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Evidence for a Role of p38 MAP Kinase in Expression of Alkaline Phosphatase During Osteoblastic Cell Differentiation

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In the present study, we investigate the implication of the mitogen-activated protein kinases (MAPKs) Erk, p38, and JNK in mediating the effect of fetal calf serum (FCS) on the differentiation of MC3T3-E1 osteoblast-like cells. Erk is stimulated by FCS in proliferating, early-differentiating, as well as in mature cells. Activation of p38 by FCS is not detected in proliferating cells but is observed as the cells differentiate. JNK is activated in response to FCS throughout the entire differentiation process, but a maximal stimulation is observed in early differentiating cells. The roles of Erk and p38 pathways in mediating MC3T3-E1 cell differentiation was determined using specific inhibitors such as U0126 and SB203580, respectively. These experiments confirmed that the Erk pathway is essential for mediating cell proliferation in response to FCS, but indicated that this MAP kinase has little effect in regulating the differentiation of MC3T3-E1 cells. In contrast, p38 only marginally influenced proliferation, but appeared to be critical for the control of alkaline phosphatase (ALP) expression in differentiating cells. Finally, results obtained with high doses of SB203580, which also affected JNK activity, suggest that p38 and/or JNK are probably also involved in the control of type 1 collagen and osteocalcin expression in differentiating cells. The data indicate that MAPKs regulate different stages of MC3T3-E1 cell development in response to FCS. Distinct MAPK pathways seem to independently modulate osteoblastic cell proliferation and differentiation, with Erk playing an essential role in cell replication, whereas p38 is involved in the regulation of ALP expression during osteoblastic cell differentiation. JNK is also probably involved in the regulation of osteoblastic cell differentiation, but its precise role requires further investigation. (Bone 30:91–98; 2002) © 2002 by Elsevier Science Inc. All rights reserved.

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Introduction

The differentiation of preosteoblasts is dependent upon the temporal regulation of multiple interacting signaling pathways. Recent discoveries of transcription factors that control either bone development or the expression of bone phenotypic markers have significantly improved our understanding of molecular mechanisms supporting osteoblast growth and differentiation.[20] Upstream cellular mechanisms responsible for either the expression or the activation of these factors remain, however, largely unknown. In particular, the role of mitogen-activated protein kinases (MAPKs) in bone forming cells is poorly understood. Three major subfamilies of structurally related MAPKs have been identified in mammalian cells: the extracellular signal-regulated kinases (Erks); the c-Jun N-terminal kinases (JNKs); and the p38 MAP kinases (p38s). MAPKs are proline-directed serine-threonine kinases that have important functions as mediators of cellular responses to a variety of extracellular stimuli.[3,24,31] Erks are characteristically activated by various growth factors and by phorbol esters. Members of the JNK and p38 subfamilies are strongly activated in response to stress stimuli (ultraviolet radiation, heat shock, and hyperosmolarity[18,26,30]), and thus proinflammatory cytokines, and have been given the name stress-activated protein kinases (SAPKs). Whereas the Erk pathway is usually associated with cell proliferation and protection from apoptosis, JNK and p38 can promote apoptosis in many systems.[10,15,16,39] Recent data suggest that, in addition to its effect on apoptosis, the p38 MAPK pathway might also be involved in the differentiation of neuronal cells[12,27] and adipocytes.[9]

In osteoblast-like cells, activation of Erks has been reported in response to several growth factors including mitogens acting either through receptor tyrosine kinases (RTKs) such as basic fibroblast growth factor (bFGF),[5,36] platelet-derived growth factor (PDGF),[5,42] insulin-like growth factor-1 (IGF-1),[14] epidermal growth factor (EGF),[25] or G-protein-coupled receptors (GPCRs) like prostaglandin $F_{2\alpha}$,[11] sphingosine 1-phosphate,[17] endothelin-1,[13] and fluoride.[4,33] Activation of Erks has also been reported in response to cross-linking of collagen with integrins,[37] 17β-estradiol,[8] interleukin-6,[28] and hypoxia.[25] As reported in other cell systems, activation of Erks by growth factors in osteoblast-like cells is associated with enhanced cell proliferation. However, recent data have suggested that this MAPK might also be involved in the regulation of bone cell differentiation.[13,37,38] Little information has been provided on the regulation and role of the JNK and p38 pathways in bone-forming cells. Recently, we found that the p38 pathway mediates the stimulation of alkaline

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phosphatase (ALP) activity induced by activation of a Gi-protein-coupled receptor agonist in osteoblast-like cells,[34,35] suggesting that this MAP kinase could be involved in the differentiation of bone-forming cells. To further determine the role of MAP kinases in the differentiation of osteoblastic cells, we used murine calvarial-derived MC3T3-E1 cells, which is an in vitro model of osteoblast development. These cells express distinct proliferative and differentiating stages. During the initial phase of culture (days 1–8), MC3T3-E1 cells actively replicate, but do not express, ALP. By days 6–9, cells undergo growth arrest and start to express osteoblastic functions, including production of ALP, processing of procollagens and incremental deposition of a collagenous extracellular matrix, as well as secretion of osteocalcin.[32] In the present work, we sought to determine which MAP kinases are activated by fetal calf serum (FCS), an essential growth factor supplement for the normal in vitro differentiation of these cells,[41] and analyzed the role of MAP kinases in osteoblast differentiation using specific inhibitors.

