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Role of c-Jun N-Terminal Kinase/p38 Stress Signaling in 1-β-D-Arabinofuranosylcytosine-Induced Apoptosis

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ABSTRACT. 1-β-D-Arabinofuranosylcytosine (ara-C) induced apoptosis in HL-60 cells, which was preceded by the activation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 mitogen-activated protein kinase (MAPK). 2′-Amino-3′-methoxyflavone (PD098059) and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) were used to inhibit the activity of ERK and p38, respectively. SEK-AL, a dominant-negative mutant of SEK1, was transfected into HL-60 cells (HL-60/SEK-AL) to assess the role of JNK/SAPK activity in apoptosis. PD098059 (25 μM) inhibited ara-C-induced caspase-3-like activity but was ineffective in altering ara-C-mediated apoptotic DNA fragmentation and clonogenicity. On the other hand, SB203580 (20 μM) inhibited ara-C-induced caspase-3-like activity, apoptotic DNA fragmentation, and clonogenicity. The inhibition of JNK1 activation in HL-60/SEK-AL cells did not block ara-C-induced apoptotic DNA fragmentation. These results suggest that ara-C-induced apoptotic DNA fragmentation and loss of clonogenicity occur through a p38-dependent pathway. BIOCHEM PHARMACOL **59**;4:407–418, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. p38; JNK; SAPK; ERK; 1-β-D-arabinofuranosylcytosine; apoptosis; PD098059; SB203580

The deoxycytidine analog ara-C† is one of the most effective agents in the treatment of acute myelogenous leukemia [1]. Whereas intracellular metabolic conversion of ara-C into ara-CTP causes DNA chain termination and DNA strand breaks [2, 3], an alternate target for ara-C involves modulation of lipid metabolism. Specifically, ara-C increases the levels of the lipid second messengers ceramide and diglyceride [4]. The latter molecule is formed through the reversal of cholinephosphotransferase [5], whereas the former can accumulate through the degradation of sphingomyelin by either neutral or acidic sphingomyelinase [6].

Ara-C promotes both cell death and differentiation in leukemia cells [7]. However, the precise signaling mechanisms that regulate these processes remain largely unknown. Recently, attention has focused on the role of MAPK signaling in coordinating such physiological events as proliferation, differentiation, and apoptosis. Currently, three MAPK signaling modules have been described: ERK, JNK/SAPK, and p38.

Each MAPK family member is controlled by an upstream dual specificity MAP kinase kinase that requires initial activation by an upstream MAP kinase kinase kinase. Thus, the Raf→MEK→ERK signaling cascade has been attributed to cellular proliferation and differentiation [8], whereas the MEKK1→MKK4/SEK1→JNK/SAPK module is believed to be important for the induction of apoptosis [9]. The third MAPK, p38, is activated by MKK3, MKK6, and in some systems MKK4/SEK1 [10, 11]. The physiological effects of p38 are debatable, however, since different reports suggest p38 to be either non- or pro-apoptotic [12–20]. Moreover, many stimuli that activate the JNK/SAPK signaling cascade appear to activate p38 also.

Previous studies report that high concentrations of ara-C induce both JNK/SAPK and p38 activities [21, 22]. Here, we studied the effects of ara-C on the activities of ERK, JNK/SAPK, and p38. We found that JNK/SAPK, and p38 to a lesser degree, were activated at time points that preceded the appearance of internucleosomal DNA fragmentation in ara-C-treated HL-60 cells. Inhibition of p38 blocked DNA fragmentation and inhibited caspase-3-like activity induced by ara-C that correlated with an increase in ERK activation. However, pharmacological inhibition of the ERK pathway did not restore apoptosis in cells treated with ara-C and SB203580. These results suggest that ara-C-induced apoptosis can be blocked by the specific inhibition of p38, whereas ERK activation does not appear to be responsible for the anti-apoptotic effects of p38 inhibition.

