Journal of Cellular Biochemistry 82:68–77 (2001)

Inhibition of p38MAP Kinase Potentiates the JNK/SAPK Pathway and AP-1 Activity in Monocytic but not in Macrophage or Granulocytic Differentiation of HL60 Cells

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Abstract Monocytic differentiation of HL60 cells induced by 1,25-dihydroxyvitamin D_3 (1,25 D_3) has been recently shown (Exp Cell Res 258, 425, 2000) to be enhanced by an exposure to SB203580 or to SB202190, specific inhibitors of p38MAP kinase, with concomitant up-regulation of the c-jun N terminal kinase (JNK) pathway. In the present study we inquired if this enhancement and the JNK up-regulation are limited to 1,25 D_3-induced differentiation, or if they occur more generally in HL60 cell differentiation. We found that dimethylsulfoxide (DMSO)-induced differentiation, and to a lesser extent tetradecanoylphorbol acetate (TPA)-induced macrophage differentiation were also potentiated by the p38MAPK inhibitors, but that granulocytic differentiation in response to all-trans retinoic acid (RA) was not. The enhancement of differentiation by p38MAPK inhibitors was accompanied by an activation of the JNK MAPK pathway, as shown by the phosphorylation levels of these kinases and by AP-1 binding, but only in 1,25 D_3-treated cells. This shows that an up-regulation of the JNK stress pathway during 1,25 D_3-induced monocytic differentiation occurs selectively in this lineage of differentiation and is not necessary for the expression of the differentiated phenotype. J. Cell. Biochem. 82: 68–77, 2001.

Key words: 1,25-dihydroxyvitamin D_3; retinoic acid; DMSO; TPA; MAP kinase pathways; differentiation

Human leukemia cells placed in culture survive and proliferate for a time, but usually cannot be maintained indefinitely [Visonneau et al., 1995]. Occasionally, however, a permanent cell line is established, and provides a source of cells for studies of cell and molecular biology of human leukemia. One such example is the HL60 cell line, a myeloblastic leukemia with promyelocytic features [Gallagher et al., 1979], which grows very well in culture without supplementation by exogenous hematopoietic growth factors, possibly because of multiple mutations that include amplification of the c-*myc* proto oncogene [Dalla-Favera et al., 1982], an activating N-ras mutation [Murray et al., 1983], and a deletion of the p53 tumor suppressor gene [Wolf and Rotter, 1985]. Remarkably, HL60 cells can be induced in vitro to differentiate towards several mature myeloid forms; the granulocyte [Collins et al., 1979], the macrophage [Rovera et al., 1979], and the monocyte [Tanaka et al., 1983; McCarthy et al., 1983]. This allows detailed studies of the mechanistic basis for each differentiation lineage, but although many such studies have been reported, the precise sequences of events that result in lineage phenotype have not been elucidated.

Recently, attention has been directed to the role of mitogen and stress activated kinase pathways (MAPKs and SAPKs) in HL60 cell differentiation. Yen et al. [1998, 1999] have reported that retinoic acid, which induces the granulocytic form of differentiation, activates the extracellular signal

Grant sponsor: NIH grant from the National Cancer Institute; Grant number: R01-CA 44722.

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Received 30 November 2000; Accepted 16 January 2001

activated kinase 2 (ERK2), but not the c-jun N-terminal kinase (JNK), or the p38MAP kinase. In contrast, we found that ERK2 has only a transient role in 1,25 D_3-induced monocytic differentiation [Wang and Studzinski, 2001], but that activation of the JNK/c-jun pathway is associated with monocytic differentiation induced by a low concentration of 1,25 D_3 [Wang et al., 2000]. Surprisingly, this enhancement of JNK/c-jun activity resulted from the inhibition of the other arm of the stress-activated pathways, the p38MAPK pathway, by its inhibitors SB203580 (SB203) and SB202190 (SB202).

We now extend these studies and demonstrate that although TPA-induced macrophage differentiation, and DMSO-induced "mixed phenotype" differentiation of HL60 cells are enhanced by a simultaneous exposure to SB203 or SB202, this is not associated with a synergistic upregulation of the JNK/c-jun pathway. Further, these inhibitors of p38MAPK did not enhance the granulocytic phenotype resulting from an exposure to RA nor upregulated the JNK/c-jun pathway in RA-treated cells, even though the upstream components of p38 pathway are upregulated under these conditions. The synergistic upregulation of JNK pathway thus appears to be specific for 1,25 D_3-induced differentiation of HL60 cells.