Materials and Methods

Chemicals

Cell culture plasticwares were purchased from Falcon (Becton-Dickinson, Franklin Lakes, NJ) and Corning-Costar (Integra Biosciences, Switzerland). Fetal calf serum (FCS), glutamine, antibiotics, and trypsin/ethylene-diamine tetraacetic acid (EDTA) were from Gibco (Life Technologies, Basel, Switzerland). α-modified essential medium (αMEM) was purchased from Amimed (Bioconcept, Allschwill, Switzerland). 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole (SB203580) and type I collagenase were from Sigma Co. (St. Louis, MO). 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercaptobutadiene) (U0126) was obtained from Tocris (Baldwin, MO). All reagents for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Munich, Germany). GeneScreen membranes were from DuPont de Nemours (Brussels, Belgium). [α-^{32}P]deoxy-CTP and [γ-^{32}P]ATP were obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). All other chemicals were from standard laboratory suppliers and were of the highest purity available.

Antibodies

Antibodies against ERK2 (sc-154-G), p-JNK (agarose-conjugated, sc-6254), c-Jun (D11:sc-7481), p-c-Jun (KM-1: sc-822), JNK (sc-571), and p38 (SC-535-G) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against p-Erk (agarose-conjugated, #9109) and p-p38 (agarose-conjugated, #9219) were purchased from New England Biolabs (Beverly, MA). The antibody against MAPKAP-K2 was from StressGen (Victoria, BC, Canada).

Cell Culture

Mouse calvaria-derived MC3T3-E1 cells were cultured in α-minimal essential medium (αMEM) containing 10% FCS (vol/vol), 0.5% nonessential amino acids (vol/vol), 100 IU/mL penicillin, and 100 μg/mL streptomycin. They were seeded at 10,000 cells/cm^2 and cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2-95% air and the culture medium was changed every 2–3 days. In the subclone of MC3T3-E1 cells used in this study, cells cultured in αMEM and 10% FCS proliferated rapidly during 6–7 days with a decline in growth between days 8 and 10. During this time period, cells began to express bone markers such as ALP, type I collagen (Coll type I), and osteocalcin (OC). Maximal expression of these markers was reached after approximately 25 days. In experiments aimed at testing the effect of the MAPK inhibitors, agents were added 1 h prior and with each medium change (every 2–3 days).

Analysis of Markers of Bone Cell Differentiation

Cells cultured in six well tissue culture clusters for various time periods were harvested in 1 mL of 0.2% Nonidet P-40 and the cell suspension was disrupted by sonication. After centrifugation at 1500*g* for 5 min, ALP activity was measured in the supernatant by the method of Lowry et al.[22]

Collagen synthesis was determined by measuring the 4-hydroxyproline content in the cell layer. Briefly, cells were hydrolyzed in 6N HCl for 16 h at 116°C. After lyophilization and reconstitution of the lysate in distilled water, the amount of 4-hydroxyproline was determined by spectrophotometry at 550 nm as previously described.[1]

Osteocalcin released in the medium was measured by radioimmunoassay using a goat anti-mouse osteocalcin antibody and a donkey anti-goat secondary antibody (Biomedical Technologies, Inc., Stoughton, MA) as previously described.[6]

DNA content was measured as described by Burton[2] with calf thymus DNA as standard and cell replication by cell counting (Coulter counter).

The amount of protein was determined using the Pierce Coomassie Plus assay reagent (Pierce, Rockford, IL).

RNA Extraction and Northern Blotting

Cells were cultured in 25 cm^2 flasks for various times and total RNA was isolated using the TriPure Isolation Reagent from Roche Molecular Biochemicals (Rotkreuz, Switzerland). Cell layers were scraped into 1 mL of TriPure Reagent and homogenized with a pipette. Cell lysates were then cleared by centrifugation before addition of 0.2 mL chloroform. After mixing, the samples were separated into three phases by centrifugation. Finally, RNA contained in the upper aqueous phase was precipitated with 0.5 mL isopropanol, washed with 75% ethanol, air-dried, and resuspended in water.

