of OC precursor cells and mature OCs [Hofbauer and Heufelder, 2001; Lerner, 2004].

RANKL interacts with RANK and recruits TNF-receptor associated factors (TRAFs) 1, 2, 3, 5, and 6. The deletion analyses of receptor genes have shown that RANK sequentially binds to TRAF6 and NF-κB-inducing kinase in order to induce activation of NF-κB, and that it binds to TRAF2 in order to induce the activation of mitogen-activated protein kinase (MAPK) pathways, such as c-Jun N-terminal kinase (JNK), p38MAPK, and extracellular signal-regulated kinase 1/2 (ERK1/2) [Lee et al., 1997; Darnay et al., 1999]. RANKL is also known to activate the transcriptional factor AP-1 by inducing c-Fos expression [Mohamed et al., 2007]. Interestingly, although RANKL is clearly important in promoting OC formation and activity, it was recently also found to trigger an autocrine negative-feedback mechanism in OC precursor cells; this mechanism ultimately limits the extent of osteoclastogenesis, which is simultaneously stimulated

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by RANKL [Hayashi et al., 2002; Takayanagi et al., 2002]. This negative feedback pathway involves the induction of interferon (IFN)-β activation by RANKL via c-Fos mRNA expression and subsequent inhibition of the RANKL-induced c-Fos mRNA expression by IFN-β, which is required for OC formation [Takayanagi et al., 2002]. Moreover, inhibitory cytokines such as interleukin (IL)-4, IL-10, and IL-13 inhibited the formation and functioning of OCs [Mohamed et al., 2007; Yamada et al., 2007]. Thus, the homeostasis of bone remodeling is maintained in normal bones by these factors.

Multiple myeloma (MM) is an incurable hematological malignancy characterized by the accumulation of malignant plasma cells in the bone marrow. MM patients have an increased risk of developing an infection, anemia, thrombocytopenia, renal failure, and bone disease. Osteolytic bone disease occurs in 70–80% of MM patients and results in severe bone pain, pathological fractures, paralysis on nerve compression, hypercalcemia, and death [Callander and Roodman, 2001]. This process of bone destruction is primarily due to an increased degree of OC activation and greater interaction between the MM cells and the bone marrow microenvironment. In MM, the degree of osteolysis is enhanced due to adhesive interactions between the bone marrow stromal cells and MM cells; these interactions induce the production of osteolytic factors, such as IL-6 and TNF-α. Nevertheless, the level of these cytokines has not been found to be consistently elevated in the peripheral blood of MM patients [Kiss et al., 1994; Anderson, 1999]. These factors probably cannot be detected because they are only secreted locally in areas of the bone marrow that contain high numbers of malignant plasma cells. Alternatively, other factors may be responsible for bone destruction in MM patients.

In recent studies, it has been reported that macrophage inflammatory protein (MIP)-1α, which is an osteoclastogenic C–C chemokine, is constitutively secreted by most MM cells in patients with multiple osteolytic lesions. In vitro and in vivo studies have shown that MIP-1α can induce OC formation in bone marrow cultures [Fuller et al., 1995; Kukita et al., 1997; Tsubaki et al., 2007]. It has also been reported that MIP-1α acts as an OC-stimulating factor in human marrow cultures, and that it is overexpressed in MM patients but not in healthy individuals. Thus, MIP-1α probably plays a major role in the microenvironment by mediating bone destruction in MM patients [Choi et al., 2000; Tsubaki et al., 2008].

With regard to normal bone homeostasis, inhibitory cytokines suppress the process of osteoclastogenesis. However, MIP-1α may inhibit these cytokines in OC precursor cells and thereby increase osteolysis in MM patients. Therefore, we investigated whether MIP-1α has an inhibitory effect on IFN-β expression in OC precursor cells and the mechanism underlying such inhibition, if present.

MATERIALS AND METHODS

MATERIALS

MIP-1α (Sigma, St. Louis, MO) was dissolved in phosphate-buffer saline (PBS, pH 7.4), filtrated through 0.45-μm syringe filters (Iwaki Glass, Tokyo, Japan), and used in various assays described below.

U0126, LY204002, and SB203580 were purchased from Promega (Southampton, Hants, UK). The abovementioned reagents were dissolved in dimethyl sulfoxide (DMSO), suspended in PBS, and filtrated through 0.45-μm syringe filters before use.

CELL CULTURE

C7 cells were provided by Dr. Shin-ichi Hayashi (Tottri University, Japan) and cultured in α-minimal essential medium (α-MEM; Sigma) supplemented with 10% foetal calf serum (FCS; Gibco, Carlsbad, CA), 50 U/ml human recombinant M-CSF (Leukoprol; Kyowa Hakko, Tokyo, Japan), 100 μg/ml penicillin (Gibco), 100 U/ml streptomycin (Gibco), and 25 mM HEPES (pH 7.4; Wako). The cells were cultured in an atmosphere containing 5% CO_2. RAW264.7 cells were purchased from DS Pharma Biomedical (Osaka, Japan) and cultured in α-MEM supplemented with 10% FCS, 100 μg/ml penicillin, and 100 U/ml streptomycin in the presence of 5% CO_2.

TARTRATE-RESISTANT ACID PHOSPHATASE STAINING

Cells were fixed with 10% formalin in PBS and subsequently rinsed with HEPES-buffered solution (0.9% NaCl and 10 mM HEPES, pH 7.1). Next, they were stained with fast red violet LB dissolved in tartrate-resistant acid phosphatase (TRAP) buffer (50 mM sodium acetate, 30 mM sodium tartrate, 0.1% Triton X-100, and 100 μg naphthol AS-MX phosphate; pH 5.0) for 45 min at 37°C. In each well, the TRAP-positive cells (>3 nuclei) were quantified under an Olympus microscope equipped with a 20× objective.

CULTURE CONDITIONS FOR THE DEVELOPMENT OF OC-LIKE MULTINUCLEATED CELLS FROM C7 OR RAW264.7 CELLS

C7 cells were cultured for 12 days in α-MEM containing 10% FCS (5,000 cells/well in 24-well plates) and different concentrations of MIP-1α. The cultures were fed every 3 days by replacing the medium with 500 μl of fresh medium containing different concentrations of MIP-1α. RAW264.7 cells were cultured for 7 days in α-MEM containing 10% FCS (10,000 cells/well in 24-well plates) and different concentrations of MIP-1α. The cultures were fed every 3 days by replacing the medium with 500 μl of fresh medium containing different concentrations of MIP-1α.

PIT FORMATION ASSAY

To analyze the bone resorption potential of TRAP-positive MNCs, C7 cells (5×10^3 cells/ml) were cultured in the presence of 10 ng/ml of MIP-1α for 12 days on dentine slices placed in 96-well plates. Cultures were fed every 3 days by replacing the spent medium with 50 μl of fresh medium containing 10 ng/ml of MIP-1α. After 12 days, cells were brushed off, and the slices were stained with 2% Coomassie Brilliant Blue R250 (Fluka Chemie, Switzerland) in methanol.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA), and an aliquot (1 μg) of purified total RNA was subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the SuperScript fist-strand synthesis system for RT-PCR (Invitrogen).