MATERIALS AND METHODS

Chemicals and Antibodies

We obtained 1,25 D_3 as a gift from Dr. Milan Uskokovic, (Hoffmann LaRoche, Nutley, NJ). A stock solution was prepared by dissolving it in absolute ethanol at 2.5×10^{-4} M. All-trans retinoic acid (RA), phorbol-12-myristate-13-acetate or tetradecanoylphorbol acetate (TPA), and dimethyl sulfoxide (DMSO) were purchased from Sigma. The p38 kinase inhibitors SB203 and SB202 were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). The antibodies to p38 (A-12, mouse monoclonal), CRK-L (c-20, rabbit polyclonal), c-fos (k-25, rabbit polyclonal) JNK1/2 (FL, rabbit polyclonal), c-jun (D-11, mouse monoclonal), and p90RSK (c-21, rabbit polyclonal) were purchased from Santa Cruz Co. (Santa Cruz, CA). Antibodies used to detect phospho-p90RSK (Ser 381), phospho-MKK3/MKK6 (Ser 189/207), phospho-p38MAPK (Thr180/Tyr182), phospho-JNK (Thr 183/Tyr 185) and phospho-c-jun (Ser 63), all rabbit polyclonal antibodies, were purchased from New England Bio Labs (Beverly, MA).

Tissue Culture

HL60-G cells [Wang et al., 1998], a subclone of human promyeloblastic leukemia HL60 cells [Gallagher et al., 1979], were grown at 37°C in suspension culture in RPMI 1640 medium (Mediatech, Washington, DC) supplemented with 1% glutamine and 10% heat-inactivated, iron-enriched bovine calf serum (Hyclone, Logan, UT). The cultures were passaged and fed 2–3 times weekly to maintain log phase growth. For cell proliferation experiments, HL60 cells were suspended at 1.5×10^5 cells/ml, while for all other experiments they were seeded at 3×10^5 cells/ml of fresh medium, at which time the 1,25 D_3 RA, TPA, and DMSO were added to obtain the concentrations indicated in he individual experiments. The p38MAPK inhibitors SB203580 or SB202190 were added to the cultures for the indicated times at the specified concentrations 1 h before the exposure to 1,25 D_3. Cell viability was determined using trypan blue (0.25%) exclusion, and cell numbers by counting cells in a Neubauer hemocytometer. Each experiment was repeated at least four times.

Determination of Markers of Differentiation

Aliquots of 1×10^6 cells were harvested at indicated times, washed twice with PBS, then incubated for 45 min at room temperature with 0.5 μl MY4-RD-1 and 0.5 μl MO1-FITC (Coulter, Miami, FL) each containing 0.5 μg of the antibody, to analyze the expression of surface cell markers CD14 and CD11b, respectively. The cells were then washed three times with ice cold 1X PBS, and resuspended in 0.5 ml 1X PBS. Two parameter analysis was performed using an Epics Profile II instrument (Coulter Electronics, Hialeah, FL). Isotypic mouse IgG1 was used to set threshold parameters.

Monocytic differentiation was also monitored by cytochemical determination of the cytoplasmic enzyme monocytic serine esterase (MSE) activity, also referred to as NSE, as described previously [Wang et al., 1997], and the general myeloid functional capability by measuring the proportion of cells exhibiting the respiratory burst as shown by the nitro-blue tetrazolium (NBT) reaction following stimulation with 200 ng/ml of TPA, following the previously described procedure [Studzinski et al., 1985].

Cell Cycle Distribution

Aliquots of 1×10^6 cells were washed twice with ice-cold 1X PBS and the cells were fixed in 75% ethanol at -20°C overnight, washed twice with PBS, and incubated with 100 U/ml of RNase (BMB, Indianapolis, IN) at 37°C for 1 h. The cell pellet was again washed twice with PBS and resuspended in 1 ml propidium iodide solution (PI, 10 μg/ml, Sigma). The cells were analyzed using an Epics Profile II instrument (Coulter), and cell cycle distribution was determined by Multicycle Software Program (Phoenix Flow System, San Diego, CA). Debris, doublets, and aggregates were gated out using a bit map surrounding the dispersed cell population [Studzinski et al., 1996].

Cell Extracts and Western Blotting

Western blotting was performed using whole cell extracts which were prepared by homogenizing frozen cell pellets in an extraction buffer (20 mM Tris-HCl, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA (pH 7.5), 1 mM PMSF and 0.02% leupeptin) and mixed with an equal amount of 3X SDS sample buffer (150 mM Tris, 30% glycerol, 3% SDS, 1.5 mg/ml bromophenol blue dye, 100 mM DTT). The proteins in 40 μg of whole cell extracts were separated using a 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech Inc, Piscataway, NJ). The membranes were blocked with 5% milk in TBS/0.01% Tween 20 for 1 h, blotted with the specified primary antibodies for 1 h, and then with a horseradish-linked secondary antibody for 1 h. The protein bands were visualized using a chemoluminescence assay system (Amersham). The optical density (OD) of each band was quantitated using an image quantitator (Molecular Dynamics, Sunnyvale, CS). The blots were stripped according to the manufacturer's protocol (Amersham) and successively reprobed for up to four times with different antibodies and finally for the constitutively present Crk-L protein.