For northern blotting analyses, 10 μg of total RNA were denatured in glyoxal and separated by electrophoresis on a 1.2% agarose gel. RNA was transferred to GeneScreen membranes by capillary transfer. Membranes were ultraviolet cross-linked and stained with methylene blue to control equal sample loading and RNA integrity. The membranes were then hybridized to the rat ALP complementary deoxyribonucleic acid (cDNA) probe labeled with 50 μCi of [α-^{32}P]deoxy-CTP by random priming (Megaprime DNA labeling system, Amersham Life Sciences). The rat ALP cDNA probe, a 2.5 kb *Eco*RI restriction fragment purified from the pBS+ plasmid, was provided by Dr. G. Rodan (Merck Sharp and Dohme Research Laboratories, West Point, PA). Prehybridization (2 h) and hybridization (3 h) were performed in QuickHyb hybridization solution (Stratagene, La Jolla, CA), which was supplemented with 100 μg/mL salmon sperm DNA. Membranes were washed twice for 15 min in 2 × standard saline citrate (SSC) and 0.1% sodium dodecylsulfate (SDS) at 25°C and for 10–30 min at 65°C in 0.1 × SSC, 0.1% SDS before exposition to Kodak X-Omat AR films for 18–72 h at −80°C.

Western Blotting and Kinase Assay

Cells cultured in 75 cm^2 flasks were exposed to fresh culture medium for various time periods and frozen in liquid nitrogen. Whole cell lysates were obtained by solubilizing the cells at 4°C

in 3 mL lysis of buffer A containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2 mmol/L Na_3VO_4, 0.01 μmol/L calyculin A, 0.1 μmol/L mycrocystin LR, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS. The cell lysates were cleared by centrifugation at 6000 rpm, 4°C, for 30 min. For western blotting analysis, an aliquot of the supernatant was diluted with an equal amount of 2 × reducing sample buffer consisting of 125 mmol/L Tris (pH 6.8), 20% glycerol, 4% SDS, 200 mmol/L dithiothreitol, and 0.025% bromophenol blue. For kinase assays, similar amounts of protein lysate were incubated with the appropriate agarose-conjugated antiphospho-MAPK antibody for 18 h at 4°C. Immunoprecipitates were washed two times in buffer A and two times in kinase buffer containing 50 mmol/L Tris (pH 7.4), 25 mmol/L β-glycerophosphate, 20 mmol/L $MgCl_2$, 1.0 mmol/L dithiotreitol, 10 μmol/L ^{32}P-ATP (50 μCi/mL), and 2.5 μg of either GST-Elk1 (New England Biolabs) for Erk assay; GST-ATF2 (Santa Cruz Biotechnology) for p38 assay, GST-c-Jun (Biomol Research Laboratories, Campus Drive, PA) for JNK assay, or recombinant HSP27 (StressGen, Victoria, BC, Canada) for MAPKAP-K2 assay, and incubated for 30 min at 30°C. The reaction was stopped by addition of 2 × reducing buffer. The samples were heated at 70°C for 30 min, fractionated by reducing SDS-PAGE on 6%–15% acrylamide gradient gels, and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA). Immobilon P membranes were finally exposed for autoradiography at −80°C. Immunoblotting was performed as described previously with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies.[4] Immunoreactive bands and biotinylated molecular weight standards were visualized by ECL (Amersham Pharmacia Biotech).

Statistical Analysis

Results are expressed as the mean ± SEM. Comparative studies of means were performed using one-way analysis of variance followed by a post hoc test (Fisher's projected least significant difference) with a statistical significance at $p < 0.05$.

Results

Growth factors contained in FCS have been shown to play a critical role in the growth and differentiation of MC3T3-E1 cells.[41] It is well documented that many growth factors can lead to the stimulation of different MAP kinases. We therefore investigated the effects of FCS on activation of the Erk, p38, and JNK pathways in these cells. Data shown in **Figure 1** indicate that addition of fresh culture medium containing 10% FCS in early differentiating (day 10) MC3T3-E1 cells induces activation of the three types of MAPKs. Activation of the Erk pathway was rapid and already maximal after 5 min. The response then gradually decreased, although slight stimulation was still detected after 3 h (Figure 1A, upper panel). The kinetics of p38 and JNK activation by FCS were quite different from that observed from Erk. The stimulation of p38 was apparent after 15 min with a maximal effect detected at 1 h. The response then disappeared rapidly (Figure 1A, middle panel). Activation of the JNK pathway was also detected after 15 min with a maximal effect at 1 h, but the JNK response was more pronounced compared with p38 (Figure 1A, lower panel). Analysis of MAPK activation in response to various doses of FCS in αMEM medium indicates that there was no activation of the Erk, p38, and JNK pathways when medium without FCS was added (Figure 1B). In this study, we observed a difference in Erk activation by various doses of FCS as compared with the stimulation of the two other MAPK

