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Role of mitogen-activated protein kinase family in serum-induced leukaemia inhibitory factor and interleukin-6 secretion by bone marrow stromal cells

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1 In the haematopoietic microenvironment, bone marrow stromal cells play an important role in regulating haematopoiesis by expressing various cytokines, including leukaemia inhibitory factor (LIF) and interleukin-6 (IL-6). However, the intracellular signal that regulates cytokine secretion in bone marrow stromal cells has not been determined. The aim of this study was to evaluate the role of mitogen-activated protein kinase (MAPK) family in serum-induced secretion of LIF and IL-6 by bone marrow stromal cells.

2 Transformed human bone marrow stromal cells (HS-5) were stimulated with foetal calf serum (FCS) to produce LIF and IL-6. FCS also induced activation of extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun NH_2-terminal kinase (JNK).

3 Both PD98059 (MAPK/ERK kinase inhibitor) and SB203580 (p38 MAPK inhibitor) attenuated FCS-induced LIF protein production and gene expression. SB203580 decreased IL-6 production and gene expression, but PD98059 had no effect on IL-6 production and gene expression.

4 Expression of a dominant-negative mutant form of JNK1 that blocked FCS-induced JNK activity had no effect on protein production and gene expression of these cytokines.

5 These findings demonstrate that both ERK and p38 MAPK are involved in FCS-induced LIF secretion, whereas only p38 MAPK is important for IL-6 secretion, and that FCS-induced activation of JNK has no effect on the production of LIF and IL-6. We conclude that, in spite of their similar biological effects, they are differentially regulated at the level of MAPK activity in bone marrow stromal cells.

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Keywords: Bone marrow stromal cells; leukaemia inhibitory factor; interleukin-6; MAPK; haematopoiesis

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CREB, cAMP-responsive element binding protein; DMSO, dimethyl sulphoxide; DN, dominant-negative; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1α, interleukin-1α; IL-6, interleukin-6; JNK, c-Jun NH_2-terminal kinase; LacZ, β-galactosidase; LIF, leukaemia inhibitory factor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MOI, multiplicity of infection; PBS, phosphate buffer saline; PMSF, phenylmethyl sulphonyl fluoride; RT, reverse transcription; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBST, tris buffered saline-Tween20; TNF-α, tumour necrosis factor-α; X-gal, β-D-galactopyranoside

Introduction

Bone marrow stromal cells play an important role in the regulation of haematopoiesis through the signals mediated by direct contact with haematopoietic progenitor cells and through the production of cytokines (Chabannon & Torok-Storb, 1992 ; Dorshkind, 1990; Greenberger, 1991; Kittler *et al*., 1992). Cytokines produced locally in the marrow microenvironment are necessary for the induction of proliferation, differentiation and maturation of haematopoietic stem cells into mature precursors and blood elements. Bone marrow stromal cells secrete numerous kinds of cytokines, including leukaemia inhibitory factor (LIF) and interleukin-6 (IL-6) (Guba *et al*., 1992; Wetzler *et al*., 1991). LIF and IL-6 are pleiotropic cytokines of the IL-6 cytokine family, sharing the common gp130 receptor subunit together with interleukin-11, oncostatin M, ciliary neurotrophic factor and cardiotrophin-1 (Kishimoto *et al*., 1995). Due to the close similarities of the gp130-related IL-6 cytokine family, cytokines of this family show partially overlapping or redundant biological effects in various tissues and cells. LIF and IL-6 also share a number of biological activities such as induction of hepatic acute phase protein synthesis, regulation of neural differentiation, stimulation of megakaryocytopoiesis, differentiation of the murine myeloid leukaemic cell line, M1 and promoting the survival and/or proliferation of primitive haematopoietic precursors *in vivo* and *in vitro* (Kishimoto *et al*., 1995; Lord

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et al., 1991; Metcalf, 1991; Taupin *et al*., 1998; Wetzler *et al*., 1991).

Mitogen-activated protein kinase (MAPK) cascade is a major signalling pathway that is shared by various types of cells (Schaeffer & Weber, 1999; Widmann *et al*., 1999). In mammalian cells, three important groups of kinases compose the MAPK family, including the extracellular signal-regulated kinase (ERK), the p38 MAPK, and the c-*jun* NH_2-terminal kinase (JNK). ERK cascade appears to mediate signals promoting cell proliferation, differentiation, or survival, whereas p38 MAPK and JNK cascades appear to be involved in the cell responses to stresses. Recent studies provide evidence that these signal transduction cascades mediate the production of cytokines in various types of cells (Carter *et al*., 1999; Hashimoto *et al*., 2000; Marin *et al*., 2001). Human bone marrow stromal cells were reported to product LIF and IL-6 spontaneously, in response to serum and monocyte-derived cytokines such as interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) (Lorgeot *et al*., 1997; Rougier *et al*., 1998). However, the intracellular signal that regulates cytokine expression in bone marrow stromal cells has not been determined. Several reports suggest that depressed haematopoiesis in immunosuppressive disorders may be due to the defective cytokine production in bone marrow stroma (Hamburger, 1995; Mayer *et al*., 1997; Stark *et al*., 1993). Therefore, it is important to clarify the mechanism of cytokine expression in bone marrow stromal cells. In this study, we have investigated the role of MAPK family in serum-induced LIF and IL-6 production by human bone marrow stromal cells.

Methods

Cell culture

Human bone marrow stromal cell line HS-5 (Roecklein & Torok-Storb, 1995) was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). We used HS-5 cells because they were capable of sustaining proliferation of haematopoietic progenitors *in vitro*. HS-5 cells express fibronectin; collagen I, III and IV; and low levels of vascular cellular adhesion molecule (VCAM-1). HS-5 cells do not express major histocompatibility complex (MHC) class II antigens, CD34, CD14, STRO-1 or FVIII (Roecklein & Torok-Storb, 1995). Cells were seeded in 6-well plates and maintained at 37°C and 5% CO_2 in RPMI medium supplemented with 10% foetal calf serum (FCS), 1% L-glutamine, 100 μg ml^{-1} streptomycin, and 100 u ml^{-1} penicillin. For all experiments, cells at 70% confluence were made quiescent by incubation with serum free RPMI medium for 48 h and then stimulated with various concentrations of FCS for specified durations. The serum starvation (48 h) was not toxic to the cells, as evaluated by Trypan blue and Hoechst 33258 staining (data not shown).

Chemicals and reagents

FCS was purchased from Equitech-Bio (Kerrville, TX, U.S.A.). The MAPK/ERK kinase (MEK) inhibitor PD98059 and the p38 MAPK inhibitor SB203580 were purchased from Calbiochem (San Diego, CA, U.S.A.). PD98059 and SB203580 were dissolved in dimethyl sulphoxide (DMSO), where final concentration of DMSO was determined to be $<0.01\%$. In each experiment using these inhibitors, the vehicle contained the same concentration of DMSO as was present in the treatment group. Rabbit polyclonal antibodies against p38 MAPK and phosphorylated p38 MAPK were purchased from New England Biolabs (Beverly, MA, U.S.A.). Rabbit polyclonal antibody against ERK1/2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and phosphorylated ERK1/2 was purchased from Promega (Madison, WI, U.S.A.).