The resultant cDNAs were used as templates for PCR in order to generate products corresponding to the mRNAs of the various proteins used in the study. Each PCR reaction mixture contained cDNA, dNTPs (TaKaRa Biomedical, Shiga, Japan), 10× PCR buffer (TaKaRa Biomedical), and Pyrobest DNA polymerase (TaKaRa Biomedical). PCR was performed as follows. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), denaturation was carried out at 94°C for 0.5 min; annealing, at 60°C for 0.5 min and extension, at 72°C for 0.5 min (30 cycles). For cathepsin K, calcitonin receptor (CTR), IFN-β, IFN-stimulated gene factor 3 γ (ISGF3γ), suppressors of cytokine signaling (SOCS)-3, c-fos, and interferon regulatory factor (IRF)-3, denaturation was carried out at 94°C for 1 min; annealing, at 50°C for 1 min; and extension, at 72°C for 1 min (35 cycles). PCR was performed using a DNA thermal cycler (PCR thermal cycler MP; TaKaRa Biomedical). The following primers were used: cathepsin K, 5′-GGA AGA AGA CTC ACC AGA AGC-3′ (5′-primer) and 5′-GTC ATA TAG CCG CCT CCA CAG-3′ (3′-primer); CTR, 5′-CCA TTC CTG TAC TTG GTT GGC-3′ (5′-primer) and 5′-AGC AAT CGA CAA GGA GTG AC-3′ (3′-primer); IFN-β, 5′-CTT CTC CAC CAC AGC CCT CTC-3′ (5′-primer) and 5′-CCC ACG TCA ATC TTT CCT CTT-3′ (3′-primer); ISGF3γ, 5′-GAA CCC TCC CTA ACC AAC CA-3′ (5′-primer) and 5′-AGG TGA GCA GCA GCG AGT AG-3′ (3′-primer); SOCS-3, 5′-GCC ATG GTC ACC CAC AGC AAG TT-3′ (5′-primer) and 5′-AAG TGG A G CAT CAT ACT GAT CCA GGA-3′ (3′-primer); c-fos, 5′-ATG ATG TTC TCG GGT TTC AAC G-3′ (5′-primer) and 5′-CAG TCT GCT GCA TAG AAG GAA CCG-3′ (3′-primer); IRF-3, 5′-CCA GGT CTT CCA GCA GAC ACT-3′ (5′-primer) and 5′-TAG GCT GGC TGT TGG AGA TGT-3′ (3′-primer); GAPDH, 5′-ACT TTG TCA AGC TCA TTT-3′ (5′-primer) and 5′-TGC AGC GAA CTT TAT TG-3′ (3′-primer). The PCR products were mixed with bromophenol blue loading buffer and separated by electrophoresis in a 2% agarose gel in TAE buffer. After staining with ethidium bromide, the PCR products were visualized under ultraviolet light and recorded with a Cool Saver (Atto, Tokyo, Japan).

WESTERN BLOTTING

C7 cells and RAW264.7 cells treated under various conditions were lysed with a lysis buffer (20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP40, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1 mM PMSF), and the protein concentrations of the resultant cell lysates were determined with a BCA protein assay kit (Pierce, Rockford, IL). An aliquot of each extract (40 μg of protein) was fractionated by electrophoresis in an SDS–polyacrylamide gel and transferred to a PVDF membrane (Amersham, Arlington Heights, IL). Membranes were blocked with a solution containing 3% skim milk and then incubated overnight at 4°C with each of the following antibodies: anti-phospho-ERK antibody, anti-phospho-Akt antibody, anti-phospho-p38MAPK antibody, anti-ERK1/2 antibody, anti-Akt antibody, anti-p38MAPK antibody, and anti-I-κB antibody (Cell Signaling Technology, Beverly, MA). Subsequently, the membranes were incubated for 1 h at room temperature with sheep anti-rabbit IgG antibody labeled with horseradish peroxidase (Amersham). Reactive proteins were visualized with a chemiluminescence kit (Amersham), according to the manufacturer's instructions. Mouse anti-β-actin monoclonal antibody (Sigma) was used as the primary antibody (internal standard) for detecting β-actin protein.

STATISTICAL ANALYSIS

All results are expressed as the mean and standard deviation (SD) of several independent experiments. Multiple comparisons of the data were made using ANOVA with Dunnet's test. *P*-values $<1\%$ were considered significant.

RESULTS

EFFECT OF MIP-1α ON OC FORMATION

To test the potential of C7 cells to differentiate into osteoclasts, we used a single-culture system in the presence of 10 ng/ml of MIP-1α. Generation of TRAP-positive osteoclasts in these cultures was observed within 12 days, and fully matured functional osteoclasts that formed pits on dentine slices were detected after 12 days under the same culture conditions (Fig. 1A,B). Next, we tested the effects of MIP-1α on OC formation. C7 cells were cultured (5×10^3 cells/ml) in the presence of MIP-1α; concentrations in the range of 0.1–10 ng/ml were used. As shown in Figure 1C, the degree of OC formation increased as the MIP-1α concentration increased from 0.1 to 10 ng/ml. MIP-1α also caused increased expression of the osteoclast-specific markers, CTR and cathepsin K (Fig. 1D,E). Moreover, we investigated the effects of MIP-1α in RAW264.7 cells. As shown in Figure 1F, the degree of OC formation in RAW264.7 cells increased as the MIP-1α concentration increased from 0.1 to 10 ng/ml. MIP-1α also caused increased expression of the osteoclast-specific markers, CTR and cathepsin K, in RAW264.7 cells (Fig. 1F–H).

MIP-1α DOWNREGULATED IFN-β mRNA EXPRESSION AND INHIBITED IFN-β SIGNALING

We found that MIP-1α augmented OC formation. Next, we investigated the effect of MIP-1α on IFN-β mRNA expression in C7 cells and RAW264.7 cells. We detected IFN-β mRNA expression in the C7 cells of the control group to which MIP-1α had not been added and observed that IFN-β mRNA expression began to decrease 2 h after the addition of MIP-1α (Fig. 2A,B). Further, we observed that the IFN-β mRNA expression decreased in a time-dependent manner from days 1 to 3 after the addition of MIP-1α in C7 cells and RAW264.7 cells (Fig. 2C,D,G). Moreover, we observed that administration of MIP-1α decreased the secretion of IFN-β in C7 cells and RAW264.7 cells (Fig. 2E,H). We also found that co-administration of IFN-β inhibited the MIP-1α-induced osteoclast formation in C7 cells (Fig. 2F).