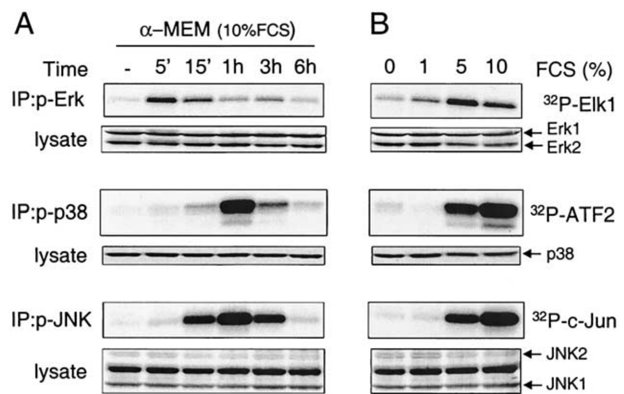

Figure 1. Activation of Erk, p38, and JNK in MC3T3-E1 cells after addition of fresh culture medium. MC3T3-E1 cells were cultured for 10 days in complete αMEM medium containing 10% FCS. Then, cells were either kept in the same medium or changed with fresh αMEM medium containing 10% FCS for various incubation times (A) or with αMEM culture medium containing different amounts of FCS (B) for 5 min (Erk determination) or 1 h (p38 and JNK determinations). At the end of the incubation period, cells were rapidly frozen in liquid nitrogen before lysis at 4°C. Lysates were then immunoprecipitated with specific antiphospho-MAPK antibodies. Immune complexes were incubated with the appropriate substrate and [γ-^{32}P]ATP in phosphorylation buffer as described in *Materials and Methods*. Following in vitro phosphorylation, proteins were heated in SDS sample buffer, subjected to SDS-PAGE electrophoresis, transferred to Immobilon P membranes, and autoradiographed. The upper panel of each pair of images is an autoradiogram of the phosphorylated kinase substrate, whereas the lower panel is a western blot of the corresponding kinase protein.

pathways. A low concentration of FCS (1%) was sufficient to slightly enhance the activity of Erk, but not that of p38 and JNK. In addition, Erk was maximally stimulated by 5% FCS, whereas this concentration of FCS was not sufficient for maximal activation of p38 and JNK (Figure 1B). This observation suggests that the growth factors contained in FCS differentially stimulate these three types of MAPKs in osteoblast-like cells.

To determine the respective role of these MAPKs in controlling the proliferation and differentiation of osteoblast-like cells, we analyzed MAPK activation by FCS at various stages of MC3T3-E1 cell differentiation. Data shown in **Figure 2** indicate that activation of the Erk pathway by FCS was detected at all stages of MC3T3-E1 cell development. In contrast, the p38 MAP kinase pathway was not activated in proliferating cells. It was, however, maximally stimulated in early-differentiating cells and marginally activated in late-differentiating MC3T3-E1 cells (Figure 2, middle panel). A stimulation of the JNK pathway by FCS was already detected during proliferation. The response was, however, maximal in early-differentiating and still present in late-differentiating cells (Figure 2, lower panel). The amounts of the various MAPKs was relatively constant throughout the culture periods except for JNK2 expression, which was increased by 2.4- and 4.1-fold in differentiating cells at days 11 and 18, respectively, as compared with levels in proliferating cells (Figure 2, lower panel).

The observation that the three types of MAPKs (Erk, p38, and JNK) are activated by FCS in differentiating MC3T3-E1 cells suggested a possible role(s) for these signaling pathways in the establishment of the osteoblastic phenotype. To test the role of Erk, we used the specific MEK inhibitor, U0126. A dose of 1 μmol/L was sufficient to block almost completely the stimulation of Erk induced by FCS in differentiating MC3T3-E1 cells without influencing the activation of p38 and JNK (**Figure 3**A).

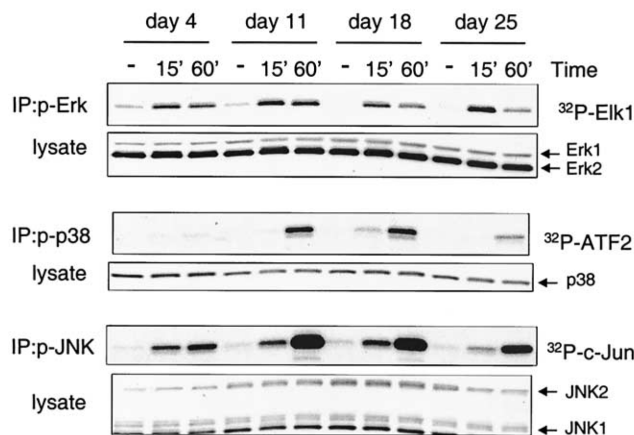

Figure 2. Activation of Erk, p38, and JNK after addition of fresh culture medium as a function of time in culture. MC3T3-E1 cells were cultured for the indicated number of days in complete αMEM medium containing 10% FCS. Then, at various time intervals, fresh αMEM medium containing 10% FCS was added for 15 min and 1 h. At the end of the incubation periods, cells were rapidly frozen in liquid nitrogen before lysis at 4°C. Lysates were then immunoprecipitated with specific antiphospho-MAPK antibodies. In vitro kinase assays and western blotting analyses were performed as described in the legend to Figure 1.