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† *Abbreviations*: ara-C, 1-β-D-arabinofuranosylcytosine; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; SEK, SAPK/ERK kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK/ERK kinase; ERK, extracellular signal-regulated kinase; MBP, myelin basic protein; PMSF, phenylmethylsulfonyl fluoride; FBS, fetal bovine serum; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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MATERIALS AND METHODS

Materials

Ara-C (Upjohn) was dissolved in PBS and stored as a 10 mM stock solution. 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) and 2′-amino-3′-methoxyflavone (PD098059) (Calbiochem) were stored in DMSO as a 20 mM and a 25 mM stock solution, respectively. pcDNA/SEK-AL was the gift of Dr. J. R. Woodgett. Zeocin was purchased from Invitrogen. Agarose-conjugated polyclonal antibodies to JNK1 (C-17), p38 (N-20), and ERK2 (C-14) as well as GST-c-Jun (1–79), ATF-2, anti-JNK1 (C-17), and anti-p38 (C-14) were from Santa Cruz Biotechnology. Anti-hemagglutinin (12CA5) was from Boehringer Mannheim. MBP was purchased from Life Technologies. [γ-^{32}P]ATP (>4500 Ci/mmol) was obtained from ICN. All other reagents were obtained through the Sigma Chemical Co.

Cell Culture and Viability

HL-60 cells, a model of human promyelocytic leukemia, were grown in RPMI 1640 supplemented with 10% FBS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin G. Cell cultures were subcultured thrice weekly to maintain logarithmic growth. Cell viability as determined by trypan blue exclusion was > 95%.

Transfection of HL-60 Cells

HL-60 cells were electroporated as described previously [23], with minor modifications. Briefly, HL-60 cells (7×10^6) were washed in RPMI 1640 and electroporated with either purified pcDNA3.1/Zeo(+) (empty vector) or pcDNA3.1/SEK-AL. A Bio-Rad Gene Pulser was used for electroporation according to the following parameters: 500 mF, 300 V, and 0.75 kV/cm. Electroporated cells were allowed to recover for 48 hr in RPMI + 10% FBS, whereupon selection began with 500 μg/mL of Zeocin for 5 weeks. Selection-resistant cells were subcloned by soft agar clonogenicity and assayed for expression of the SEK-AL mutant protein.

Internucleosomal DNA Fragmentation Assay

The procedure for analyzing internucleosomal DNA fragmentation was performed as reported previously [24]. Briefly, HL-60 cells (2.5×10^6) were washed and resuspended in lysis buffer (10 mM Tris, 20 mM EDTA, 0.1% NP-40, pH 7.4) for 20 min at 25°, followed by incubation in the presence of RNase A (0.1 mg/mL) for 30 min at 37°. Proteinase K (0.5 mg/mL) then was added to the lysates for 16 hr at 55°. DNA was extracted by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the lysates, followed by ethanol precipitation. Purified DNA was resuspended in 10 mM Tris:1 mM EDTA (pH 8.0). Then DNA samples were fractionated by agarose gel electrophoresis using a 2% agarose gel in Tris:acetate:EDTA running buffer. Internucleosomal DNA fragmentation was visualized by ethidium bromide staining (0.5 μg/mL) and UV illumination. Each experiment was repeated at least two times with similar results.