The above results indicate that MIP-1α suppresses IFN-β mRNA expression and IFN-β secretion in C7 cells and RAW264.7 cells. Next, we investigated any changes that may occur in IFN-β signaling when the expression of IFN-β is inhibited. MIP-1α suppressed ISGF3γ mRNA expression within a short duration from when it was added (Fig. 3A,B). Accordingly, the ISGF3γ mRNA expression decreased in a time-dependent manner from days 1 to –3 after the addition of MIP-1α (Fig. 3C,D). However, SOCS-3 mRNA expression was not affected by MIP-1α.

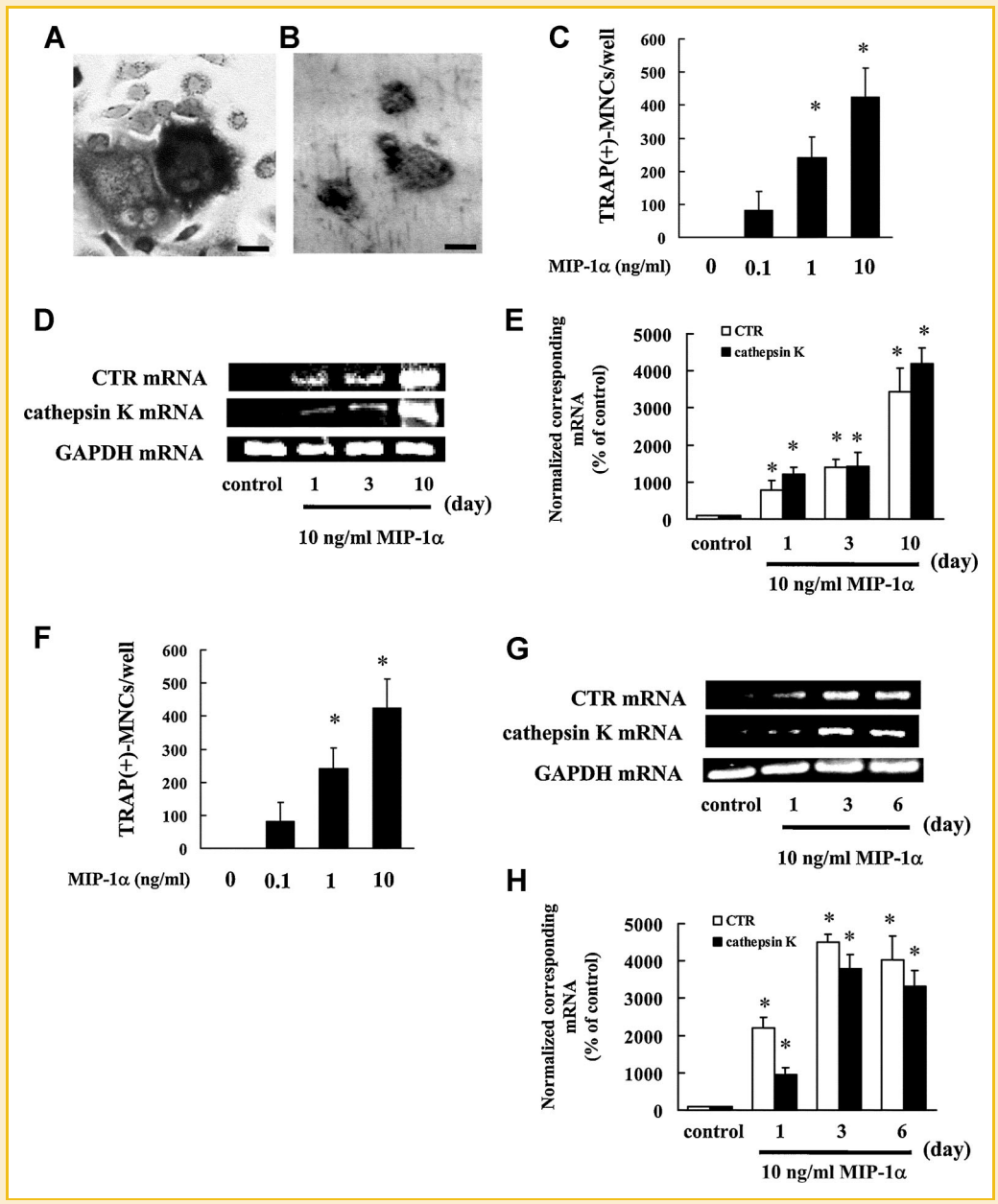

Fig. 1. Effects of recombinant mouse MIP-1α on OC formation in mouse macrophage-like C7 cells and RAW264.7 cells. A: C7 cells differentiate into osteoclasts. C7 cells (5×10^3 cells/ml) were cultured in 24-well plates. Cells were treated with 10 ng/ml of MIP-1α. Cultures were fed every 3 days. After 12 days, cells were fixed and stained for TRAP. C7 cells form TRAP-positive MNCs. Scale bar indicates 50 μm. B: For the pit formation assay, C7 cells (5×10^3 cells/well) were cultured in a 96-well plate on a dentine slice for 12 days. Pits on the dentine slice were stained with Coomassie Brilliant Blue R250. C: C7 cells and F: RAW264.7 cells were incubated with various concentrations of MIP-1α. MIP-1α significantly stimulated OC formation in a concentration-dependent manner. Results are presented as the mean (SD) of quadruplicate determinations in a typical experiment. A similar pattern of results was obtained in five independent experiments. $^*P < 0.01$ versus absence of MIP-1α (ANOVA with Dunnet's test). D,G: Equal amounts of total RNA were reverse-transcribed to generate cDNAs that were used for PCR analysis of CTR and cathepsin K mRNA expressions in (D) C7 cells and (G) RAW264.7 cells. E,H: Quantification of the amount of CTR and cathepsin K mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^*P < 0.01$ versus controls (ANOVA with Dunnet's test).