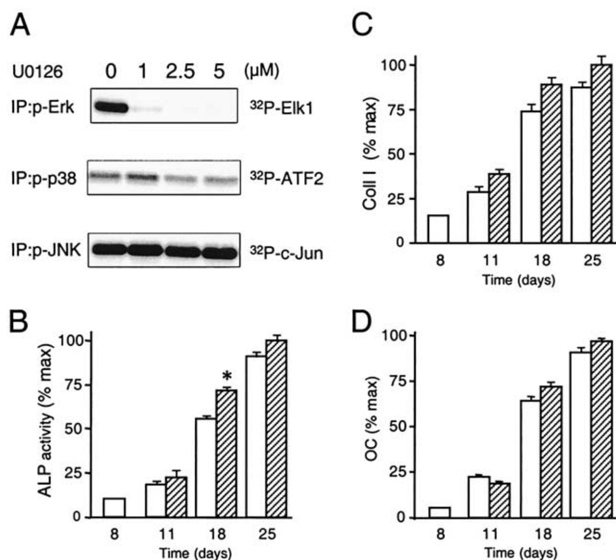

Figure 3. Effect of the MEK inhibitor, U0126, on activation of Erk, p38, and JNK and on MC3T3-E1 cell differentiation. (A) MC3T3-E1 cells were cultured for 8 days in complete αMEM medium containing 10% FCS. Then, cells were preincubated with various concentrations of U0126 for 1 h before exposure to fresh culture medium containing 10% FCS for 5 min (Erk determination) or 1 h (p38 and JNK determinations). (B)–(D) To analyze the effect of U0126 on cell differentiation, cells were treated with 1 μmol/L of U0126 (hatched bars) or its vehicle (open bars) as described in *Materials and Methods*. Expression of the osteoblastic markers alkaline phosphatase (ALP [100% = 76.4 nmol/min per milligram]) (B), type I collagen (Coll I [100% = 9.53 μg/mg]) (C), and osteocalcin (OC [100% = 0.68 ng/mg]) (D) was measured after various times. In (B)–(D), values are the mean ± SEM of four or five determinations. Each determination was corrected by the amount of protein and expressed in percent of the maximal value recorded at day 25. $^{*}p < 0.01$ compared with the respective time control.

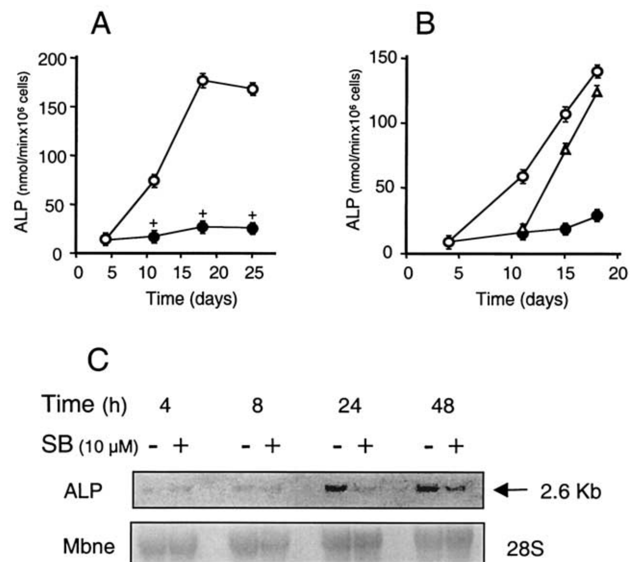

Figure 4. Effect of the p38 MAPK inhibitor, SB203580, on alkaline phosphatase enzyme activity and mRNA expression in MC3T3-E1 cells. (A) MC3T3-E1 cells were cultured for 4 days and exposed to either 10 μmol/L SB203580 (filled circles) or its vehicle (open circles) for 8 days as described in *Materials and Methods*. Then, the inhibitor was either kept in the culture medium (filled circles) or removed (open triangles) for various times before the determination of ALP activity. (C) The influence of SB203580 (SB) on ALP mRNA expression was studied by northern blotting using total RNAs (10 μg) obtained from MC3T3-E1 cells preincubated with either 10 μmol/L SB203580 (SB) or its vehicle and exposed to fresh culture medium containing 10% FCS for various times. A picture of the methylene blue-colored membrane (Mbne) indicating the amount of 28S rRNAs is shown below the blot. In (A) and (B), values are the mean ± SEM of four determinations of a representative experiment. $^{+}p < 0.01$ compared with vehicle.

This dose of U0126 was also sufficient to completely block the increase in DNA content observed between day 8, when cells reach the end of their proliferative phase, and day 11, when cells stop proliferating (day 8: vehicle, 80.1 ± 1.0; day 11, vehicle, 101.4 ± 2.0; day 11: U0126, 84.5 ± 1.4 μg DNA/well). This latter observation confirms that the Erk pathway is involved in the control of cell proliferation in response to growth factors in osteoblastic cells. Associated with the selective inhibition of Erk activation and cell proliferation by U0126, we found that the activity of ALP and the deposition of type 1 collagen were, in general, slightly more elevated in differentiating cells treated with this inhibitor as compared with controls (Figure 3B,C). A very small, nonsignificant decrease in the stimulation of osteocalcin secretion was deteced in early-differentiating cells treated with the inhibitor, but this effect was no longer present in late-differentiating cells (Figure 3D).