Immunocomplex Kinase Assays for the MAPK

Cells (2.5×10^6) were washed twice in ice-cold PBS, pH 7.2, and then lysed in a buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM Na_2EDTA, 0.5 mM 2-mercaptoethanol, 0.5% NP-40, 1 mM PMSF, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, and 10 mM *p*-nitrophenol phosphate for 30 minutes at 4°. Subsequently, lysates were centrifuged (12,000 *g*, 10 min at 4°), and the supernatants were collected. To the supernatants, 5 μg of agarose-conjugated anti-JNK1, anti-ERK2, or anti-p38 polyclonal antibody was added in 200 μL of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris, pH 7.2, 1% deoxycholic acid, 1% Triton X-100, and 0.1% SDS) and incubated with rocking for 1 hr at 4°. Following two washes in radioimmunoprecipitation assay buffer and then two washes in kinase wash buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 10 mM *p*-nitrophenol phosphate, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 50 μM Na_3VO_4), protein–antibody complexes were resuspended in 30 μL of kinase buffer (kinase wash buffer plus 40 μM [γ-^{32}P]ATP (2.5 Ci/mmol) and 0.5 μg of GST-c-Jun (1–79) substrate for JNK/SAPK, 10 μg of MBP for ERK, and either 10 μg of MBP or 1 μg of ATF-2 for p38). Kinase reactions were allowed to proceed for 20 min at 30°. Reactions were terminated by the addition of Laemmli sample buffer and heated for 5 min at 95°. Following separation by 12% SDS–PAGE, phosphorylated substrate was analyzed with a PhosphorImager (Molecular Dynamics) and ImageQuant software. Results shown are representative of at least three independent assays with similar outcomes.

Western Blotting

Cells were lysed in a buffer containing 20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM Na_2EDTA, 0.5 mM 2-mercaptoethanol, 0.5% NP-40, 1 mM PMSF, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, and 10 mM *p*-nitrophenol phosphate followed by centrifugation (12,000 *g*, 5 min at 4°). Whole cell lysates (50 μg) were collected and mixed with Laemmli sample buffer, heated for 5 min at 95°, and then separated by 12% SDS–PAGE. Following SDS–PAGE, proteins were transferred to polyvinylidene difluoride using a Semi-Dry Transblot (Bio-Rad) according to the manufacturer's instructions. After blocking, the membrane was incubated with either anti-JNK1 (1:2000), anti-ERK1/2 (1:1000), or anti-p38 (1:2000) for 2 hr at 25°. The membrane was washed extensively and then

incubated with anti-rabbit IgG horseradish peroxidase conjugate (1:3000) for 30 min at 25°. Finally, the membrane was developed using enhanced chemiluminescence (New England Nuclear). The immunoblots shown are representative of at least two independent experiments with comparable results.

Caspase-3-Like Activity Assay

Caspase-3-like protease activity was measured using z-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (z-DEVD-AFC) (Enzyme Systems) as a substrate in a fluorogenic assay. Cells (5×10^6) were treated, washed with PBS (pH 7.2, 4°), and then lysed in 100 μL of a buffer containing 100 mM HEPES (pH 7.5), 1% Triton X-100, 10 mM dithiothreitol, 1 mM EDTA, 1 mM PMSF, 2.5 μg/mL of leupeptin, and 1 μg/mL of aprotinin. Cleavage of z-DEVD-AFC into the free fluorogenic AFC product was monitored at λ_{excit} = 400 nm and λ_{emit} = 505 nm for 15 min at 25°. Total protein from the cell lysates was quantitated by Bradford analysis (Bio-Rad). Results are reported as caspase-3-like activity and represent the means from at least two independent experiments.

Soft Agar Clonogenicity Assay

Cells were washed with RPMI 1640 and resuspended in RPMI 1640 supplemented with 10% FBS. Cell density was determined with a Coulter Counter (Coulter Electronics). Cells (4.5×10^3) from each group were mixed with 1.5 mL of cloning medium (RPMI 1640, 25% FBS, 100 μg/mL of streptomycin, 100 U/mL of penicillin G, and 0.3% Bacto-agar) in a cloning dish (35 mm diameter). Cloning dishes were incubated (37°, 5% CO_2, humidified air) for 7–10 days, at which time colonies (>50 cells) were scored using an inverted light microscope. Results are based on at least three independent experiments assayed in quadruplicate.

Statistical Analysis

Where indicated, one-way ANOVA and Tukey's post test were used to test for statistical significance ($P < 0.05$).