MIP-1α INCREASED THE DEGREE OF ERK1/2 AND Akt ACTIVATION AND INHIBITED p38 MAPK ACTIVATION

In order to investigate which signaling pathways are induced when MIP-1α augments OC formation and suppresses IFN-β expression in C7 cells and RAW267.4 cells, we studied the changes that occur in the phosphorylation of ERK, Akt, and p38 MAPK after the addition of MIP-1α. In C7 cells, unlike the control, a transient increase in the phosphorylation of ERK1/2 and Akt was observed 5, 15, and 30 min after the addition of MIP-1α. The activation of NF-κB was examined as the decomposition of I-κB protein. Using the control as a reference, we observed no substantial change in the levels of I-κB protein. On the other hand, the degree of p38MAPK phosphorylation

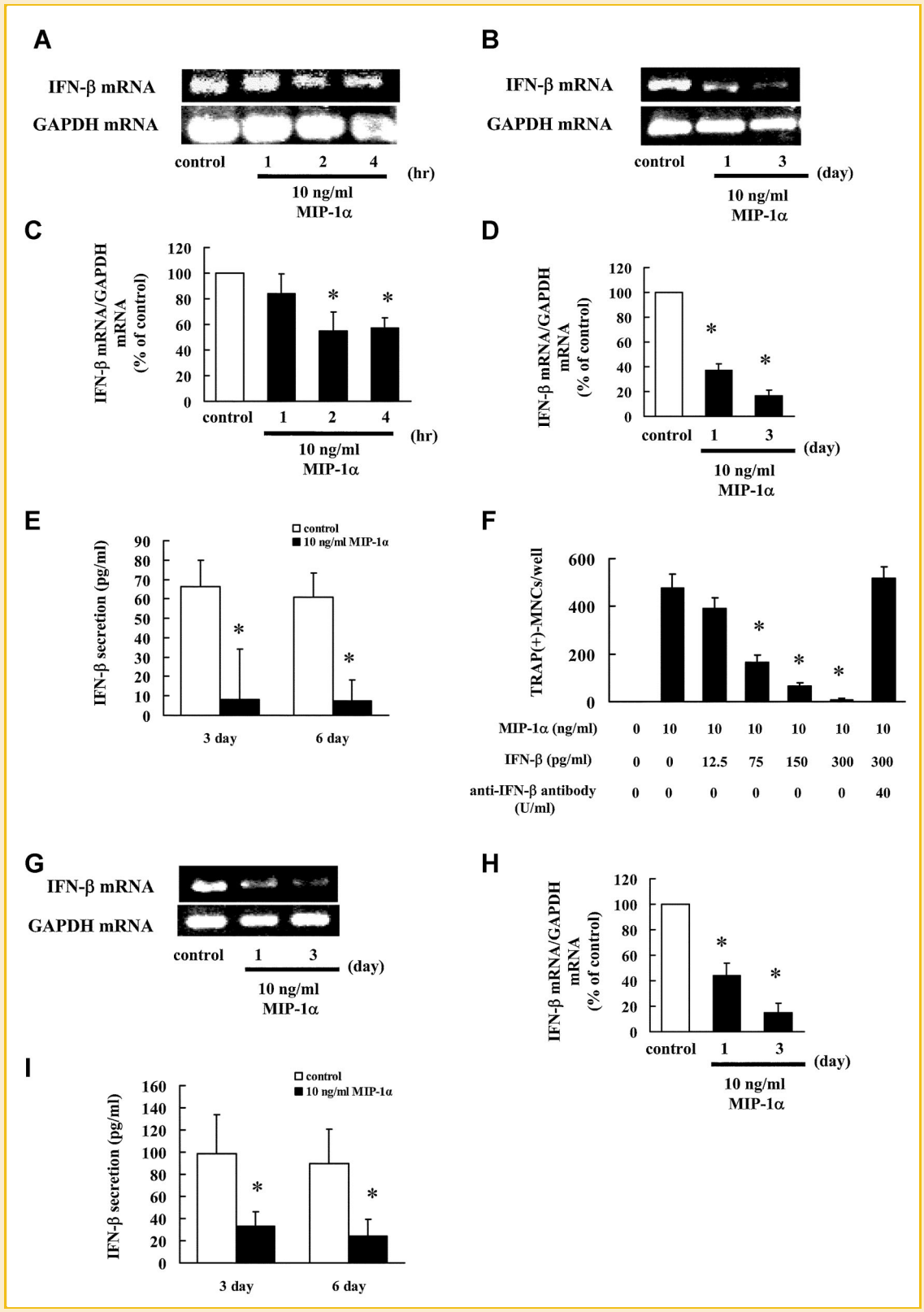

Fig. 2. Inhibitory effects of MIP-1α on the expression of IFN-β. A,B,G: The same quantity of total RNA was reverse-transcribed in each reaction to generate cDNAs that were used in PCR for analyzing IFN-β mRNA expression in (A,B) C7 cells and (G) RAW264.7 cells. C,D,H: Quantification of the amount of IFN-β mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test). E,I: Cells were treated with MIP-1α for 3 and 6 days. Culture supernatant was collected and analyzed by enzyme-linked immunosorbent assay (ELISA). These results are representative of five independent experiments. $^{*}P < 0.01$ versus control. F: C7 cells were incubated with MIP-1α and various concentrations of IFN-β. Administration of IFN-β significantly inhibited OC formation in a concentration-dependent manner. Results are presented as the mean (SD) of quadruplicate determinations in a typical experiment. A similar pattern of results was obtained in five independent experiments. $^{*}P < 0.01$ versus administration of MIP-1α (ANOVA with Dunnet's test).

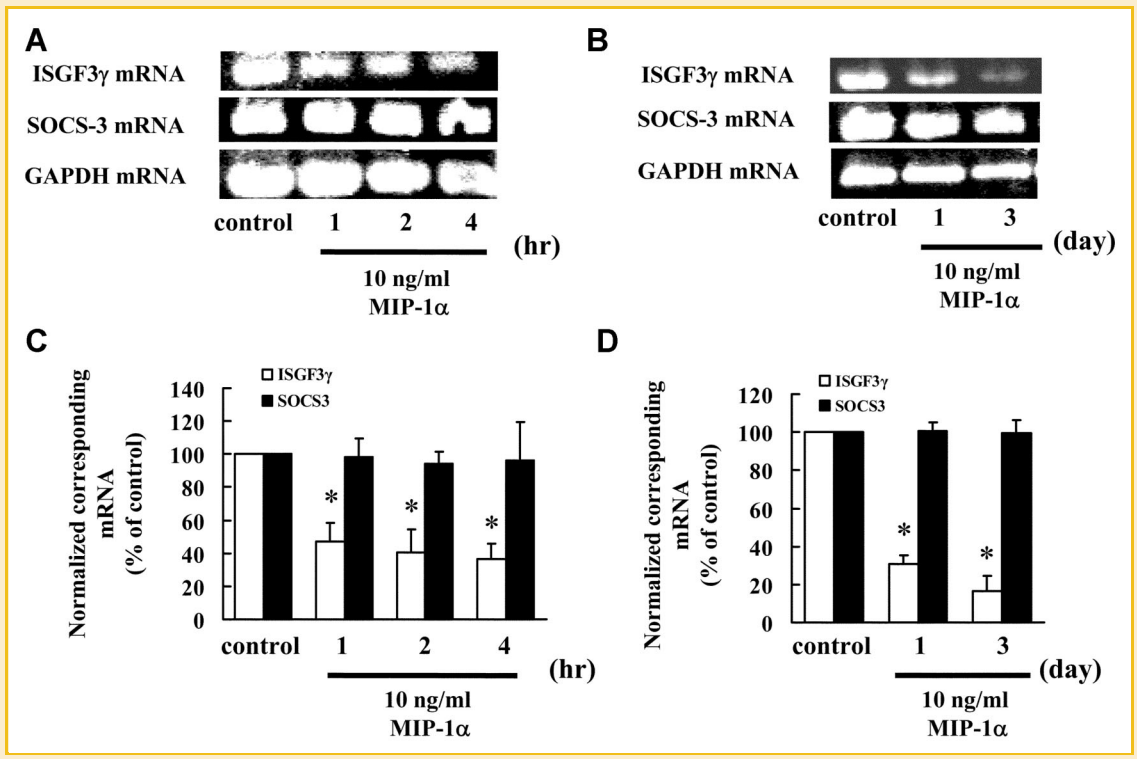

Fig. 3. Inhibitory effects of MIP-1α on the expression of ISGF3γ. A,B: The same quantity of total RNA was reverse-transcribed in each reaction to generate cDNAs that were used in PCR for analyzing ISGF3γ and SOCS3 mRNA expressions in C7 cells. C,D: Quantification of the amount of ISGF3γ and SOCS-3 mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test).