The role of the p38 pathway was investigated with the SB203580 inhibitor.[21] Data shown in **Figure 4** indicate that 10 μmol/L SB203580 almost completely blocked expression of ALP activity in differentiating MC3T3-E1 cells (Figure 4A). This effect was observed in the presence of a slightly higher proliferation and a slight decrease in the protein content (**Table 1**), suggesting that this inhibitor had no cytotoxic effect. This was further confirmed by the nearly complete reversibility of inhibition when SB203580 was removed (Figure 4B). Associated with its effect on ALP activity, SB203580 also blocked the increase in ALP mRNA expression induced by 10% FCS (Figure 3C),

Table 1. Effect of SB203580 on MC3T3-E1 cell growth and alkaline phosphatase activity

Time (days)	DNA content (μg/well)		Protein content (mg/well)		Alkaline phosphatase activity (nmol/mL per minute)	
	Veh	SB	Veh	SB	Veh	SB
4	21.4 ± 0.4	—	0.20 ± 0.01	—	13.7 ± 6.7	—
11	46.6 ± 1.7	50.2 ± 1.9[a]	0.76 ± 0.01	0.76 ± 0.01	203.7 ± 7.7	53.3 ± 0.8[b]
18	41.7 ± 1.3	50.2 ± 0.3[a]	1.08 ± 0.01	0.93 ± 0.02[a]	518.9 ± 22	102.5 ± 3.0[b]
25	42.0 ± 1.1	46.6 ± 1.3	1.27 ± 0.01	1.15 ± 0.01[a]	611.7 ± 4.0	101.7 ± 7.5[b]

MC3T3-E1 cells were cultured for 4 days before exposure to 10 μmol/L SB203580 (SB) or its vehicle (Veh). At various timepoints, cells were harvested for the determination of DNA and protein content as well as alkaline phosphatase activity. Each value represents mean ± SEM of four determinations of a representative experiment.
[a]$p < 0.01$ compared with vehicle.
[b]$p < 0.001$ compared with vehicle.

suggesting that the increase in p38-induced ALP expression in differentiating cells occurs at the mRNA level.

SB203580 was reported to inhibit not only p38 but also particular JNK isoforms.[7] We thus verified whether there was a good correlation between p38 inhibition in the cells and the decrease in ALP activity in response to various doses of SB203580. Data shown in **Figure 5** indicate that there was effectively a good relationship between the doses of SB203580 that inhibit FCS-induced expression of ALP activity, on the one hand (Figure 5A), and those that decrease the activity of MAPKAP-K2, on the other (Figure 5B). MAPKAP-K2 is a selective and physiologic downstream effector of p38 and has been used as a read-out to monitor cellular p38 activity.[23] This observation therefore strongly correlates p38 activity with ALP expression in differentiating osteoblasts. In contrast, there was no apparent relationship between changes in ALP expression in the presence of low doses (1–10 μmol/L) of SB203580 and inhibition of JNK activity assessed by measuring the level of phosphorylated c-Jun (Figure 5A and **Figure 6**B). However, we observed that Coll type I deposition or OC secretion were inhibited only by higher doses (10–30 μmol/L) of SB203580. These alterations correlated with a decrease in JNK activity (Figure 6), suggesting that this MAPK may also be involved in controlling the expression of osteoblastic differentiation markers other than ALP, and thus may also play a role in osteoblastic differentiation. This relationship, however, cannot be investigated at present because of a lack of reagent for JNK.

Discussion

The results of the present study strongly suggest that the MAPKs Erk and p38 are involved in the control of osteoblast proliferation and differentiation. As mentioned earlier, the role of JNK could not be investigated because of a lack of reagent. Activation of these signaling pathways in differentiating MC3T3-E1 cells after addition of fresh culture medium containing 10% FCS is mediated by factors contained in the serum, because no stimulation of these MAPKs was detected when fresh culture medium containing no FCS was used (Figure 1, right panel). The serum factors responsible for the stimulation of these MAPKs in osteoblastic cells are not known, but likely involve ligands for different types of receptors such as RTKs, GPCRs, and possibly cytokine receptors.

Kinetic analysis, as well as the dose response to FCS, suggest the implication of different signaling complexes for activation of the three types of MAPKs by serum growth factors (Figure 1, left panel). We observed that activation of p38 and JNK was significantly delayed compared with the rapid stimulation of Erk by FCS (Figure 1A). This observation and recent data indicating that