Preparation of Nuclear Extract

Nuclear extracts were prepared by the procedure described before [Andrews and Faller, 1991]. Briefly, 2×10^7 cells were harvested and washed twice with ice-cold PBS, and resuspended in 0.2 ml ice-cold cell extract buffer (10 mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KC1, 0.5 mM dithiothreitol (DDT), and 0.2 mM phenylmethysulfonyl fluoride (PMSF). The cells were kept on ice for 10 min to allow them to swell, vortexed for 10 sec, and microfuged at 4°C at 16,000*g* for 30 sec. The supernatant was saved as the cytoplasmic extract, and the pellet was resuspended in 30 μl of nuclear extraction buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgC1_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF), placed on ice for 20 min, and centrifuged at 4°C at 16,000*g* for 2 min. The supernatant was saved as the nuclear extract and stored at -80°C.

Gel Mobility Shift Assay

The gel mobility shift assay was performed by using Stratagene Gelshift Assay Kit (La Jolla, CA). The AP-1 oligonucleotide 5′-CGCTTGATGAGTCAGCCGGAA-3′ (Santa Cruz, Santa Cruz, CA) was 5′ -end labeled by phosphorylation with T4 polynucleotide kinase in the presence of γ^{32}P-ATP (Amersham). Five μg of nuclear extract and 20 pg of labeled AP1 probe were incubated for 30 min at room temperature in a binding buffer (1 μg poly(dI-dC), 20 mM Hepes, pH 7.9, 5 mM $MgC1_2$, 50 mM KC1, 1 mM DTT, 0.25 mg/ml BSA, 4% glycerol), then the samples were subjected to electrophoresis using a 4% polyacrylamide gel with a constant current of 22 mA for 3 h. The gel was dried and exposed to Kodak film.

Statistical Methods

All experiments were repeated at least four times. Differences between means of various subgroups was assessed by two-tailed Student's *t*-test. The computations were performed with an IBM-compatible personal computer using Microsoft EXCEL program.

RESULTS

Exposure of HL60 Cells to p38 Inhibitor SB203 Results in Activation of an Upstream Regulator of p38MAPK Pathway

To determine if the previously described [Wang et al., 2000] effects of the inhibitors of p38MAPK pathway on its upstream components are modified by a simultaneous exposure to diverse differentiation agents, we exposed HL60 cells to 1,25 D_3, RA, TPA, and DMSO for 96 h in the presence or absence of SB203. This time period was chosen to allow the expression of the differentiated phenotype in cells treated

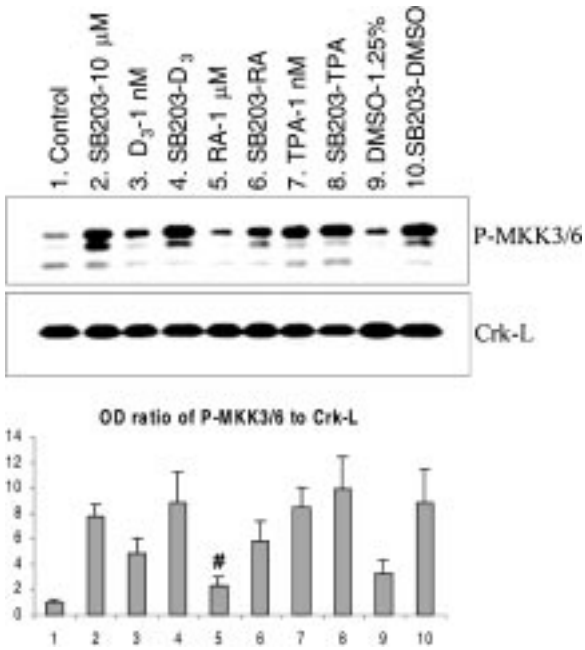

Fig. 1. Upregulation of the MKK3/6, the upstream regulator of p38MAPK, by SB203, in the absence or presence of inducers of differentiation of HL60 cells. The immunoblot shown in the upper panel was stripped and reprobed for a constitutively expressed protein, Crk-L. Control = untreated HL60 cells. Optical densities (OD) of the protein levels were determined by Flourimage with Image Quant Software (Molecular Dynamics, Sunnyvale, CA). The ratios of optical density (OD) of phospho-MKK3/6 to Crk-L were plotted for the different treatment groups. Means ± SEM are shown, n=3. # = not significantly different from control ($P > 0.05$), all other groups were significantly different from the control.

with RA and DMSO, which induce differentiation slowly, and in cells treated with low (1 nM) concentrations of 1,25 D_3 and TPA. Figure 1 shows that when SB203 was added to the medium alone or together with the differentiation agents the extracts of the treated cells contained increased levels of the phosphorylated form of MKK3/6, a kinase upstream of p38 in this arm of the MAPK pathway [Enslen et al., 1998]. The P-MKK3/6 levels were similar in all SB203-containing groups, showing that while SB203 inhibits p38 kinase activity, as previously shown in these cells [Wang et al., 2001], it also activates P-MKK3/6 irrespective of the differentiation status of the cells. It can also be noted that treatment of HL60 cells with 1,25 D_3, TPA, or DMSO alone also induced P-MKK3/6 (Fig. 1), suggesting that unlike RA these inducers also activate the upstream components of the p38MAPK pathway.