was found to decrease when examined 5, 15, 30, 60, and 120 min after MIP-1α stimulation; the same was not observed in the control (Fig. 4A,B). In RAW264.7 cells, unlike the control, a transient increase in the phosphorylation of ERK1/2 was observed 5, 15, 30, 60, and 120 min after the addition of MIP-1α. The levels of phosphorylated Akt consistently increased above the levels seen in the control group at 5 and 15 min after the addition of MIP-1α. Using the control as a reference, we observed no substantial change in the levels of the I-κB protein. On the other hand, the degree of p38MAPK phosphorylation was found to decrease when examined 5, 15, 30, 60, and 120 min after MIP-1α stimulation; the same was not observed in the control (Fig. 4C,D).

U0126 INHIBITED MIP-1α-INDUCED OC FORMATION

The results described thus far have shown that MIP-1α induced OC formation via the activation of the MAPK kinase (MEK)/ERK and phosphatidylinositol-3 kinase (PI3K)/Akt pathways and the inhibition of p38MAPK activation. Next, we added U0126 (a MEK inhibitor), LY294002 (a PI3K inhibitor), or SB203580 (a p38MAPK inhibitor) to the C7 cells and RAW264.7 cells in order to determine whether the suppression of ERK1/2, Akt or p38MAPK functions would inhibit OC formation. We observed that U0126 significantly inhibited MIP-1α-induced OC formation, while LY294002 had no effect on the same. Interestingly, the addition of SB203580 augmented OC formation without requiring MIP-1α stimulation (Fig. 5).

MIP-1α UPREGULATED C-Fos EXPRESSION IN C7 CELLS THROUGH THE MEK/ERK PATHWAY

To elucidate the mechanism underlying the effect of MIP-1α in the process of OC formation, the effect of MIP-1α on the expressions of c-fos mRNA and protein was evaluated. MIP-1α was found to induce the expressions of the c-fos mRNA and protein (Fig. 6A–H). In addition, pretreatment with U0126 before adding MIP-1α suppressed MIP-1α-induced c-fos mRNA expression (Fig. 6I–L). However, U0126 did not affect IFN-β mRNA expression (data not shown). The results indicate that MIP-1α elevated the c-fos mRNA and c-Fos protein expressions via the activation of the MEK/ERK pathway, and subsequently induced OC formation.

MIP-1α DECREASED IFN-β mRNA EXPRESSION THROUGH SUPPRESSION OF IRF-3 mRNA EXPRESSION

We found that MIP-1α suppressed p38MAPK activation and that SB203580 induced OC formation. Next, we investigated the effect of MIP-1α and SB203580 on the expressions of IRF-3 and IRF-7 mRNAs, which are the transcription factors of IFN-β mRNA. MIP-1α was found to decrease IRF-3 mRNA expression in a time-dependent manner, but it had no effect on IRF-7 mRNA expression (Fig. 7A–D). In addition, SB203580 was also found to suppress the expressions of IFN-β and IRF-3 mRNAs, but it did not affect IRF-7 mRNA expression (Fig. 7E–H). Interestingly, SB203580 induced higher c-fos mRNA expression in the absence of MIP-1α (Fig. 7I–L). These results indicate that MIP-1α decreased the IFN-β mRNA expression