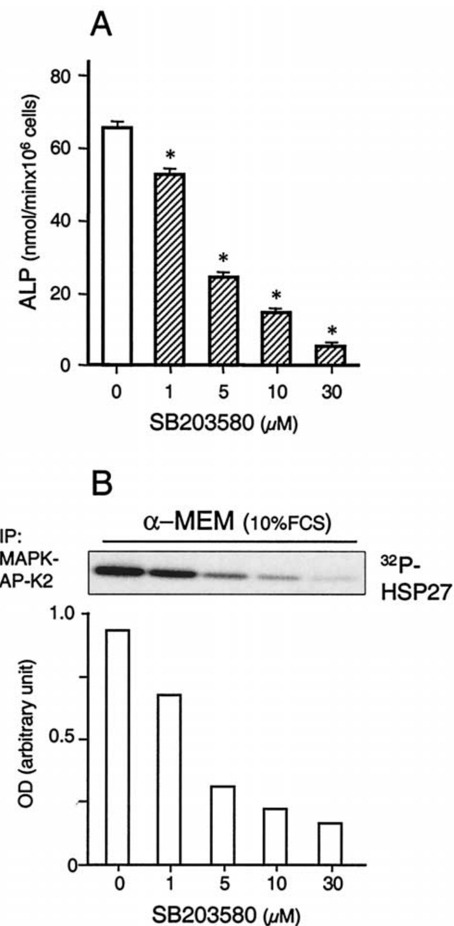

Figure 5. Dose-dependent effect of SB203580 on alkaline phosphatase and p38 MAPK activities. (A) MC3T3-E1 cells were cultured for 4 days before exposure to various concentrations of SB203580 (hatched bars) or its vehicle (open bars) for 7 days and ALP activity was determined as described in *Materials and Methods*. Values are the mean ± SEM of four determinations of a representative experiment. *$p < 0.01$ compared with vehicle. (B) MC3T3-E1 cells cultured for 10 days were preincubated with various concentrations of SB203580 for 1 h. Then, fresh αMEM + 10% FCS culture medium containing either SB203580 or its vehicle were added for a further 60 min and cells were rapidly frozen in liquid nitrogen before lysis at 4°C. Lysates were then immunoprecipitated with a specific anti-MAPKAP-K2 antibody and in vitro MAPKAP-K2 activity was assessed by phosphorylation of HSP27 as described in *Materials and Methods*. Changes in HSP27 phosphorylation content were quantified by densitometry (OD).

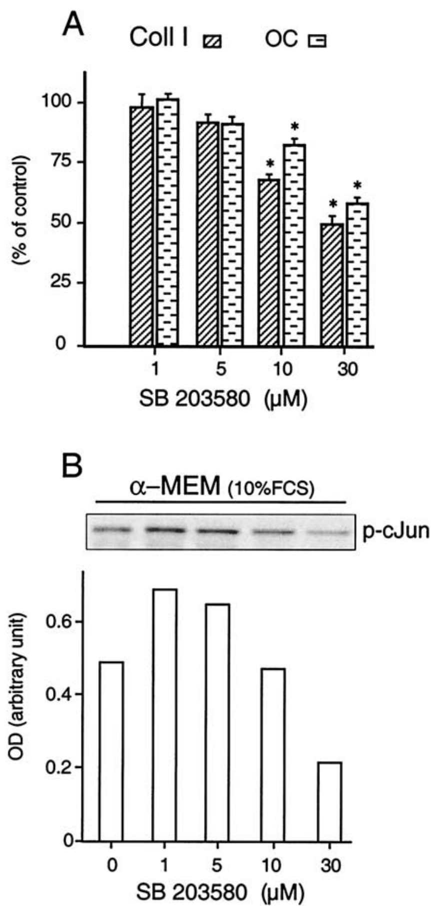

Figure 6. Dose-dependent effect of SB203580 on collagen deposition, osteocalcin production, and JNK activity in MC3T3-E1 cells. (A) MC3T3-E1 cells were cultured for 4 days before exposure to various concentrations of SB203580 (hatched bars, dashed bars) or its vehicle (open bars) for 7 days and the amount of deposited collagen (hatched bars) and the production of osteocalcin (dashed bars) were determined as described in *Materials and Methods*. Values are the mean ± SEM of four determinations of a representative experiment. $*p < 0.01$ compared with control. (B) MC3T3-E1 cells cultured for 10 days were preincubated with various concentrations of SB203580 for 1 h. Then fresh αMEM + 10% FCS culture medium containing either SB203580 or its vehicle were added for a further 60 min and cells were rapidly frozen in liquid nitrogen before lysis at 4°C. Phosphorylated c-Jun (p-c-Jun) in cell lysates was determined by western blotting using a specific antibody. Changes in c-Jun phosphorylation were quantified by densitometry (OD).

Erk can mediate activation of JNK induced by growth factors in endothelial cells[29] suggest that Erk could mediate the activation of JNK and/or of p38 by FCS in MC3T3-E1 cells. However, our results obtained with the specific MEK inhibitor appear to exclude such a cross-talk between Erk and either JNK or p38 in MC3T3-E1 cells. Indeed, the nearly complete inhibition of Erk activation by 1 μmol/L U0126 did not influence the stimulation of either p38 or JNK by FCS (Figure 3A). Thus, the Erk pathway does not seem to be involved in the activation of p38 and JNK and the underlying molecular mechanism of activation of these MAPKs in MC3T3-E1 cells by FCS remains to be investigated.