Protein isolation and Western blot analysis

After treatment, the cells were washed with precooled phosphate buffer saline (PBS) and lysed with the lysis buffer ((mM) HEPES 20, pH 7.2, NaCl 25, EGTA 2, NaF 50, Na_3VO_4 1, β-glycerophosphate 25, dithiothreitol [DTT] 0.2, phenylmethyl sulphonyl fluoride [PMSF] 1, aprotinin 60 μg ml^{-1} and Triton X-100 0.1%). After a brief sonication, the cells were centrifuged, and the supernatants were stored -80°C until use. The protein concentration was determined using bicinchoninic acid assay (Pierce Chemical, Rockford, IL, U.S.A.). Protein extracts (20 μg) from HS-5 were boiled for 5 min in Laemmli sample buffer (Laemmli, 1970), then separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE), and the separated proteins were electrophoretically transferred to Hybond–PVDF membranes (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Nonspecific background was blocked by incubating the membrane in Tris buffered saline-Tween20 (TBST) buffer ((mM) Tris 25, pH 7.8, NaCl 125, and Tween-20 0.1%) containing 5% bovine serum albumin (BSA) for 2 h at room temperature. The membrane was washed three times in TBST buffer and then incubated overnight at 4°C with the appropriate dilution of primary antibody. After three washes in TBST buffer, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin (Amersham Pharmacia Biotech), and the antibody complexes were visualized by the ECL detection system (Amersham Pharmacia Biotech) as directed by the manufacturer.

Activity assay of JNK

The enzymatic activity of JNK was determined by using the JNK assay kit from New England Biolabs. Briefly, cells were lysed with lysis buffer ((mM) Tris 20 pH 7.4, NaCl 150, EDTA 1, EGTA 1, Triton 1%, sodium phosphate 2.5, β-glycerophosphate 1, Na_3VO_4 1, $MgCl_2$ 10, PMSF 1). Cell lysates, containing 250 μg total proteins, were incubated overnight at 4°C with the N-terminal c-*jun* (1-89) fusion protein bound to glutathione-Sepharose beads. The N-terminal 89 amid acid segment of c-Jun contains a high-affinity binding site for JNK just N-terminal to the 2 phosphorylation sites, Ser 63 and Ser 73. It can selectively pull down JNK from the cell lysates. After washing twice with lysis buffer and twice with kinase buffer ((mM) Tris 2.5 at pH 7.5, β-glycerophosphate 5, DTT 2, Na_3VO_4 0.1, $MgCl_2$ 10) to remove nonspecifically bound proteins, the beads were resuspended in the kinase buffer with 100 μM ATP and incubated for 30 min at 30°C. Phosphorylation of c-Jun was

measured by Western blot analysis with a phospho-specific c-Jun antibody that specifically detects Ser63-phosphorylated c-Jun, a site important for c-JUN-dependent transcriptional activity.

Extraction of RNA and reverse transcription (RT)

RNA was extracted from HS-5 cells using Isogen (Nippongene, Toyama, Japan). cDNA was produced with the Omniscript RT kit (Qiagen, Valencia, CA, U.S.A.) using oligo (dT) primers (Applied Biosystems, Foster City, CA, U.S.A.). One microgram total RNA was reverse-transcribed and stored at -20°C until use in PCR.

Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time polymerase chain reaction (real-time PCR) was performed using the LightCycler (Roche Diagnostic, Mannheim, Germany) technology. Primers and probes, described in Table 1, were designed and synthesized by Nihon Gene Research Lab's (Sendai, Japan). Reactions were performed using 2 μl LightCycler DNA Master Hybridization Probes (Roche Diagnostics), $MgCl_2$ 3 mM, 0.5 μM of 3′ and 5′ oligonucleotide primers, 0.2 μM of fluorescein probe, 0.4 μM of LightCycler Red640 probe, 1 μl cDNA, and water to a final volume of 20 μl. After an initial denaturation step at 95°C for 60 s, cDNA were amplified for 40 cycles. Amplification of each sample and each standard curve dilution was as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 95°C for 0 s, 58°C for 15 s, 72°C for 13 s; LIF, 95°C for 0 s, 58°C for 15 s, 72°C for 11 s; IL-6, 95°C for 0 s, 55°C for 15 s, 72°C for 11 s. Standard curves for quantification of cytokines or GAPDH cDNA were generated by serial dilution of cDNA from FCS-stimulated or GAPDH cDNA were generated by serial dilution of cDNA from FCS-stimulated HS-5 cells. Data acquisition and analysis was performed using LightCycler software (version 3.39; Roche Diagnostics). To control for variation in the input cDNA quantity, expression of the target cytokine mRNA was normalized to GAPDH expression, a house keeping gene, in the same sample by dividing the quantity of PCR product of interest by the quantity of GAPDH product. Normalized values were either plotted directly, or plotted as relative expression determined by dividing the quantity of PCR product in FCS-stimulated cells by the quantity of PCR product in unstimulated controls, using the normalized values.

Quantitation of cytokines by ELISA

Cell-free supernatants from HS-5 cultures were assayed for the presence of LIF and IL-6 using enzyme-linked immunosorbent assay (ELISA) kits purchased from R&D Systems (Minneapolis, MN, U.S.A.). All assays were conducted according to manufacturer's specifications. Concentrations of each cytokine were calculated based on standard curves generated from recombinant cytokines provided with kits.

Dominant-negative mutant of JNK

Dominant-negative (DN) mutant of JNK was produced as previously described in detail (Izumi *et al*., 2001). Briefly, cDNA fragment containing the full-length coding regions of rat wild-type 46-kDa JNK was obtained from mRNA of rat vascular smooth muscle cells by RT–PCR method, using RT–PCR kit (Toyobo, Osaka, Japan). DN mutant of 46-kDa JNK (DN–JNK) was constructed by PCR using primers designed to produce a lysine (AAG)→arginine (CGG) substitution at lysine 52 in the ATP-binding site of wild-type JNK. The dual-activating phosphorylation sites in 46 kDa JNK (T183 and Y185) were not mutated. DN–JNK cannot transfer phosphate and so have negligible catalytic efficiency of the enzyme.

Recombinant adenovirus and gene transfer

The recombinant adenovirus expressing β-galactosidase (LacZ) or DN–JNK was generated by the cosmid cassette and adenovirus DNA-terminal protein complex method, using an Adenovirus Expression Vector Kit (Takara Biomedicals, Osaka, Japan). For adenovirus-mediated gene transfer, HS-5 cells were exposed to adenoviral vectors carrying DN–JNK at the multiplicity of infection (MOI) of 10 for 1 h. To evaluate LacZ expression, cells were stained by β-D-galactopyranoside (X-gal) and examined under light microscopy. The transfection efficiency was consistently $>60\%$ (data not shown).