Effects of Inhibition of p38 Kinase on Cell Cycle and Viability of Differentiating HL60 Cells

Addition of SB203 to undifferentiated HL60 cells had little effect on cell cycle traverse and survival, as shown by the proportions of cells in G1 phase and the S phase compartments, and by trypan blue permeability (Table I). It has been previously reported that induction of differentiation in HL60 cells by 1,25 D_3 results in delayed effects on cell cycle and cell proliferation [Studzinski et al., 1987; Zhang et al., 1994b]. Similarly, in the experiments described here, cell proliferation continued during the first 96 h of exposure to 1,25 D_3 (data not shown), though at this time a G1 to S phase block was beginning to be observed in 1,25 D_3-treated cells, which was potentiated by the presence of SB203 (Table I). Interestingly, addition of SB203 to the inducers of differentiation RA or TPA had little effect on the cell cycle parameters, exemplified by cells in the G1 and S phase compartments shown in Table I, but potentiated the effects on the cell cycle produced by 1,25 D_3 and DMSO (Table I).

TABLE I. Effect of the p38 Inhibitor SB203580 and/or Differentiation Agents on Cell Viability, G1 Phase, and S Phase

	Viability (%)	G1 phase (%)	S phase (%)
Control	95.0 ± 2.8	55.2 ± 0.9	41.3 ± 1.0
SB203-10 μM	93.0 ± 1.4	57.5 ± 2.6	40.0 ± 1.0
D_3-1 nM	94.0 ± 2.8	59.7 ± 1.3	35.2 ± 4.1
SB203-D3	95.5 ± 1.7	71.5 ± 6.6	23.0 ± 7.0
RA-1 μM	93.5 ± 0.7	75.5 ± 5.8	19.7 ± 5.4
SB203-RA	92.5 ± 0.7	74.5 ± 4.1	21.3 ± 2.6
TPA-1 nM	90.0 ± 1.4	87.1 ± 3.0	7.7 ± 4.0
SB203-TPA	91.0 ± 2.8	85.3 ± 7.0	9.3 ± 6.8
DMSO-1.25%	92.0 ± 2.8	70.4 ± 6.6	22.7 ± 4.0
SB203-DMSO	92.0 ± 1.4	87.1 ± 1.3	8.7 ± 0.1

The cells were seeded at 150 k cells/ml and exposed to the compounds at indicated concentrations for 96 h. Control = untreated HL60 cells.

Effects of SB203 on Surface Markers of Differentiation

Treatment of HL60 cells with SB203 and/or inducers of differentiation under conditions described in Table I showed that the effects of 1,25 D_3 and DMSO on differentiation markers CD14 and CD11b were potentiated by this p38MAPK inhibitor, but the effects of RA were not significantly affected (Fig. 2, and data not shown). Since 1 nM 1,25 D_3 alone induced CD14 expression in most cells, the potentiating effect of SB203 can be observed by the increased intensity of CD14 expression (the clustering of cells at the upper register of the vertical axis), and by the increase in the proportion of CD11b positive cells (Fig. 2). In cells treated with TPA SB203 increased the expression of CD11b, but not the expression of CD14 (Fig. 2).

Since it has been reported that at concentrations higher than 3 μM SB203 inhibits activity of the kinase PDK1 [Lali et al., 2000; Rane et al., 2000], we tested the effects of lower concentrations of SB203 on the potentiation of differentiation by DMSO. As previously shown for 1,25 D_3 [Wang et al., 2000], DMSO-induced differentiation was increased by SB203 at concentrations lower than 3 μM (Table II). This suggests that inhibition by SB203 of p38MAP kinase potentiates DMSO-induced differentiation.

Comparison of the Effects of SB203 on Myelo-Monocytic Markers and JNK Pathway

The NBT reaction, a measure of superoxide production used as a general marker of phagocytic phenotype [Hua et al., 2000], was enhanced by all four inducers of differentiation studied here (Fig. 3). This increase was potentiated by simultaneous exposure to SB203 and 1,25 D_3, TPA or DMSO, but not by an exposure to RA. However, only 1,25 D_3-SB203 combination enhanced activation of JNK 1/2, as shown by its phosphorylation status (Fig. 3).

The MSE reaction is considered to be a specific marker of monocytic phenotype [Yam et al., 1971]. As shown in Figure 4, 1,25 D_3 and TPA increased the expression of this marker. In addition, DMSO, originally described an as inducer of granulocytic differentiation [Collins et al., 1979], also induced this marker (Fig. 4). Co-administration of SB203 and 1,25 D_3 or DMSO markedly enhanced the expression of MSE, but again only SB203-1,25 D_3 combination enhanced the activation of the JNK/c-jun pathway, shown in this instance by phosphorylation of c-jun (Fig. 4). In contrast, activation of another component of signal transducing cascades, p90RSK, was not enhanced by SB203. Thus, in the context of these experiments activation of the JNK pathway is specific for 1,25 D_3-SB203 combination, while TPA or DMSO-induced differentiation is phenotypically enhanced by SB203, but the JNK pathway is not enhanced.