RESULTS

Clinically achievable concentrations of ara-C induce cells to undergo apoptosis [7, 25]. Internucleosomal DNA fragmentation is mediated by a DNase [26] and is a biochemical hallmark of apoptosis in HL-60 cells. We first established concentration–response and time–course profiles for HL-60 cells treated with ara-C (Fig. 1). HL-60 cells were treated with increasing concentrations of ara-C for 4 hr and then assayed for internucleosomal DNA fragmentation. Both 1 and 10 μM concentrations of ara-C induced similar levels of apoptotic DNA fragmentation, whereas concentrations below 1 μM revealed no DNA fragmentation (Fig. 1A). Next, we investigated the kinetics of DNA fragmentation with 1 μM ara-C, a clinically achievable concentration

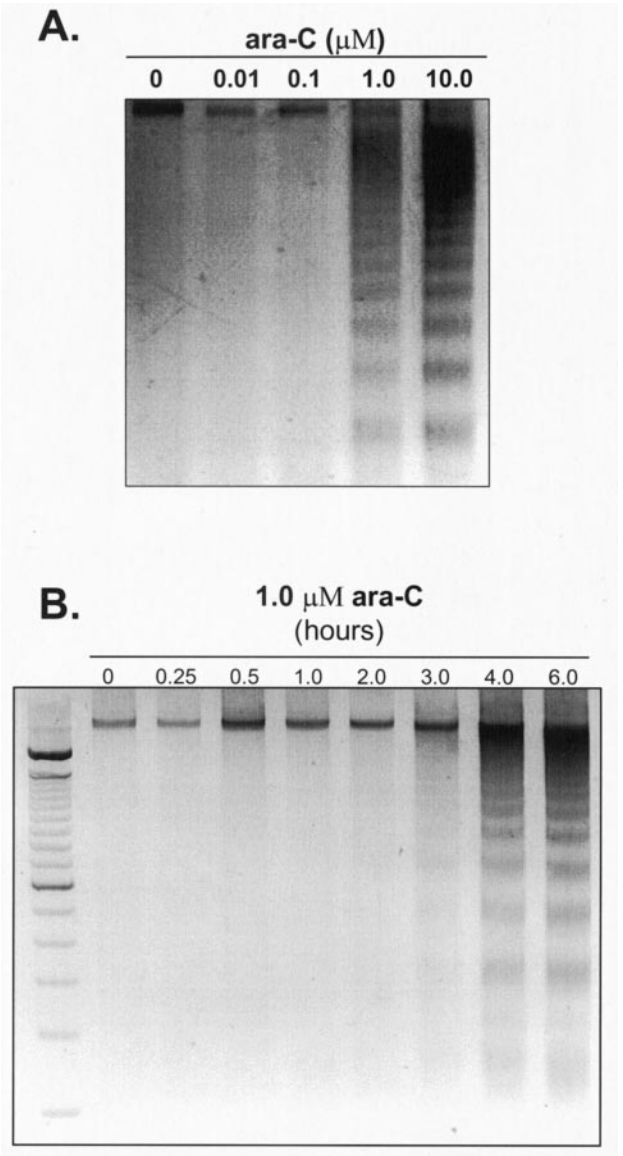

FIG. 1. Concentration and time dependence of ara-C-induced internucleosomal DNA fragmentation. (A) HL-60 cells were exposed to increasing concentrations of ara-C (0.01, 0.1, 1.0, and 10.0 μM) for 4 hr. (B) HL-60 cells were incubated with 1.0 μM ara-C for different times (0.25 to 6.0 hr), followed by lysis and extraction of genomic DNA. Purified DNA was separated by electrophoresis in a 2% agarose gel at 125 V for 45 min. Ethidium bromide staining and UV illumination were used to visualize the DNA fragmentation. The experiments shown are representative of three independent determinations with similar results.

obtained in situations where standard dose ara-C is administered [27]. Apoptotic DNA fragmentation was not detected until after 3 hr of treatment with ara-C (Fig. 1B).