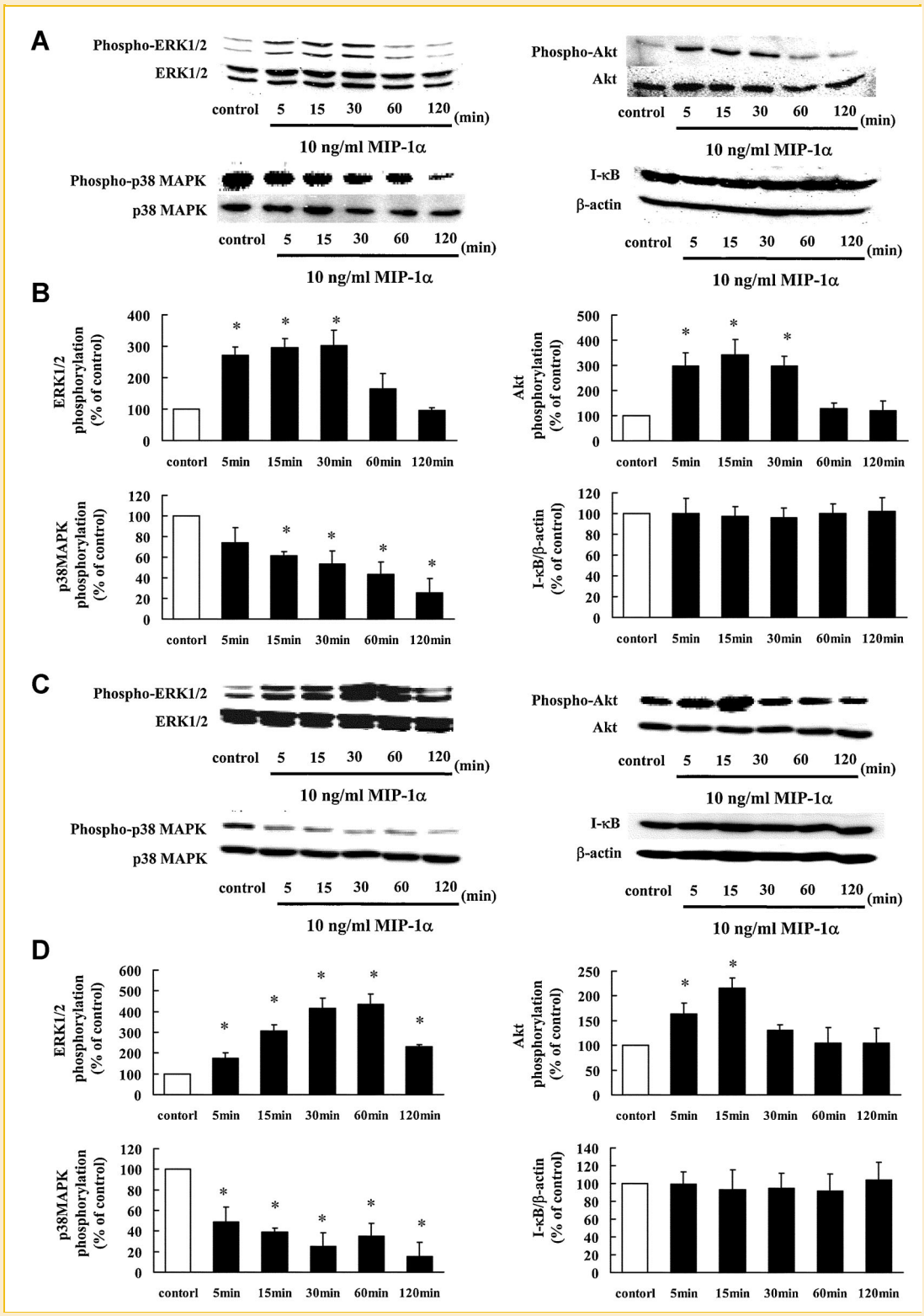

Fig. 4. MIP-1α induces the activation of ERK and Akt, but suppresses p38MAPK activation. A,C: Whole-cell lysates were extracted and immunoblotted with antibodies against phosphorylated ERK1/2 (phospho-ERK1/2), phosphorylated Akt (phospho-Akt), and phosphorylated p38MAPK (phospho-p38MAPK), and against ERK, Akt, I-κB, and p38MAPK in (A) C7 cells or (C) RAW264.7 cells. B,D: Quantification of the amount of phospho-ERK1/2, phospho-Akt, phospho-p38MAPK, or I-κB normalized to the amount of total ERK, Akt, p38MAPK, or β-actin, respectively. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test).

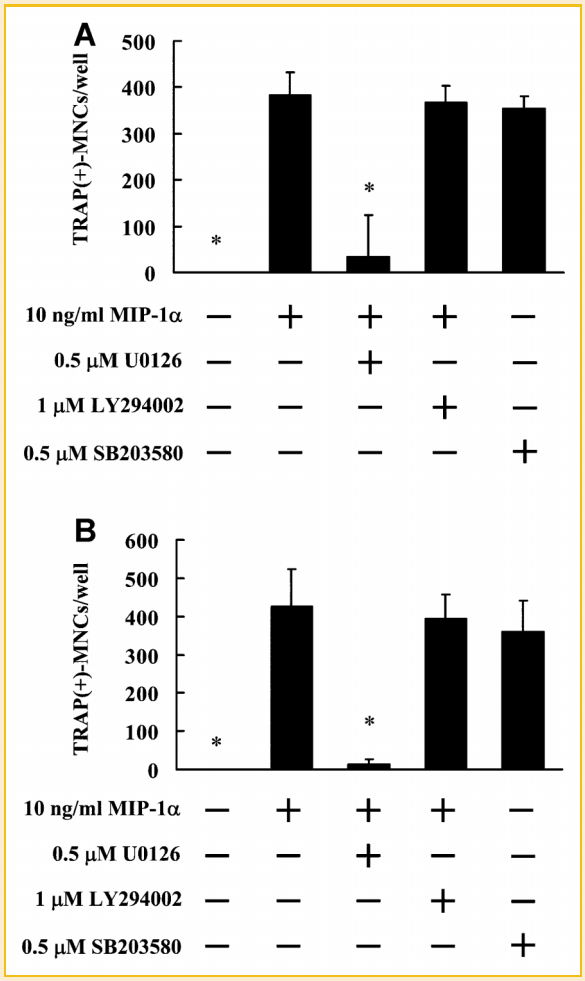

Fig. 5. U0126 inhibited MIP-1α-induced OC formation and SB203580 augmented OC formation in C7 and RAW264.7 cells. C7 cells and RAW264.7 cells were incubated with U0126, LY294002, or SB203580 in the presence or absence of MIP-1α for 12 days. U0126 significantly inhibited MIP-1α-induced OC formation, while SB0203580 augmented OC formation in C7 cells and RAW264.7 cells. The results are representative of five independent experiments. $^{*}P < 0.01$ versus presence of MIP-1α (ANOVA with Dunnet's test).

by inhibiting p38MAPK activation, and that SB203580 induced OC formation by upregulating c-fos mRNA expression and suppressing IFN-β mRNA expression.

DISCUSSION

In this study, we showed that MIP-1α induces OC formation and suppresses IFN-β mRNA expression and IFN-β secretion in C7 cells and RAW264.7 cells. IFN-β, produced by a RANKL-induced mechanism, inhibits osteoclastogenesis by suppressing c-Fos expression [Takayanagi et al., 2002]. In addition, IFN-β inhibits OC formation through the upregulation of *CXCL11*, which is an IFN-stimulated gene in human CD14-positive monocytes [Coelho et al., 2005], whereas MIP-1α directly induces OC formation [Choi et al., 2000]. These results indicate that MIP-1α may suppress IFN-β mRNA expression and IFN-β secretion in C7 cells and RAW264.7 cells and induce OC formation.

We found that MIP-1α suppressed ISGF3γ mRNA expression. Binding of IFN-β to its receptor induces transcription of the gene encoding ISGF3γ, which is a member of the ISGF3 heterotrimeric complex. The inhibitory effect of IFN-β on osteoclastogenesis is absent in the bone marrow monocyte/macrophage precursor cells of ISGF3γ-deficient mice [Takayanagi et al., 2002] suggesting that MIP-1α inhibits the autocrine signaling of IFN-β by suppressing IFN-β mRNA expression and IFN-β secretion.

With regard to MIP-1α-mediated signal transduction, it has been reported that the activation of ERK1/2 and Akt promotes cell growth and survival [Lentzsch et al., 2003]. Furthermore, we found that OC formation is necessary for the activation of signal transduction factors such as ERK1/2, Akt, NF-κB, and p38MAPK. Our results clearly showed that MIP-1α activates ERK1/2 and Akt but inhibits p38MAPK activation.

ERK1/2 was found to induce the transcription of the gene encoding c-fos, which is an important transcription factor in osteoclastogenesis. RANKL-induced expression of c-Fos in turn upregulates the expression of IFN-β. In this study, we showed that MIP-1α induces c-fos mRNA transcription and c-Fos expression, and that U0126 (a MEK1/2 inhibitor) inhibits OC formation and suppresses c-fos mRNA expression but does not affect IFN-β mRNA expression; the concentration of U0126 used in this study suppressed MIP-1α-induced ERK activation to levels similar to those shown by the control (data not shown). A previous study has already reported that U0126 inhibits OC formation, and that ERK1/2 is an essential signal transduction factor involved in GM-CSF-induced osteoclastogenesis [Lee et al., 2009]. A recent study has shown that the nuclear factor of activated T cells c1 (NFATc1) is located downstream to c-Fos and that it associates with the c-Fos protein to induce the transcription of OC-specific genes such as those encoding TRAP, CTR, and cathepsin K [Wagner and Eferl, 2005]. Moreover, we found that MIP-1α induces the expression of CTR and cathepsin K mRNA in C7 and RAW264.7 cells. These results suggest that MIP-1α induces OC formation through the MEK/ERK/c-Fos pathway, and that MIP-1α-induced upregulation of c-Fos expression does not affect IFN-β mRNA expression.