An incidental finding in this experiment was the increase in phosphorylation of c-jun in cells treated with TPA alone (Fig. 4, lane 7). Since no activation of JNK1/2 was observed in these cells (Fig. 3, lane 7), this suggests that another c-jun phosphorylating kinase is activated by TPA. It is not clear if this activation is related to the activation of MKK3/6 noted in Figure 1.

Enhancement of c-jun Activation and AP-1 Binding by SB202 in 1,25 D_3-Induced Differentiation

SB202 is a more potent inhibitor of p38MAPK activity than SB203 [Cuenda et al., 1995; Frantz et al., 1998], so it was of interest to determine if this compound increases the activation of the JNK/c-jun pathway during differentiation of HL60 cells induced by compounds other than 1,25 D_3. As shown above for treatment with SB203 (Fig. 1), exposure of undifferentiated and differentiated cells to the more potent SB202 also activated the upstream components of the p38 MAP kinase pathway, demonstrated in this case by the levels of phosphorylated p38 (Fig. 5). Also similar was the finding that inducers of differentiation other than RA also modestly upregulated this pathway in the absence of SB202 (Fig. 5). It can also be observed in Figure 5 that the effect of SB202 on c-jun was similar to the effect of SB203 shown in Figure 4, and the presence of SB202 produced little or no change in the levels of two members of the fos family, c-Fos and Fra-1, while Fos-B levels were markedly increased by the addition of SB202 together with 1,25 D_3. Further examination of this system for binding of AP-1 transcription factors to the AP-1 (or TRE) DNA element showed that only 1,25 D_3 synergises with SB202 to increase AP-1 DNA binding (Fig. 6), an evidence of AP1 activation. Thus, inhibition of the p38MAPK pathway has a significance for 1,25 D_3-induced differentiation not shared with the other inducers.