Since apoptosis is regulated through intracellular signaling mechanisms, we investigated whether or not ara-C affects the ERK, JNK/SAPK, and p38 signaling cascades (Fig. 2). An immunocomplex kinase assay was used to measure the activities of the ERK, JNK/SAPK, and p38 MAPKs in HL-60 cells treated with 1 μM ara-C. We observed an increase in ERK2 activity after 15 min of ara-C

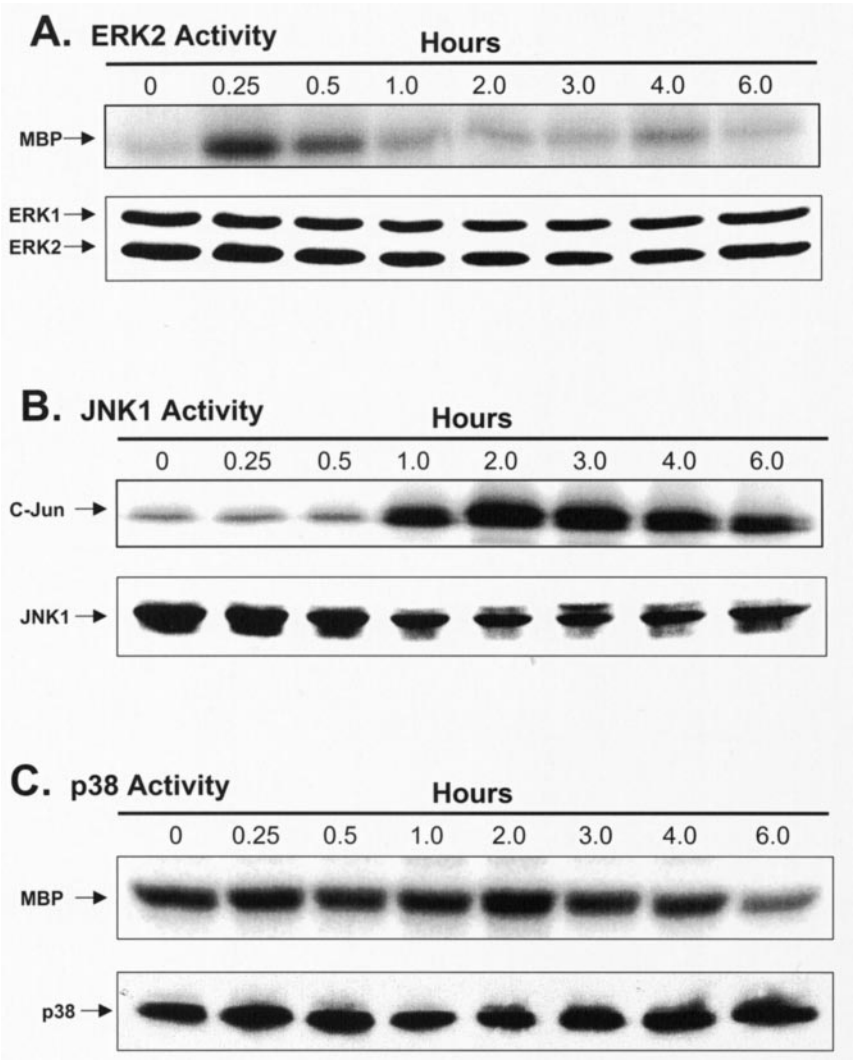

FIG. 2. Effect of ara-C on ERK, JNK/SAPK, and p38 activity. HL-60 cells were treated with 1.0 μM ara-C for 0.25 to 6.0 hr. Whole cell lysates were prepared and immunoprecipitated with (A) anti-ERK2, (B) anti-JNK1, or (C) anti-p38 followed by an *in vitro* immunocomplex kinase assay using MBP (for ERK and p38) and GST-c-Jun (1–79) (for JNK1) as substrate (upper panel). The substrates subsequently were heated at 95° for 5 min and then separated by 12% SDS–PAGE. The degree of phosphorylated substrate was analyzed with a PhosphorImager. (A) ERK2, (B) JNK1, or (C) p38 protein expression was determined by western blotting of whole cell lysates with anti-ERK1/2 (1:1000), anti-JNK1 (1:2000), and anti-p38 (1:2000), respectively; blots were exposed to anti-rabbit horseradish peroxidase conjugate and developed by enhanced chemiluminescence (lower panel). The experiments shown are from the same whole cell lysates and are representative of at least two additional experiments with similar results.