In this study, we observed the MIP-1α-stimulated activation of Akt. We also found that LY294002 (a PI3K inhibitor) does not affect MIP-1α-induced OC formation; the concentration of LY294002 used in this study suppressed MIP-1α-induced Akt activation to levels similar to those shown by the control (data not shown). PI3K inhibitor has a potent inhibitory effect on OC differentiation; this effect may be due to the reduced survival of OC precursor cells during differentiation [Lee and Kim, 2003]. The activation of Akt by activin A, a stimulator of osteoclastogenesis, promotes the survival of OC precursor cells [Sugatani et al., 2003]. We also found that a high concentration of LY294002, capable of completely inhibiting Akt activation, also inhibited OC formation by stimulating MIP-1α and induced cell death in the cell lines used in the study (data not shown). These results suggest that MIP-1α-induced activation of Akt does not affect OC formation.

IFN-β is produced by the activation of signal transduction factors such as p38MAPK, NF-κB, and JNK. Lipopolysaccharides (LPSs) are

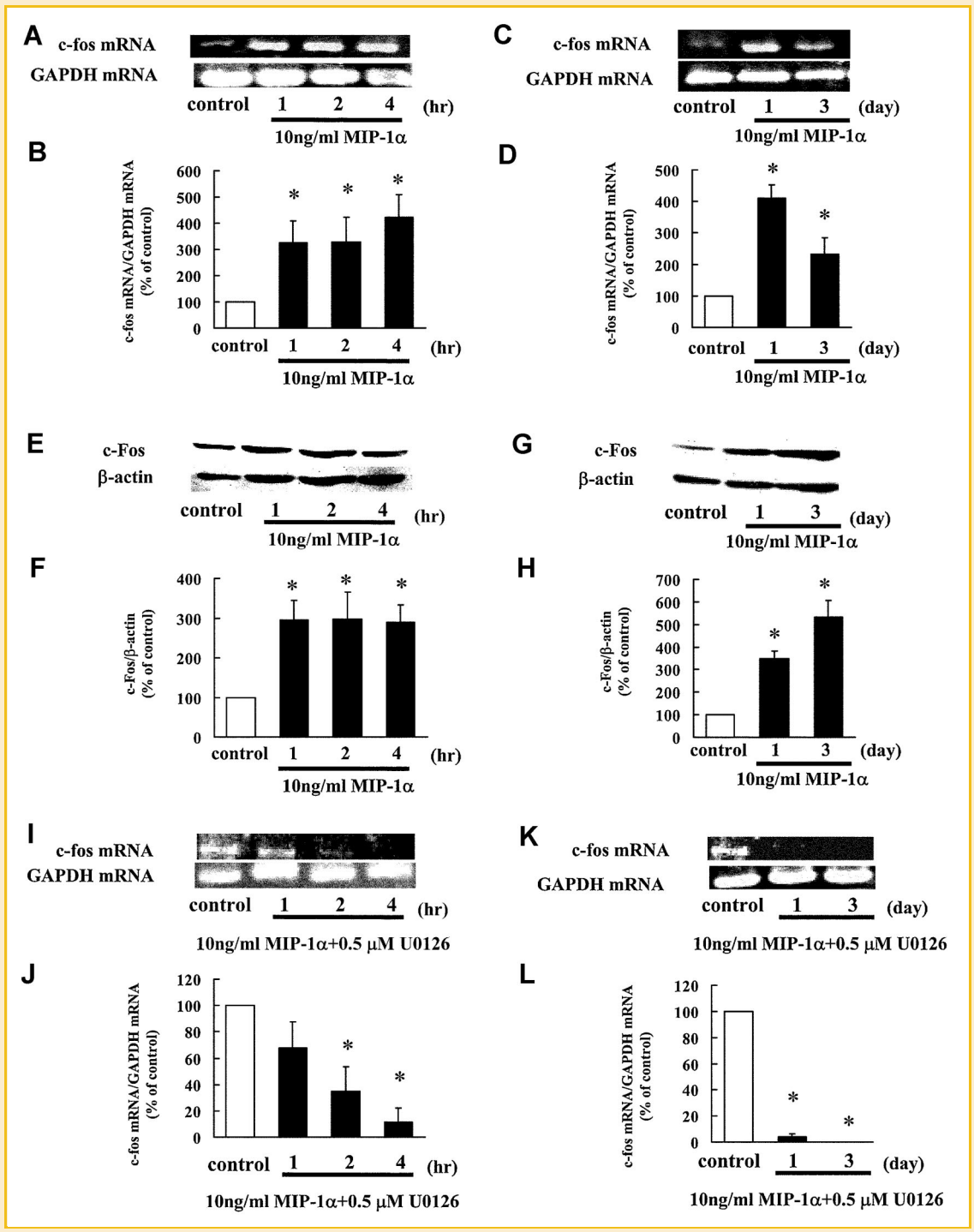

Fig. 6. MIP-1α induced c-Fos mRNA expression through the activation of the MEK/ERK pathway. A,C: The same quantity of total RNA was reverse-transcribed to generate cDNAs that were used in PCR for analyzing c-fos mRNA expressions in C7 cells. B,D: Quantification of the amount of c-fos mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test). E,G: Whole-cell lysates were extracted and immunoblotted with antibodies against the c-Fos protein. F,H: Quantification of the amount of c-Fos normalized to the amount of β-actin. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test). I,K: C7 cells were treated with U0126. The same quantity of total RNA was reverse-transcribed in each reaction to generate cDNAs that were used in PCR for analyzing c-fos mRNA expressions in C7 cells. J,L: Quantification of the amount of c-fos mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test).

known to induce IFN-β production via p38MAPK activation [Reimer et al., 2007], and LPSs activate the Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)-related adaptor molecule (TRAM) in order to induce the expression of IRF-3, a transcription factor of IFN-β [Brzezinska et al., 2009]. We found that SB203580 down-regulates the expression of IFN-β and IRF-3 mRNA to the same level