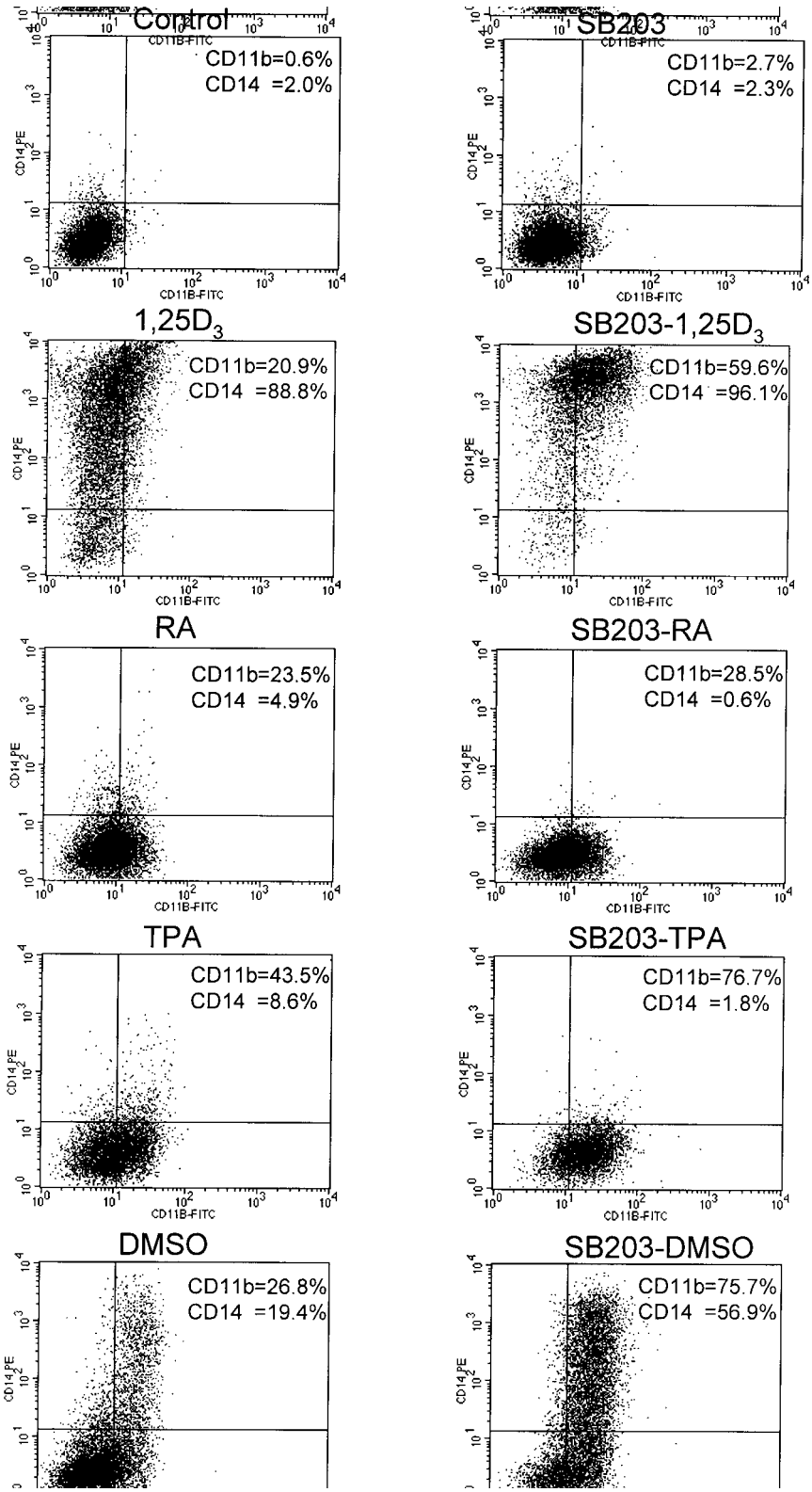

Fig. 2. The effect of SB203 on the cell cycle phenotype of HL60 cells induced to differentiate by several compounds. The inducers of differentiation were used in concentrations and under conditions described in Table I, and markers of differentiation were determined by flow cytometry. Note the potentiation by SB203 of differentiation induced by 1,25 D_3, TPA and DMSO, as shown by the values shown as insets in each panel, and by the intensity of fluorescence, particularly along the y axis. The slight increase in SB203-induced expression of CD11b in RA-treated cells was not found to be significant in subsequent experiments, which were repeated three times. Control = untreated HL60 cells.

TABLE II. Effect of Different Concentrations of p38 Inhibitors on 1,25D_3-Induced Differentiation

	CD11b %+	CD14 %+	MSE %+
Control-96h	2.1 ± 0.7	0.6 ± 0.2	0.7 ± 0.5
DMSO	31.1 ± 6.9	18.2 ± 5.0	4.0 ± 1.4
0.6 μM-SB203-DMSO	56.4 ± 4.9	21.2 ± 0.9	4.5 ± 0.7
1.2 μM-SB203-DMSO	72.0 ± 4.4	29.4 ± 2.4	8.0 ± 1.7
2.4 μM-SB203-DMSO	73.3 ± 2.5	31.4 ± 0.5	11.0 ± 2.8
5.0 μM-SB203-DMSO	80.8 ± 2.0	39.3 ± 0.7	14.5 ± 2.4
0.3 μM-SB202-DMSO	64.2 ± 6.1	24.7 ± 1.7	6.0 ± 1.4
1.2 μM-SB202-DMSO	73.6 ± 2.3	33.1 ± 3.7	9.0 ± 4.2
2.4 μM-SB202-DMSO	75.6 ± 1.8	34.1 ± 4.6	14.0 ± 2.7
5.0 μM-SB202-DMSO	85.7 ± 2.8	45.6 ± 2.7	19.5 ± 2.4
1,25D_3	54.0 ± 7.2	53.6 ± 1.2	21.0 ± 3.7

A constant concentration (1.25%) of DMSO was added into all groups except the control. Cells treated with 1 nM 1,25 D_3 were used as the positive control. Control = untreated HL60 cells. The values represent the mean ± SEM (n = 3).

DISCUSSION

The data presented here show that in addition to 1,25 D_3, which induces a monocytoid phenotype in HL60 cells [McCarthy et al., 1983; Tanaka et al., 1983; Studzinski et al., 1985], TPA, which induces macrophage-like phenotype, [Rovera et al., 1979] is a more effective inducer of differentiation in the presence of compounds SB203 or SB202, the inhibitors of p38MAP kinase, than in their absence. In contrast, the RA-induced granulocytic phenotype of HL60-G cells is clearly unaffected by the presence of SB203 or SB202, while differentiation by another compound which induces gran-

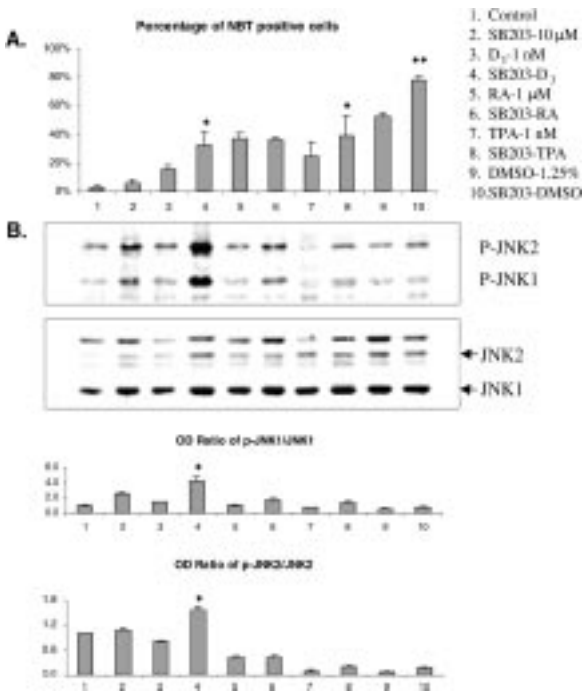

Fig. 3. Effects of 96 h treatment with SB203 on **(A)** differentiation of HL60 cells, shown by the NBT reaction, and **(B)**, upregulation of JNK1/2, shown by immunoblotting for JNK1/2 levels of expression and their activation by phosphorylation. Note that whereas SB203 potentiates both the NBT reaction and JNK activation in 1,25 D_3-treated cells, it has neither of these effects in RA-treated cells, and potentiates the NBT positivity but not JNK activation in TPA or DMSO-treated cells. In (A) and (B) * indicates significant difference at $P < 0.05$ level between SB203-treated and untreated pairs, ** indicates $P < 0.01$ (n = 4). In (B) equal loading of the gel was shown by stripping and reblotting as illustrated in Figure 4. Control = untreated HL60 cells.