treatment (Fig. 2A, top panel). This activation quickly subsided and was no longer detectable after a 1-hr exposure to ara-C. Moreover, ara-C-induced ERK activation was relatively small (~2-fold) when compared with TPA, which potently activates ERK (~6-fold, data not shown). Western blotting with an antibody against ERK1/2 (p44/p42) indicated that ERK protein expression remained unchanged in response to ara-C (Fig. 2A, bottom panel).

Ara-C caused a delayed but robust increase in JNK1 activity that remained elevated for the length of the time course (Fig. 2B, top panel). The kinetics observed for JNK/SAPK activity in ara-C-treated cells were similar to those reported by others using UV irradiation or doxorubicin in T-cell leukemia cell lines [28, 29]. Thus, increases in JNK1 activity preceded the appearance of DNA fragmentation (compare Fig. 1B with Fig. 2B). JNK1 protein levels were measured by western blotting and revealed that ara-C did not affect protein expression (Fig. 2B, bottom panel). These results temporally correlated JNK/SAPK activation with the induction of apoptotic DNA fragmentation.

p38 is activated by many of the same stimuli that activate JNK/SAPK. It has been reported that high concentration ara-C (50 μM) causes the activation of p38 [22]. However, the biological effect of p38 activation has not been investigated. Therefore, we tested whether or not 1 μM ara-C could stimulate p38 activity. Using an immunocomplex kinase assay and MBP as a substrate, we found that p38 activity in response to ara-C was elevated slightly during a 6-hr time course (Fig. 2C, top panel). The maximum level of ara-C-induced p38 activation occurred between 1 and 3