Analyses of the activation of the three types of MAPKs by FCS during the various phases of proliferation and differentiation of MC3T3-E1 cells indicate that the Erk pathway was stimulated at all stages, whereas p38 and JNK were preferentially activated during differentiation. The stimulation of p38 was observed in the absence of any change in the amount of p38 expressed in differentiating cells, suggesting that this response results mainly from activation of upstream elements in this signal transduction pathway. In contrast, the increase in JNK activity observed in differentiating cells was associated with higher expression of JNK2, suggesting that this MAPK may play a particular role in osteoblastic cell differentiation.

To determine the role of Erk and p38 in regulating growth and differentiation of MC3T3-E1 osteoblast-like cells, we used specific inhibitors such as U0126 for the Erk pathway and SB203580 to inhibit p38 activity. As mentioned earlier, Erk is activated by growth factors acting through different types of receptors and mediates their mitogenic effects in bone-forming cells. As expected from this information, inhibiting the stimulation of Erk in response to FCS in late-proliferating MC3T3-E1 cells stopped their replication (see *Results*), thus confirming the importance of this pathway in controlling bone cell proliferation. A role of Erk in osteoblastic cell differentiation has also been proposed. Essentially, Takeuchi and coworkers suggested that, in MC3T3-E1 cells, the binding of type I collagen to $\alpha_2\beta_1$ integrin receptors activates Erk, and that this signaling pathway mediates extracellular matrix-dependent stimulation of ALP.[37] More recently, Xiao et al. reported that, in MC3T3-E1 cells, the Erk pathway is essential for the regulation of Cbfa1, a transcription factor involved in osteoblast differentiation, inferring from these results that the Erk pathway may play an important role in the establishment of the osteoblastic phenotype.[40] Our data indicate that the selective inhibition of Erk activity in early-differentiating MC3T3-E1 cells had no major effect on expression of the main markers of osteoblastic cell differentiation, namely ALP, Coll I, and OC. If anything, it slightly enhanced the expression of ALP and Coll I and induced a small and transient reduction in the production of osteocalcin in early differentiating cells (Figure 3D). From this observation, we conclude that Erk is not essential for expression of the three well-characterized differentiation markers in MC3T3-E1 cells. However, because Erk is activated by serum factors throughout the differentiation of MC3T3-E1 cells, this MAPK pathway is likely to play some yet unknown role(s) during osteoblast maturation. For instance, it has been described recently that Erk is involved in the synthesis of interleukin-6 induced by either prostaglandin $F_{2\alpha}$[38] or endothelin-1,[13] suggesting that Erk could play a role in mediating osteoblast-osteoclast interaction.

Recent information from our laboratory has suggested that the p38 pathway is involved in the differentiation of osteoblastic cells. Indeed, we observed that p38 mediates the stimulation of ALP activity induced by an agonist of Gi-protein-coupled receptors in MC3T3-E1 cells.[34,35] Results from the present study suggest that p38 controls the expression of ALP in differentiating MC3T3-E1 cells, as indicated by the good correlation between the inhibition of ALP expression and that of p38 activity by various doses of SB203580. Whether this signaling pathway plays any significant role in controlling the expression of ALP in vivo remains to be documented. In MC3T3-E1 cells, inhibition of p38 activity was associated with a slight increase in their proliferation, indicating that the p38 pathway is probably not involved in mediating the growth arrest that precedes the onset of preosteoblastic cell differentiation. These results further suggest that the actions of the Erk and p38 pathways do not overlap. Whether p38 also influences the expression of osteoblastic differentiation markers other than ALP remains unclear. At high doses (10–30 μmol/L) of SB203580, the p38 activity was almost completely inhibited and this effect was associated with a significant reduction in Coll I and OC expression. However, because these doses of SB203580 also inhibited JNK activity, confirming a previous report that this inhibitor can also affect

some particular JNKs,[19] it is difficult to assess which of these two MAPKs regulates Coll I and OC expression during osteoblastic cell differentiation. In favor of a role for JNK, we found a relatively good correlation between inhibition of either Coll I deposition or OC secretion and inhibition of JNK activity by high doses of SB203580, suggesting that JNK is likely to also participate in the control of osteoblastic cell differentiation. This hypothesis, however, could not be investigated because of a lack of reagent for this MAP kinase.

In conclusion, the results of the present study indicate that MAPKs are essential mediators of MC3T3-E1 osteoblastic cell differentiation induced by serum growth factors. The Erk pathway is activated throughout the various development stages and is critical for cell proliferation. In addition, Erk probably plays other, presently undefined roles in osteoblastic cells. The p38 pathway is activated when cells start to differentiate and it controls ALP expression. Whether this MAPK also mediates the regulation of other osteoblastic markers remains to be further investigated. Finally, the JNK pathway is activated by growth factors throughout osteoblastic cell development and its activity is highest during differentiation, but its functional role also requires further investigation. Altogether, our observations suggest that the stimulation of proliferation and differentiation of osteoblastic cells by serum growth factors involves the coordinate activation of several MAPKs that have specific and distinct roles in the regulation of these cellular processes.

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