Table 1 PCR primer sequences and probes for use in real-time PCR

Gene	*Sequence (5′-3′)*	*GenBank accession number*
GAPDH		M33197
Primers Sense	TGACGGGAAGCTCACTGG	
Antisense	TCCACCACCCTGTTGCTGTA	
Probes FITC	TCAACAGCGACACCCACTCCT	
LightCycler Red 640	CACCTTTGACGCTGGGGCT	
LIF		M63420
Primers Sense	AACGCCACCTGTGCCATA	
Antisense	CAGGGAGGTGCCAAGGTA	
Probes FITC	CCGTTCCCCAACAACCTGGA	
LightCycler Red 640	AAGCTATGTGGCCCCAACGTGA	
IL-6		
Primers Sense	AAGAGGCACTGGCAGAAAAC	M14584
Antisense	GTCAGGGGTGGTTATTGCAT	
Probes FITC	CAGATTTGAGAGTAGTGAGGAACAAGCC	
LightCycler Red 640	GAGCTGTCCAGATGAGTACAAAAGTCCT	

Statistical analysis

Results are presented as mean ± s.e.mean. Statistical significance was determined with one-way analysis of variance (ANOVA) followed by Duncan multiple-range test. Differences were considered statistically significant at a value of $P<0.05$.

Results

FCS activates ERK, p38 MAPK and JNK in HS-5

To determine whether FCS could induce the activation of ERK, p38 MAPK and JNK in HS-5 cells, we exposed cells to 10% FCS for desired times after 48 h of serum starvation, and then we measured phosphorylation of ERK and p38 MAPK by Western blot analysis and performed an *in vitro* kinase assay by using a c-Jun NH_2-terminal fusion protein as a substrate. Amounts of phosphorylated form of ERK1 (44 kDa) and ERK2 (42 kDa) in FCS-stimulated cells rapidly increased, peaked at 5 min, and then gradually declined to basal level (Figure 1A, upper panel). No phosphorylated form of p38 MAPK was detected in unstimulated cells, whereas FCS induced rapid phosphorylation of p38 MAPK, with a peak occurring 15 min after FCS addition (Figure 1B, upper panel). *In vitro* kinase assay showed that FCS enhanced JNK activity, peaked at 60 min (Figure 1C). Then we stimulated HS-5 cells with various concentrations of FCS. Figure 2 demonstrates that concentrations of FCS from 0.01% to 10% clearly increased the amounts of ERK and p38 MAPK phosphorylation and JNK activity than seen in the absence of FCS.

FCS induces LIF and IL-6 production

We stimulated serum-starved (48 h) HS-5 cells with 10% FCS for various periods, and measured the protein level of LIF and IL-6 in supernatant. As shown in Figure 3A, LIF was not detected in the supernatant of unstimulated HS-5 cells for 24 h. A significant increase of LIF in the supernatant of FCS-stimulated HS-5 cells was observed after 6 h. Figure 3B demonstrates that the protein levels of IL-6 in the tissue culture medium of FCS-stimulated and unstimulated HS-5 cells were both increased in a time dependent manner. However, FCS induced significant IL-6 production compared with unstimulated HS-5 cells.

FCS increases LIF and IL-6 mRNA expression

We examined the time course of FCS-induced LIF and IL-6 mRNA expression by using quantitative real-time PCR. As shown in Figure 4, LIF (A) and IL-6 (B) mRNA expression

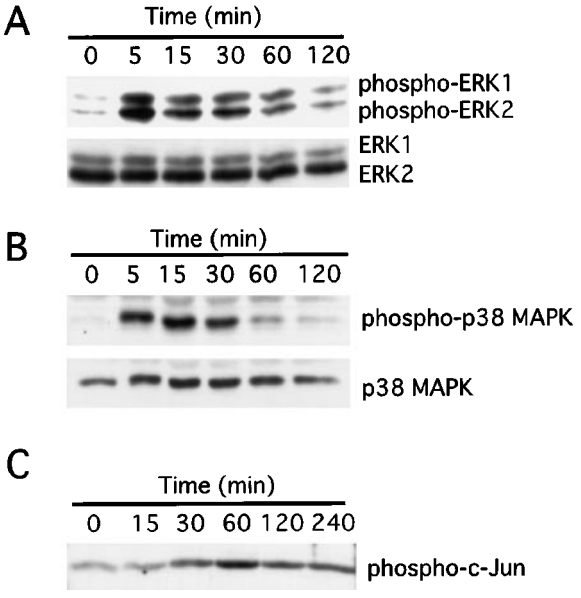

Figure 1 Activation of MAPKs in HS-5 cells stimulated by FCS: a time course. After 48 h of serum starvation, HS-5 cells were stimulated with 10% FCS for the indicated periods of time. (A,B) HS-5 cell lysates (containing 20 μg of total proteins) were separated by 12% SDS–PAGE and then transferred onto nitrocellulose membranes. Phosphorylated and native ERK were analysed using specific antiphosphorylated ERK (phospho-ERK1/phospho-ERK2; upper panel of A) and anti-ERK (ERK1/ERK2; lower panel of A) antibodies. Phosphorylated and native p38 MAPK were analysed using specific antiphosphorylated p38 MAPK (phospho-p38 MAPK; upper panel of B) and anti-p38 MAPK (p38 MAPK; lower panel of B) antibodies. (C) HS-5 cell lysates (containing 250 μg of total proteins) were incubated with an N-terminal c-Jun (1-89) fusion protein bound to glutathione-Sepharose beads for JNK kinase activity assay. Phosphorylation of c-Jun was determined by Western blot analysis with the phospho-specific c-Jun antibody.

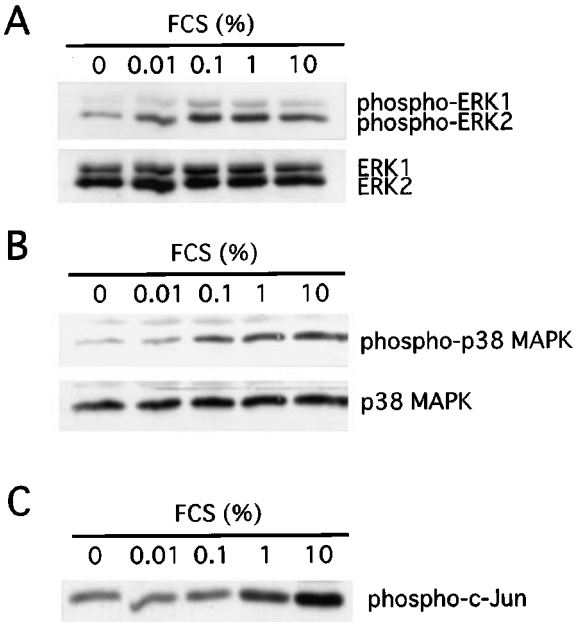

Figure 2 Activation of MAPKs in HS-5 cells stimulated by FCS: a dose response. After 48 h of serum starvation, HS-5 cells were stimulated with various concentrations (0, 0.01, 0.1, 1 or 10%) of FCS. (A) HS-5 cells were stimulated for 5 min and cell lysates were harvested. Using specific antibodies, phosphorylated ERK (phospho-ERK1/phospho-ERK2; upper panel) and native ERK (ERK1/ERK2; lower panel) were detected in HS-5 cell lysates. (B) HS-5 cells were stimulated for 15 min and cell lysates were harvested. Using specific antibodies, phosphorylated p38 MAPK (phospho-p38 MAPK; upper panel) and native p38 MAPK (p38 MAPK; lower panel) were detected in HS-5 cell lysates. (C) HS-5 cells were stimulated for 60 min and cell lysates were harvested. Cell lysates were incubated with an N-terminal c-Jun (1-89) fusion protein bound to glutathione-Sepharose beads for JNK kinase activity assay. Phosphorylation of c-Jun was determined by Western blot analysis with the phospho-specific c-Jun antibody.