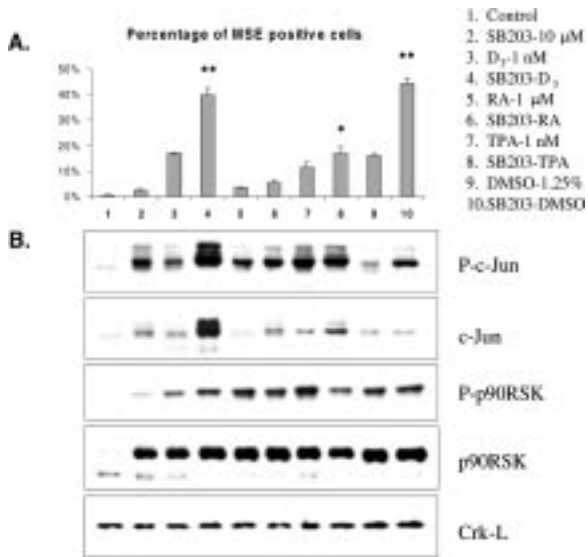

Fig. 4. Effects of SB203 on **(A)** the monocytic marker MSE also known as NSE, and **(B)** on the expression and activation of c-jun and the kinase p90RSK. These data were obtained as part of the experiments shown in Figure 3, and the same cell extracts were successively probed, and finally probed for Crk-L, a protein constitutively expressed in HL60 cells. Note that as in Figure 3, activation of the JNK/c-jun pathway parallels 1,25 D_3-induced differentiation, but not differentiation induced by RA, TPA, or DMSO. An incidental finding was activation of JNK1/2 by TPA alone. Control = untreated HL60 cells. *Significance as designated in the legend to Figure 3.

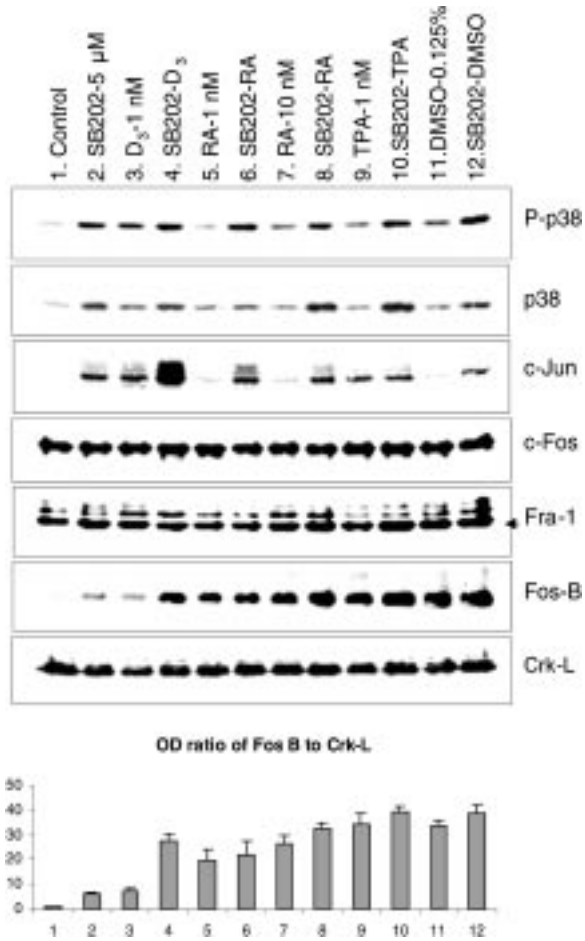

Fig. 5. Increased phosphorylation of p38MAPK induced by another inhibitor, SB202, in the absence or presence of inducers of differentiation, and its effects on c-jun and fos family of proteins. Similar to SB203, this inhibitor of p38MAPK activity potentiates 1,25 D_3-induced upregulation of c-jun, but not differentiation induced by RA, TPA, or DMSO. The c-Fos and Fra-1 proteins were unaffected by experimental manipulations, while the increases in Fos-B levels were synergistic between SB202 and 1,25 D_3. Control = untreated HL60 cells. Protein levels of Crk-L served as loading controls. The ratios of optical density of Fos B to Crk-L were determined, and means ± SEM, n = 3, are shown.