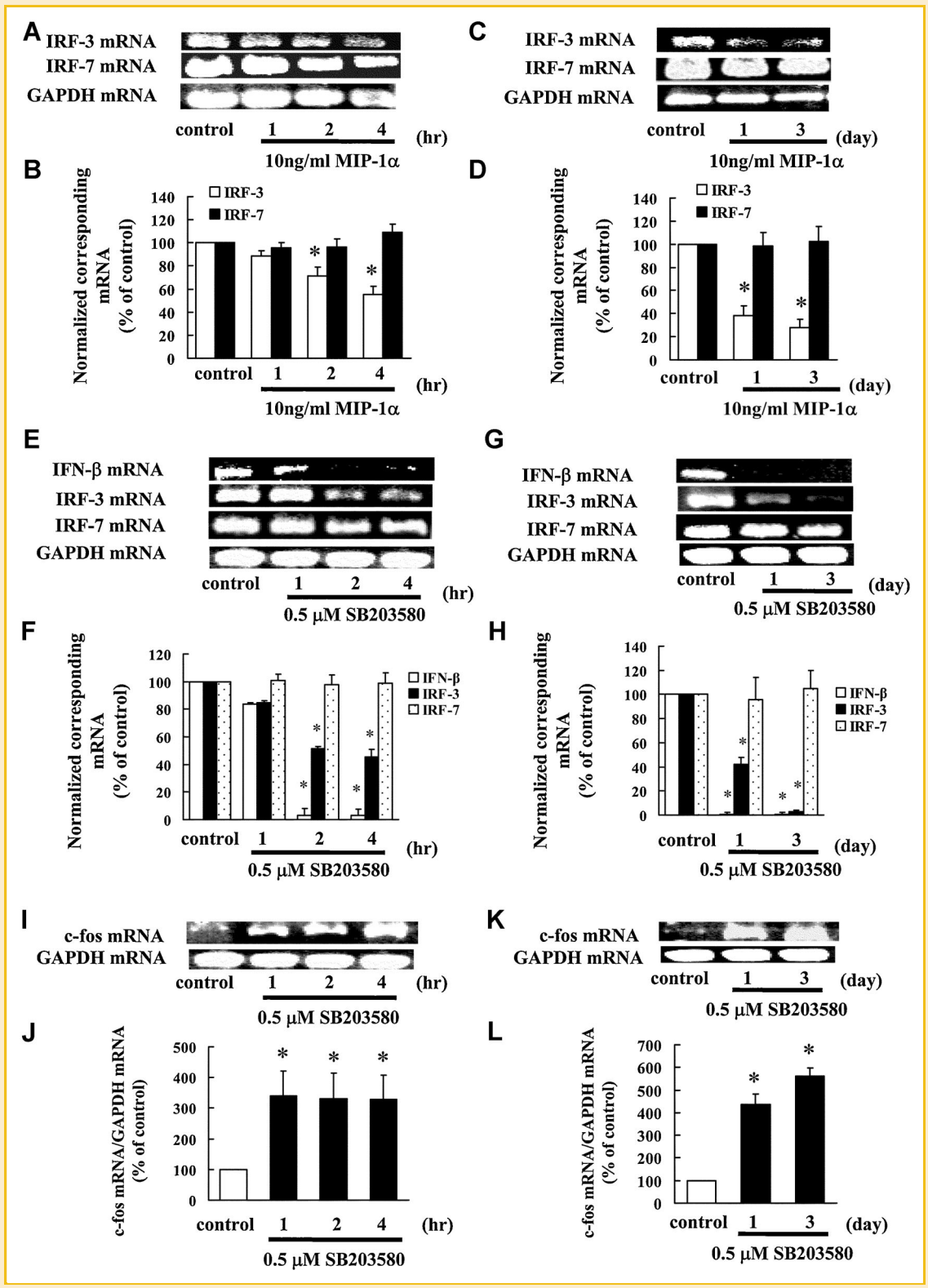

Fig. 7. MIP-1α suppressed IFN-β mRNA expression through inhibition of p38MAPK activation. A,C: Equal amounts of total RNA were reverse-transcribed to generate cDNAs that were used in PCR for analyzing IRF-3 and IRF-7 mRNA expressions in C7 cells. B,D: Quantification of the amount of IRF-3 and IRF-7 mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test). E,G: C7 cells were treated with SB203580. The same quantity of total RNA was reverse-transcribed in each reaction. F,H: Quantification of the amount of IFN-β, IRF-3, and IRF-7 mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test). I,K: C7 cells were treated with SB023580. The same quantity of total RNA was reverse-transcribed in each reaction to generate cDNAs that were used in PCR for analyzing c-fos mRNA expressions in C7 cells. J,L: Quantification of the amount of c-fos mRNA normalized to the amount of GAPDH mRNA. The results are representative of five independent experiments. $^{*}P < 0.01$ versus controls (ANOVA with Dunnet's test).

as that by MIP-1α. Moreover, SB203580 induces c-fos mRNA expression and OC formation. These results prove that MIP-1α suppresses IFN-β mRNA expression and IFN-β secretion through MIP-1α-induced inhibition of p38MAPK activation in C7 cells and RAW264.7 cells, and that MIP-1α-induced expression of c-Fos accelerates OC formation.

However, RANKL-mediated p38MAPK activation induces the expression of MITF, which is an important factor for osteoclastogenesis [Mansky et al., 2002]. Further, p38MAPK-induced MITF expression is associated with one of the later stages of OC formation [Hershey and Fisher, 2004]. We observed that although MIP-1α inhibits p38MAPK during the early stages of OC formation, p38MAPK is activated during the later stages (data not shown). Moreover, the concentration of SB203580 used in this study inhibited p38MAPK activation from 24 to 36 h, and a sufficient recovery of p38MAPK activation could be detected after 48 h (data not shown). These results suggest that p38MAPK inactivation by MIP-1α plays a role in the suppression of IFN-β mRNA expression.

The findings of this study suggest that MIP-1α induces OC formation without RANKL stimulation through the activation of the MEK/ERK/c-Fos pathway. We observed that C7 and RAW264.7 cells express chemokine receptors (CCR5 or CCR1), which are very essential for MIP-1α activity (data not shown). We have previously reported that MIP-1α interacts with CCR5 to induce the activation of the MEK/ERK pathway [Tsubaki et al., 2007]. The inhibition of CCR1 suppresses the activation of ERK1/2 and the expression of c-Fos in OC precursor cells [Vallet et al., 2007]. The findings from our study prove that MIP-1α directly induces OC formation through CCR5 or CCR1-induced activation of the MEK/ERK/c-Fos/NFATc1 pathway via a RANKL-independent mechanism.

In conclusion, we state that MIP-1α-induced OC formation is associated with the MEK/ERK/c-Fos pathway, and that MIP-1α inhibits p38MAPK activation and suppresses IFN-β expression. These findings may prove useful in the development of an OC inhibitor that targets intracellular signaling factors. If OC formation is induced by MIP-1α, then the use of an OC inhibitor can help control bone destruction in myeloma patients, and thereby improve the quality of life of these patients.

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