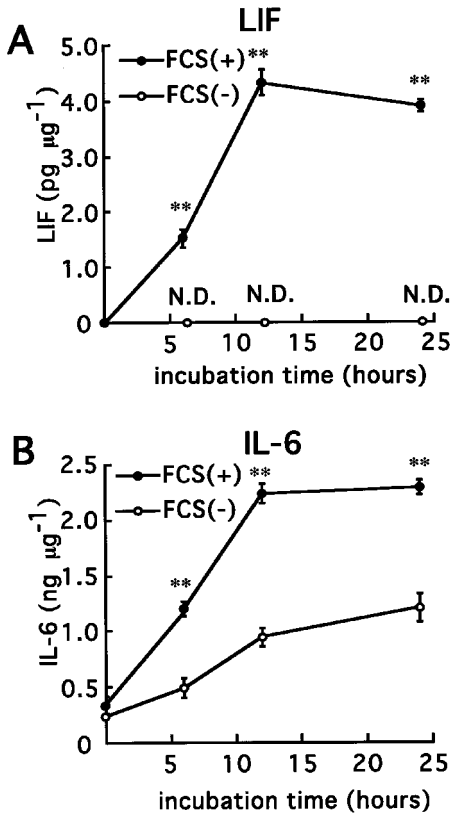

Figure 3 FCS induces LIF and IL-6 production. After 48 h of serum starvation, HS-5 cells were cultured either with 10% FCS (closed circles) or without FCS (open circles) and the concentrations of LIF (A) or IL-6 (B) in the culture supernatant were determined at 0, 6, 12 and 24 h after cultivation by ELISA. The amount of cytokine expressed was normalized to the total protein concentration in the cells of each sample. Results are expressed as mean ± s.e.mean ($n=4$). $^{**}P<0.01$ compared with unstimulated cells. N.D. = not detected.

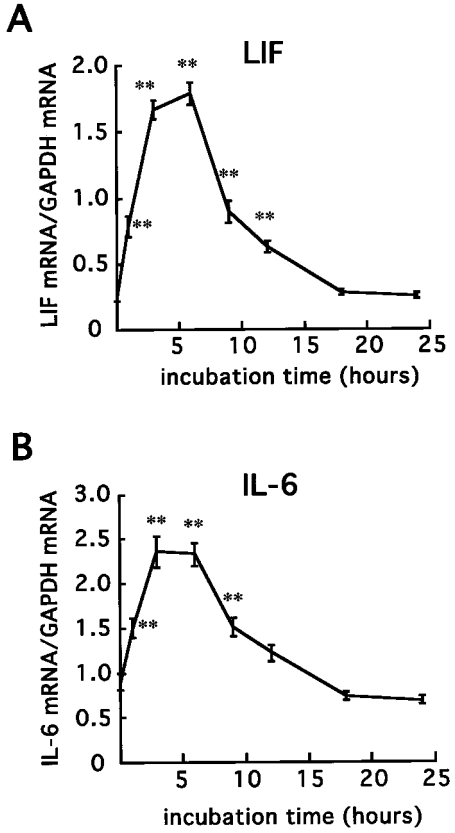

Figure 4 FCS increases LIF and IL-6 mRNA expression. After 48 h of serum starvation, HS-5 cells were stimulated with 10% FCS for the indicated periods of time. RNA (1 μg) was isolated and reverse transcribed to cDNA, which was quantified by real-time PCR, using primers specific for LIF, IL-6 and GAPDH. Values are expressed as the ratio of LIF (A) or IL-6 (B) cDNA to GAPDH cDNA. Results are expressed as mean ± s.e.mean ($n=6$). $^{**}P<0.01$ compared with cells at 0 h.

were significantly increased by FCS stimulation, reached the peak at 3–6 h and gradually returned to the basal level within 24 h.

Effects of PD98059 and SB 203580 on cytokine production

FCS stimulated HS-5 cells to produce LIF and IL-6 along with activation of ERK, p38 MAPK and JNK (Figures 1 and 2). These observations suggested that FCS-induced LIF and IL-6 production might be mediated through these pathways. To evaluate the role of these pathways in regulating the production of cytokines from FCS-stimulated HS-5 cells, it was necessary to inhibit each pathway selectively. Serum-starved (48 h) cells were incubated with various concentrations of the MEK inhibitor PD98059 or the p38 MAPK inhibitor SB302580 for 1 h. These concentrations of inhibitors were not toxic to the cells, as evaluated by Trypan blue stain. Cells were then stimulated with 10% FCS and the concentrations of LIF and IL-6 in the culture supernatant were determined at 24 h after stimulation. As shown in Figure 5A,B, increasing concentrations of PD98059 and SB203580 induced a dose-dependent decrease of LIF production. On the other hand, SB203580 significantly decreased FCS-induced IL-6 production, however, PD98059 demonstrated no inhibitory effect on IL-6 production (Figure 6A,B).

Expression of dominant negative JNK does not affect FCS-induced LIF and IL-6 production

To investigate the role of JNK pathways in regulating the production of cytokines from FCS-stimulated HS-5 cells, cells were infected with recombinant adenovirus containing DN–JNK, followed by measurements of JNK activity and cytokine concentration. As shown in Figure 7A, expression of DN–JNK resulted in a marked decrease in JNK activity in FCS-stimulated HS-5 cells (lane 4), under conditions where infection with recombinant adenovirus containing LacZ had no effect on kinase activity (lane 3). The functional role of the JNK activation was investigated by measuring the FCS-induced cytokine production. As shown in Figure 7B,C, expression of DN–JNK had no effect on FCS-induced LIF or IL-6 production in HS-5 cells.

Effects of MAPK inhibition on FCS-induced cytokine mRNA expression

To further evaluate the effects of blockade of MAPK family on cytokine production, we also wanted to determine whether

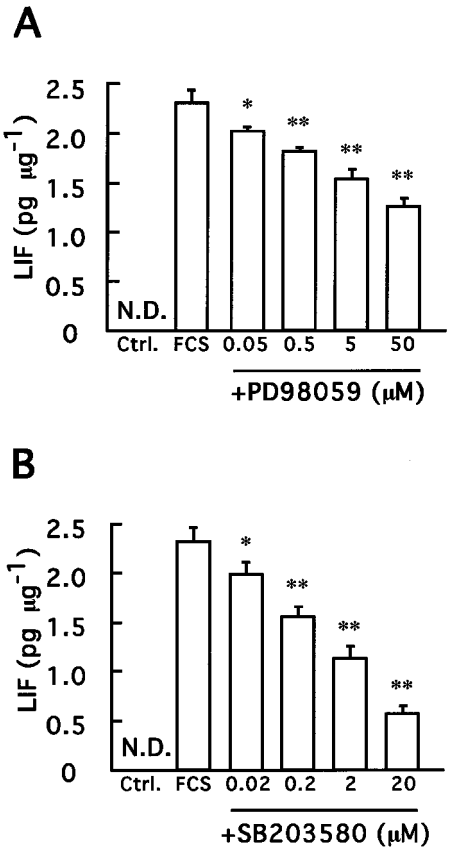

Figure 5 PD98059 and SB203580 inhibit FCS-induced LIF production in a dose dependent manner. After 48 h of serum starvation, HS-5 cells were cultured with or without increasing concentration of PD98059 (A) or SB203580 (B) for 1 h. Then cells were stimulated with 10% FCS for 24 h and LIF concentrations were determined by ELISA. The amount of cytokine expressed was normalized to the total protein concentration of each sample. Results are expressed as mean ± s.e.mean ($n=4$). $^*P<0.05$, $^{**}P<0.01$ compared with FCS alone. N.D. = not detected.