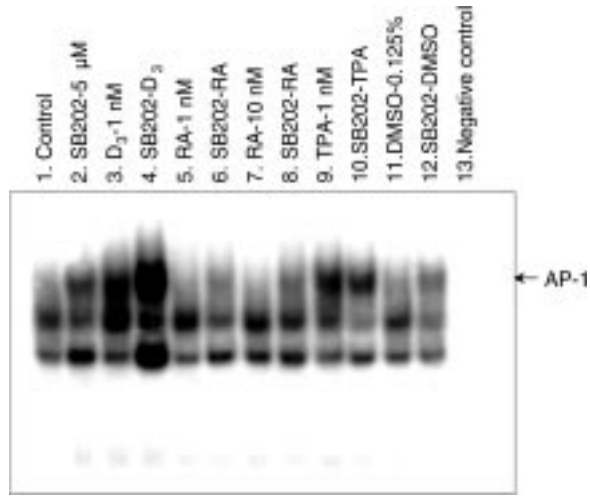

Fig. 6. Electrophoretic mobility shift assay for binding of the AP-1 transcription factor to its cognate TRE DNA element. The intensity of AP-1 binding approximately parallels the levels of phospho-c-jun shown in Figure 4B, and demonstrates that SB202 potentiates activation of JNK/c-jun pathway by 1,25 D_3, but not by RA, TPA, or DMSO. Control = untreated HL60 cells. Negative control=-labeled AP-1 oligonucleotide only, no nuclear extract.

ulocytic phenotype, DMSO, was markedly enhanced by the p38MAPK inhibitors.

However, although DMSO was originally described as an inducer of granulocytic differentiation of HL60 cells on morphological grounds [Collins et al., 1979], it has been observed that HL60 cells exposed to DMSO undergo a defective form of neutrophil maturation, in that there is a coordinate failure of expression of secondary granule proteins [Khanna-Gupta et al., 1994]. This suggests that DMSO-induced phenotype is atypical rather than representative of normal granulocytic lineage, consistent with data shown here in Figures 2–4. These figures show that DMSO induces the expression of CD14 and MSE markers, which are characteristic of the monocyte phenotype [Yam et al., 1971; Ziegler-Heitbrock and Ulevitch, 1993; Zhang et al., 1994a], as well as a more robust expression of the general myeloid markers CD11b and NBT [Babior et al., 1975; Socinski et al., 1988], indicating a mixed monocyte–granulocyte phenotype. Thus, the present studies reveal that inhibitors of p38MAPK activity enhance monocytic, macrophage, and granulocytic differentiation with monocytic features, but not pure granulocytic differentiation of HL60 cells.

All three principal MAPK cascades, the ERK, JNK, and p38MAPK pathways have been implicated in differentiation of mammalian cells. Several previous studies concluded that differentiation is regulated by the p38MAPK signal transduction pathway. For example, SB203 blocks fusion of myoblasts to multinucleated myotubules in skeletal muscle differentiation of L8 cells [Zetser et al., 1999], and abolishes induction of hemoglobin expression by butyrate in K562 leukemia cells [Witt et al., 2000], while another inhibitor of p38MAPK, L-790,070 prevents monocyte chemotaxis [Ayala

et al., 2000]. However, it should be noted that the effects of the p38MAPK inhibitors in these studies of differentiation were attributed solely to specific inhibition of the activity of p38MAPK pathway. The data presented in the present study, and in previous reports [Birkenkamp et al., 2000; Lali et al., 2000; Wang et al., 2000], indicate that the inhibitors elicit activation of JNK and ERK MAPK pathways, suggesting that collateral effects are responsible for the alterations in the differentiation process by MAPK inhibitors. A possible explanation for these collateral effects is provided by the recent finding that in 3T3 cells MK2 and PRAK, downstream effectors of p38MAP kinase pathway, provide negative feedback to ras signaling through JNK [Chen et al., 2000]. Thus, abrogation by SB203 and SB202 of this negative feedback can explain the JNK activation by the SB compounds observed in our experiments, though the participation of ras in signaling of differentiation-related events remains to be demonstrated.

The involvement of MAPK cascades in differentiation shows clear cell specificity. For instance, in contrast to the studies mentioned above [Zetser et al., 1999; Ayala et al., 2000; Witt et al., 2000], inactivation of p38MAPK is required for differentiation of immature thymocytes to $CD4^+/CD8^+$ thymocytes [Diehl et al., 2000]. Another example is the requirement for JNK1 for differentiation of T helper (Th) cells to Th1 effector cells [Dong et al., 1998]. Additionally, we show here that the involvement of MAPK cascades in differentiation is inducer-specific, as 1,25 D_3, but not TPA-induced differentiation, was accompanied by activation of JNK MAP kinase (Fig. 4).

Thus, these studies demonstrate that there are profound differences between mechanisms of 1,25 D_3 and TPA-induced differentiation, and that while the monocytic and macrophage phenotypes are enhanced by p38MAPK inhibitors, only the pure monocytic differentiation induced by 1,25 D_3 is associated with upregulation of the JNK/c-jun pathway. It is also clear that neither monocytic nor granulocytic differentiation of HL60 cells requires the activity of the p38MAP kinase.

The delineation of similarities and differences in mechanisms of differentiation should aid further work to understand the nature of blocks to differentiation that result in development of leukemia.

ACKNOWLEDGMENTS

We are grateful to Dr. Milan Uskokovic for the gift of 1,25 D_3, and to Dr. Jie Rao for assistance with experimental work.

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