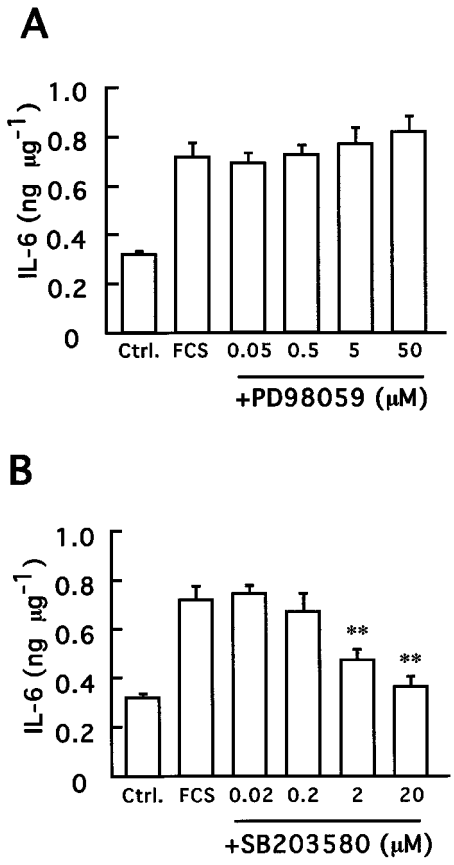

Figure 6 SB203580 but not PD98059 inhibits FCS-induced IL-6 production. After 48 h of serum starvation, HS-5 cells were cultured with or without increasing concentration of PD98059 (A) or SB203580 (B) for 1 h. Then cells were stimulated with 10% FCS for 24 h and IL-6 concentrations were determined by ELISA. The amount of cytokine expressed was normalized to the total protein concentration of each sample. Results are expressed as mean ± s.e.mean ($n=4$). $^*P<0.05$, $^{**}P<0.01$ compared with FCS alone.

inhibitors regulated LIF and IL-6 production at the level of mRNA transcription. FCS induced a significant increase in mRNA concentrations for both cytokines after 3 h of stimulation (Figure 4). We therefore studied effects of PD98059, SB203580 or DN–JNK on LIF and IL-6 mRNA concentrations after 3 h of FCS stimulation. As shown in Figure 8, FCS-induced LIF mRNA expression was significantly decreased by PD98059 or SB203580. IL-6 mRNA expression was decreased by SB203580, but not by PD98059. Inhibition of JNK activity using a dominant negative mutant of JNK1 had no effect of LIF and IL-6 mRNA expression (Figure 9). These results are consistent with the data of protein production.

Discussion

In the present study, we examined the signal transduction pathways in serum-induced LIF and IL-6 production by bone marrow stromal cell line, HS-5. Bone marrow stromal cells were reported to produce haematopoietic cytokines in response to lipopolysaccharide or inflammatory mediators such as IL-1α and TNF-α (Fibbe *et al*., 1988; Lorgeot *et al*., 1997; Rougier *et al*., 1998). However, in the absence of such inflammatory stimuli, many kinds of haematopoietic cytokines are secreted for steady-state haematopoiesis. Although immortalized cell lines might not always reflect primary cells, the availability of cell lines would allow a stable and a well-defined tool to study the mechanism of cytokine production. We therefore studied the response of the HS-5 cells to foetal calf serum, which exists normally in the marrow microenvironment and contains an undefined cocktail of growth factors and other mitogens.

Treatment of bone marrow stromal cells with FCS resulted in enhanced phosphorylation of ERK and p38 MAPK and increased kinase activity of JNK. FCS also enhanced LIF and IL-6 protein production and their mRNA expression. Kinetic analysis revealed that LIF and IL-6 mRNA expression was optimal at 3 h after stimulation and returned to basal levels after 12 h, whereas their protein production seemed to peak 12–24 h after stimulation. There are two possibilities. The first, in our experiments, when we collected conditioned media to measure cytokine production at indicated time points, we did not replace with fresh media, so the cytokine continued to accumulate throughout this time period and kept high cytokine levels for over 12 h.

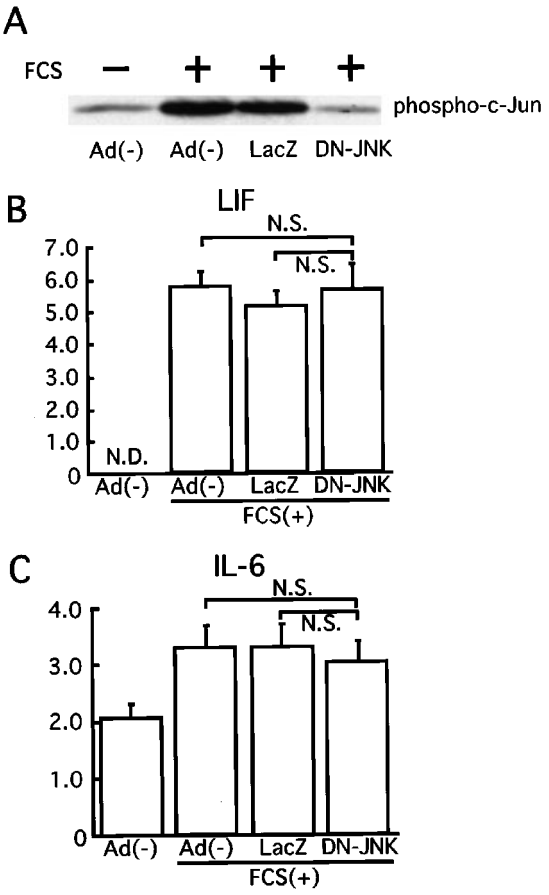

Figure 7 Effect of recombinant adenoviral infection containing DN–JNK or LacZ on response to FCS-stimulated HS-5 cells. (A) *In vitro* kinase activity in uninfected and recombinant adenovirus infected cells. HS-5 cells were infected with recombinant adenovirus containing DN–JNK or LacZ at 10 MOI. Uninfected cells were used as control. Cells were cultured with serum-free medium for 48 h, and stimulated with 10% FCS for 1 h. Cell lysates were harvested and subjected to measurement of JNK activity. (B,C) Infected and uninfected cells were made quiescent by incubation with in serum-free medium for 48 h, and then stimulated with 10% FCS for 24 h. Concentrations of LIF (B) and IL-6 (C) in the culture supernatant were determined by ELISA. The amount of cytokine expressed was normalized to the total protein concentration of each sample. In all experiments, adenoviral infection was carried out at 10 MOI. Each bar represents mean ± s.e.mean ($n=4$). AD(−) indicates no recombinant adenoviral infection; LacZ, infection with recombinant adenovirus containing LacZ; DN–JNK, infection with recombinant adenovirus containing DN–JNK. N.D. = not detected, N.S. = not significant.

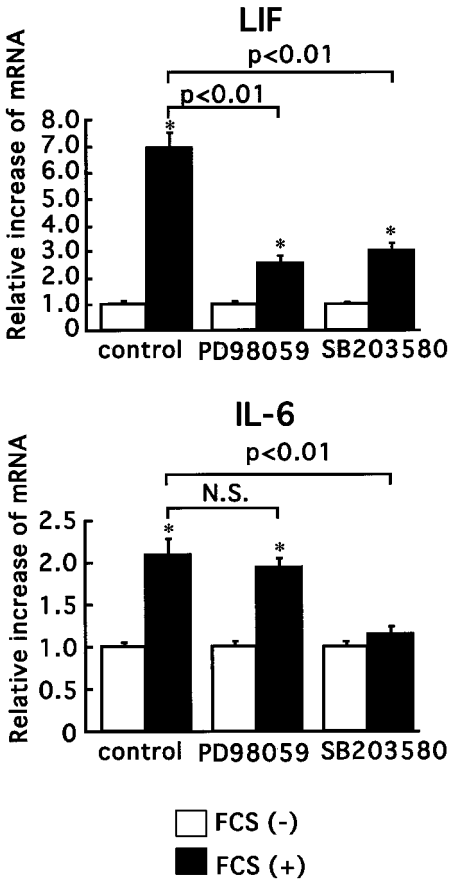

Figure 8 Effect of PD98059 or SB203580 on cytokine mRNA expression in FCS-stimulated HS-5 cells. After 48 h of serum starvation, HS-5 cells were cultured with PD98059 (50 μM) or SB203580 (20 μM) for 1 h. Untreated cells were used as controls. Cells were then stimulated or unstimulated with FCS for 3 h and RNA was isolated. RNA was reverse transcribed to cDNA, which was quantified by real-time PCR, using primers specific for LIF, IL-6 and GAPDH. Results are expressed as increase in mRNA levels as compared with unstimulated cells after normalization to GAPDH. Each bar represents mean ± s.e.mean ($n=6$). *$P<0.05$ compared with FCS (−).

Second, the cytokine protein secretion follows mRNA transcription, translation of the nucleotide sequence of mRNA into a protein and transport of the protein to the cell surface. The delay of the peak of protein production might reflect these processing. Our next goal was to investigate in which way FCS-induced MAPK activation was causally related to LIF and IL-6 production. In this study, SB203580 and PD98059 were used as the specific pharmacological inhibitors for p38 MAPK activity and MEK activity, respectively, in order to elucidate the biological functions of p38 MAPK and ERK. Pretreatment of bone marrow stromal cells with 20 μM of SB203580 showed a 75% decrease of FCS-induced LIF production and an 83% decrease in IL-6 production, with concomitantly decreased LIF and IL-6 mRNA expression. On the other hand, 50 μM of PD98059 caused a 46% decrease in LIF production, whereas PD98059 had no effect on IL-6 protein production and mRNA expression. 20 μM of SB203580 and 50 μM of PD98059 were used in this study to examine the inhibitory effect of these inhibitors on cytokine production since the previous reports with analysis of the role of p38 MAPK and ERK in eliciting various biological responses showed that these concentrations of inhibitors almost completely inhibited cytokine expression (Alessi *et al*., 1995; Jacobs-Helber *et al*., 2000; Maruoka *et al*., 2000; Matsumoto *et al*., 1998). A specific and potent pharmacological inhibitor of JNK was not commercially available, we introduced dominant negative mutant of JNK1 into bone marrow stromal cells, using the adenoviral gene transfer method. Although expression of dominant-negative mutant of JNK1 clearly inhibits FCS-induced JNK activation in bone marrow stromal cells, cytokine production and gene expression were not inhibited. These data demonstrate that

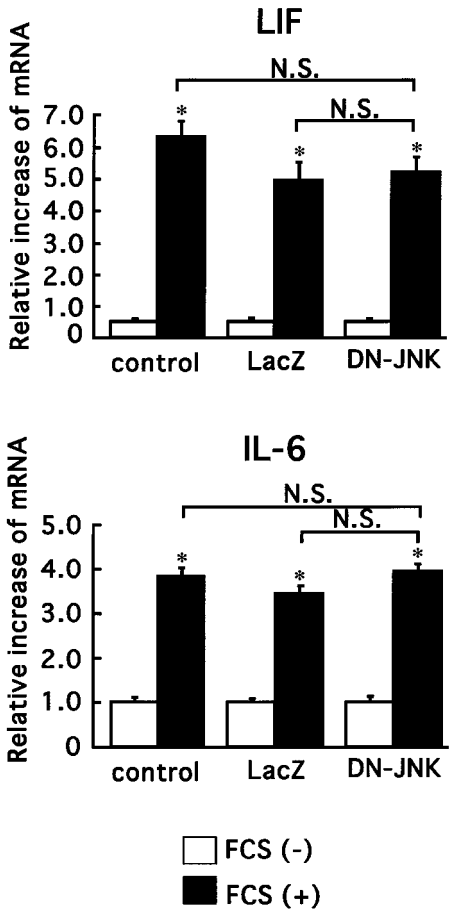

Figure 9 Effect of DN−JNK gene transfer on cytokine mRNA expression in FCS-stimulated HS-5 cells. Uninfected HS-5 cells or cells infected with LacZ of DN−JNK adenoviruses (10 MOI) were cultured in serum-free medium for 48 h, and stimulated or unstimulated with 10% FCS for 3 h. RNA was isolated and expression of cytokine mRNA was determined by real-time PCR. Results are expressed as increase in mRNA levels as compared with unstimulated cells after normalization to GAPDH. Each bar represents mean ± s.e.mean ($n=6$). *$P<0.05$ compared with FCS (−). N.S. = not significant.

activation of p38 MAPK, but not ERK activity, is essential for FCS-induced IL-6 production, whereas both ERK and p38 MAPK are involved in FCS-induced LIF production by human bone marrow stromal cells. The involvement of p38 MAPK and ERK in IL-6 production has been studied in various types of cells including human bronchial epithelial cells (Laan *et al*., 2001), human keratinocytic cells (Wery-Zennaro *et al*., 2000), human primary mesangial and proximal tubular cells (Leonard *et al*., 1999), human airway monocytes (Hedges *et al*., 2000), human astrocytoma cells (Fiebich *et al*., 2000) and others (Kondo *et al*., 2001; Suzuki *et al*., 2000). In many of these studies, IL-6 production was inhibited by SB203580 and PD98059, whereas in several types of cells PD98059 did not inhibit IL-6 production. Two previous reports demonstrated the involvement of cAMP-responsive element binding protein (CREB), a transcription factor which is a substrate of both ERK and p38 MAPK, in IL-6 gene expression by angiotensin II (Funakoshi *et al*., 1999; Sano *et al*., 2001). In our cell system, we could not elucidate the transcription factor involved in IL-6 production in bone marrow stromal cells. The signalling modules involved in IL-6 production are cell type specific and depend on the stimulatory signal. More detailed study is required to elucidate the role of MAPK pathways in IL-6 secretion. In the present study, we have shown that the activation of two distinct pathways, ERK and p38 MAPK, are critical for LIF production in HS-5 cells. Most of previous reports demonstrated that ERK and p38 MAPK were distinct parallel pathways (Widmann *et al*., 1999). However, recently, Ueda *et al*. (2002) demonstrated that p38 MAPK induced Ca^{2+} influx, thereby leading to ERK activation in SCF-stimulated BAF3 cells. We could not elucidate whether ERK and p38 MAPK pathways were parallel or in a specific order in HS-5 cells. Further study is required to clarify this point. This is the first report to show that ERK and p38 MAPK are important pathways in regulating LIF secretion. Although LIF and IL-6 both belong to the IL-6 cytokine family and they share striking functional similarities, their secretion is regulated by different signal transduction pathways in bone marrow stromal cells. Normal haematopoiesis is charcterized by a balanced interplay between haematopoietic progenitor cells and bone marrow stromal cells. This distinct usage of MAPK pathways in the production of haematopoietic cytokines may reflect the tightly controlled regulation of cytokine production and haematopoiesis within the marrow microenvironment.

Recently, Milella *et al*. (2001) reported that MEK inhibitors (PD98059 and PD184352) profoundly impaired the growth and survival of acute myeloid leukaemia cells with constitutive ERK activation, while these agents had minimal effects on normal haematopoietic progenitors *in vitro*; they concluded that disruption of MEK signalling might represent a relatively specific therapeutic strategy for acute myeloid leukaemia. However, our data suggest that disruption of MEK signalling attenuate LIF production in the marrow microenvironment. LIF are known to play a role in the haematopoiesis acting synergistically with other cytokines to induce proliferation of haematopoietic progenitor cells (Keller *et al*., 1996). In addition, LIF was reported to upregulate haematopoietic cytokine expression by murine stromal cell lines (Shih *et al*., 2000; Szilvassy *et al*., 1996). Therefore, MEK inhibition therapy for acute myeloid leukaemia could be responsible for the impairment of normal haematopoiesis in the marrow micro-environment. Further study is needed to clarify this point.

In summary, this study demonstrates that MAPK family cascades are activated in response to serum in immortalized bone marrow stromal cell line, HS-5. The activation of ERK and p38 MAPK plays an important role for LIF secretion, whereas only p38 MAPK is crucial for IL-6 secretion. Activation of JNK pathway has no effect on protein production and gene expression of these cytokines. These findings should aid in the understanding of cellular mechanisms that regulate haematopoietic cytokine production in the marrow micro-environment. However, MAPK family also plays a pivotal role in proliferation and differentiation of many types of cells. In our present study, we did not provide evidences for these points. Future studies focused on these points will further clarify the biology of haematopoietic micro-environment.

References

ALESSI, D.R., CUENDA, A., COHEN, P., DUDLEY, D.T. & SALTIEL, A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.,* **270,** 27489 – 27494.

CARTER, A.B., MONICK, M.M. & HUNNINGHAKE, G.W. (1999). Both Erk and p38 kinases are necessary for cytokine gene transcription. *Am. J. Respir. Cell. Mol. Biol.,* **20,** 751 – 758.

CHABANNON, C. & TOROK-STORB, B. (1992). Stem cell-stromal cell interactions. *Curr. Top. Microbiol. Immunol.,* **177,** 123 – 136.

DORSHKIND, K. (1990). Regulation of hemopoiesis by bone marrow stromal cells and their products. *Annu. Rev. Immunol.,* **8,** 111 – 137.

FIBBE, W.E., VAN DAMME, J., BILLIAU, A., GOSELINK, H.M., VOOGT, P.J., VAN EEDEN, G., RALPH, P., ALTROCK, B.W. & FALKENBURG, J.H. (1988). Interleukin 1 induces human marrow stromal cells in long-term culture to produce granulocyte colony-stimulating factor and macrophage colony-stimulating factor. *Blood,* **71,** 430 – 435.

FIEBICH, B.L., SCHLEICHER, S., BUTCHER, R.D., CRAIG, A. & LIEB, K. (2000). The neuropeptide substance P activates p38 mitogen-activated protein kinase resulting in IL-6 expression independently from NF-kappa B. *J. Immunol.,* **165,** 5606 – 5611.

FUNAKOSHI, Y., ICHIKI, T., ITO, K. & TAKESHITA, A. (1999). Induction of interleukin-6 expression by angiotensin II in rat vascular smooth muscle cells. *Hypertension,* **34,** 118 – 125.

GREENBERGER, J.S. (1991). The hematopoietic microenvironment. *Crit. Rev. Oncol. Hematol.,* **11,** 65 – 84.

GUBA, S.C., SARTOR, C.I., GOTTSCHALK, L.R., JING, Y.H., MULLIGAN, T. & EMERSON, S.G. (1992). Bone marrow stromal fibroblasts secrete interleukin-6 and granulocyte-macrophage colony-stimulating factor in the absence of inflammatory stimulation: demonstration by serum-free bioassay, enzyme-linked immunosorbent assay, and reverse transcriptase polymerase chain reaction. *Blood,* **80,** 1190 – 1198.

HAMBURGER, A.W. (1995). Impaired cytokine production by bone marrow stromal cells of immunodeficient mice. *Br. J. Haematol.,* **90,** 750 – 756.

HASHIMOTO, S., GON, Y., MATSUMOTO, K., MARUOKA, S., TAKESHITA, I., HAYASHI, S., ASAI, Y., JIBIKI, I., MACHINO, T. & HORIE, T. (2000). Selective inhibitor of p38 mitogen-activated protein kinase inhibits lipopolysaccharide-induced interleukin-8 expression in human pulmonary vascular endothelial cells. *J. Pharmacol. Exp. Ther.,* **293,** 370 – 375.

HEDGES, J.C., SINGER, C.A. & GERTHOFFER, W.T. (2000). Mitogen-activated protein kinases regulate cytokine gene expression in human airway myocytes. *Am. J. Respir. Cell. Mol. Biol.,* **23,** 86 – 94.

IZUMI, Y., KIM, S., NAMBA, M., YASUMOTO, H., MIYAZAKI, H., HOSHIGA, M., KANEDA, Y., MORISHITA, R., ZHAN, Y. & IWAO, H. (2001). Gene transfer of dominant-negative mutants of extracellular signal-regulated kinase and c-Jun NH2-terminal kinase prevents neointimal formation in balloon-injured rat artery. *Circ. Res.,* **88,** 1120 – 1126.

JACOBS-HELBER, S.M., RYAN, J.J. & SAWYER, S.T. (2000). JNK and p38 are activated by erythropoietin (EPO) but are not induced in apoptosis following EPO withdrawal in EPO-dependent HCD57 cells. *Blood,* **96,** 933 – 940.

KELLER, J.R., GOOYA, J.M. & RUSCETTI, F.W. (1996). Direct synergistic effects of leukemia inhibitory factor on hematopoietic progenitor cell growth: comparison with other hematopoietins that use the gp130 receptor subunit. *Blood,* **88,** 863 – 869.

KISHIMOTO, T., AKIRA, S., NARAZAKI, M. & TAGA, T. (1995). Interleukin-6 family of cytokines and gp130. *Blood,* **86,** 1243 – 1254.

KITTLER, E.L., MCGRATH, H., TEMELES, D., CRITTENDEN, R.B., KISTER, V.K. & QUESENBERRY, P.J. (1992). Biologic significance of constitutive and subliminal growth factor production by bone marrow stroma. *Blood,* **79,** 3168 – 3178.

KONDO, A., MOGI, M., KOSHIHARA, Y. & TOGARI, A. (2001). Signal transduction system for interleukin-6 and interleukin-11 synthesis stimulated by epinephrine in human osteoblasts and human osteogenic sarcoma cells. *Biochem. Pharmacol.,* **61,** 319 – 326.

LAAN, M., LOTVALL, J., CHUNG, K.F. & LINDEN, A. (2001). IL-17-induced cytokine release in human bronchial epithelial cells *in vitro*: role of mitogen-activated protein (MAP) kinases. *Br. J. Pharmacol.,* **133,** 200 – 206.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680 – 685.

LEONARD, M., RYAN, M.P., WATSON, A.J., SCHRAMEK, H. & HEALY, E. (1999). Role of MAP kinase pathways in mediating IL-6 production in human primary mesangial and proximal tubular cells. *Kidney Int.,* **56,** 1366 – 1377.

LORD, K.A., ABDOLLAHI, A., THOMAS, S.M., DEMARCO, M., BRUGGE, J.S., HOFFMAN-LIEBERMANN, B. & LIEBERMANN, D.A. (1991). Leukemia inhibitory factor and interleukin-6 trigger the same immediate early response, including tyrosine phosphorylation, upon induction of myeloid leukemia differentiation. *Mol. Cell. Biol.,* **11,** 4371 – 4379.

LORGEOT, V., ROUGIER, F., FIXE, P., CORNU, E., PRALORAN, V. & DENIZOT, Y. (1997). Spontaneous and inducible production of leukaemia inhibitory factor by human bone marrow stromal cells. *Cytokine,* **9,** 754 – 758.

MARIN, V., FARNARIER, C., GRES, S., KAPLANSKI, S., SU, M.S., DINARELLO, C.A. & KAPLANSKI, G. (2001). The p38 mitogen-activated protein kinase pathway plays a critical role in thrombin-induced endothelial chemokine production and leukocyte recruitment. *Blood,* **98,** 667 – 673.

MARUOKA, S., HASHIMOTO, S., GON, Y., TAKESHITA, I. & HORIE, T. (2000). PAF-induced RANTES production by human airway smooth muscle cells requires both p38 MAP kinase and Erk. *Am. J. Respir. Crit. Care Med.,* **161,** 922 – 929.

MATSUMOTO, K., HASHIMOTO, S., GON, Y., NAKAYAMA, T. & HORIE, T. (1998). Proinflammatory cytokine-induced and chemical mediator-induced IL-8 expression in human bronchial epithelial cells through p38 mitogen-activated protein kinase-dependent pathway. *J. Allergy Clin. Immunol.,* **101,** 825 – 831.

MAYER, A., PODLECH, J., KURZ, S., STEFFENS, H.P., MAIBERGER, S., THALMEIER, K., ANGELE, P., DREHER, L. & REDDEHASE, M.J. (1997). Bone marrow failure by cytomegalovirus is associated with an in vivo deficiency in the expression of essential stromal hemopoietin genes. *J. Virol.,* **71,** 4589 – 4598.

METCALF, D. (1991). The leukemia inhibitory factor (LIF). *Int. J. Cell. Cloning,* **9,** 95 – 108.

MILELLA, M., KORNBLAU, S.M., ESTROV, Z., CARTER, B.Z., LAPILLONNE, H., HARRIS, D., KONOPLEVA, M., ZHAO, S., ESTEY, E. & ANDREEFF, M. (2001). Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. *J. Clin. Invest.,* **108,** 851 – 859.

ROECKLEIN, B.A. & TOROK-STORB, B. (1995). Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. *Blood,* **85,** 997 – 1005.

ROUGIER, F., CORNU, E., PRALORAN, V. & DENIZOT, Y. (1998). IL-6 and IL-8 production by human bone marrow stromal cells. *Cytokine,* **10,** 93 – 97.

SANO, M., FUKUDA, K., SATO, T., KAWAGUCHI, H., SUEMATSU, M., MATSUDA, S., KOYASU, S., MATSUI, H., YAMAUCHI-TAKIHARA, K., HARADA, M., SAITO, Y. & OGAWA, S. (2001). ERK and p38 MAPK, but not NF-kappaB, are critically involved in reactive oxygen species-mediated induction of IL-6 by angiotensin II in cardiac fibroblasts. *Circ. Res.,* **89,** 661 – 669.

SCHAEFFER, H.J. & WEBER, M.J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.,* **19,** 2435 – 2444.

SHIH, C.C., HU, M.C., HU, J., WENG, Y., YAZAKI, P.J., MEDEIROS, J. & FORMAN, S.J. (2000). A secreted and LIF-mediated stromal cell-derived activity that promotes *ex vivo* expansion of human hematopoietic stem cells. *Blood,* **95,** 1957 – 1966.

STARK, R., ANDRE, C., THIERRY, D., CHEREL, M., GALIBERT, F. & GLUCKMAN, E. (1993). The expression of cytokine and cytokine receptor genes in long-term bone marrow culture in congenital and acquired bone marrow hypoplasias. *Br. J. Haematol.,* **83,** 560 – 566.

SUZUKI, M., TETSUKA, T., YOSHIDA, S., WATANABE, N., KOBAYASHI, M., MATSUI, N. & OKAMOTO, T. (2000). The role of p38 mitogen-activated protein kinase in IL-6 and IL-8 production from the TNF-alpha- or IL-1beta-stimulated rheumatoid synovial fibroblasts. *FEBS Lett.,* **465,** 23 – 27.

SZILVASSY, S.J., WELLER, K.P., LIN, W., SHARMA, A.K., HO, A.S., TSUKAMOTO, A., HOFFMAN, R., LEIBY, K.R. & GEARING, D.P. (1996). Leukemia inhibitory factor upregulates cytokine expression by a murine stromal cell line enabling the maintenance of highly enriched competitive repopulating stem cells. *Blood,* **87,** 4618 – 4628.

TAUPIN, J.L., PITARD, V., DECHANET, J., MIOSSEC, V., GUALDE, N. & MOREAU, J.F.(1998). Leukemia inhibitory factor: part of a large ingathering family. *Int. Rev. Immunol.,* **16,** 397 – 426.

UEDA, S., MIZUKI, M., IKEDA, H., TSUJIMURA, T., MATSUMURA, I., NAKANO, K., DAINO, H., HONDAZI, Z., SONOYAMA, J., SHIBAYAMA, H., SUGAHARA, H., MACHII, T. & KANAKURA, Y. (2002). Critical roles of c-Kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration. *Blood,* **99,** 3342 – 3349.

WERY-ZENNARO, S., ZUGAZA, J.L., LETOURNEUR, M., BERTOGLIO, J. & PIERRE, J. (2000). IL-4 regulation of IL-6 production involves Rac/Cdc42- and p38 MAPK-dependent pathways in keratinocytes. *Oncogene,* **19,** 1596 – 1604.

WETZLER, M., KURZROCK, R., LOWE, D.G., KANTARJIAN, H., GUTTERMAN, J.U. & TALPAZ, M. (1991). Alteration in bone marrow adherent layer growth factor expression: a novel mechanism of chronic myelogenous leukemia progression. *Blood,* **78,** 2400 – 2406.

WIDMANN, C., GIBSON, S., JARPE, M.B. & JOHNSON, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.,* **79,** 143 – 180.

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