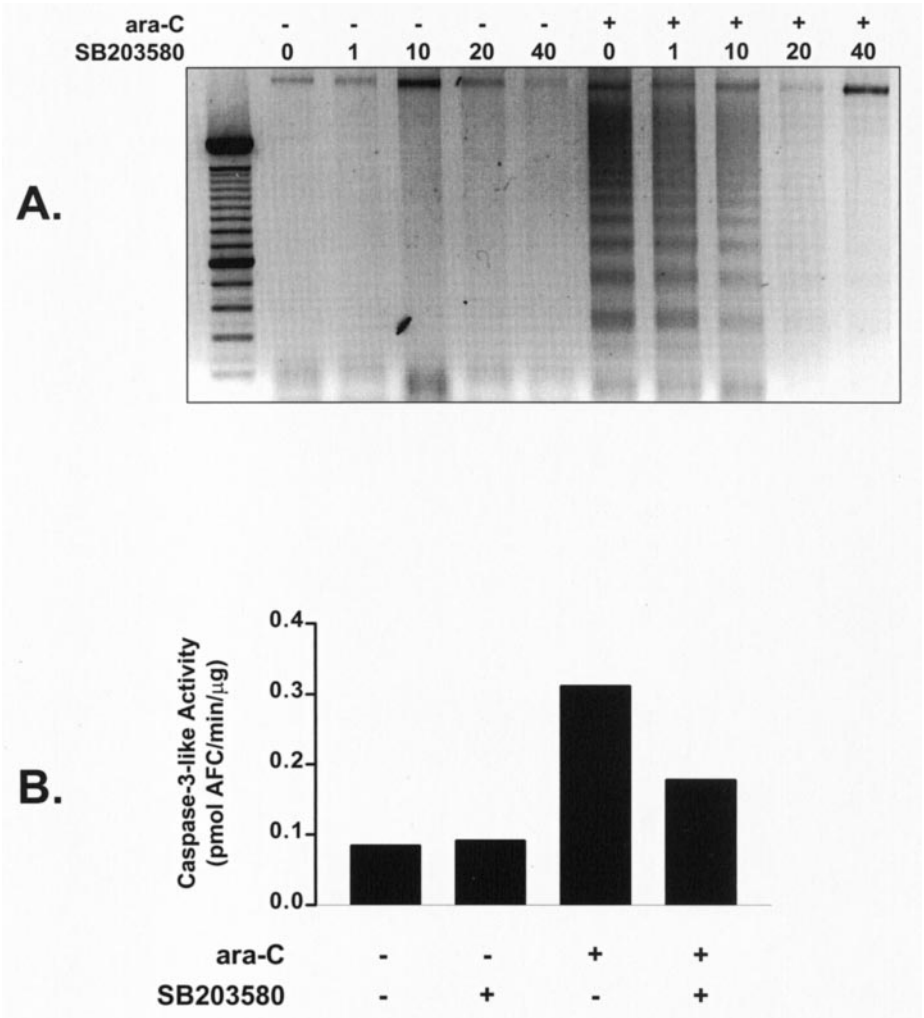

FIG. 3. Effect of SB203580 on ara-C-induced apoptotic DNA fragmentation and caspase-3-like activity in HL-60 cells. (A) HL-60 cells were treated with increasing concentrations of SB203580 for 30 min followed by vehicle (PBS) or 1 μM ara-C exposure for 4 hr. Cell lysates were prepared, and DNA was extracted, purified, and separated by 2% agarose gel electrophoresis. DNA fragmentation was visualized by ethidium bromide staining and UV illumination. Each group was normalized with 0.2% DMSO, which corresponds to the concentration of DMSO in 40 μM SB203580. (B) HL-60 cells were treated with 0.1% DMSO (vehicle) or 20 μM SB203580 for 30 min followed by PBS or 1 μM ara-C for 4 hr. Subsequently, cells were washed, and whole cell lysates were prepared. A portion (one-half) of the whole cell lysate was diluted in assay buffer with z-DEVD-AFC, and the fluorescence of liberated AFC was measured over 15 min. Total protein was determined by Bradford analysis. The results are depicted as caspase-3-like activity and represent the means of two independent experiments.

hr (1.36 ± 0.14-fold, average of seven experiments ± SEM at 3 hr). Similar results were obtained when another p38 substrate, ATF-2, was used (data not shown). Furthermore, treatment with a known p38 activator, sorbitol, as well as 50 μM ara-C, revealed similar low levels of activation (~1.5-fold, data not shown). Western blotting confirmed that p38 protein was indeed present in HL-60 cells (Fig. 2C, bottom panel). This small but consistent degree of p38 activity preceded the formation of apoptotic DNA fragmentation.

Since p38 is important for the development of apoptosis in certain systems, we investigated the apoptotic significance of p38 activation in HL-60 cells with the specific p38 inhibitor SB203580. SB203580 is a cell-permeable pyridinyl imidazole that inhibits p38 activity [30, 31]. HL-60 cells were exposed to increasing concentrations of SB203580 for 30 min prior to the addition of 1 μM ara-C for 4 hr. As illustrated in Fig. 3A, SB203580 abrogated ara-C-induced internucleosomal DNA fragmentation in a concentration-dependent manner. Whereas some inhibition of ara-C-mediated apoptosis could be detected with an SB203580 concentration of 10 μM, 20 μM SB203580 markedly reduced the appearance of DNA fragmentation. Therefore, this concentration was used in subsequent experiments.

We next investigated the means by which p38 inhibition blocked ara-C-induced apoptotic DNA fragmentation by measuring the activation of caspase-3-like proteases in a fluorogenic assay. Caspases are cysteine proteases that recognize a specific 4-amino-acid peptide sequence and cleave after an aspartate residue. Caspase-3 is believed to be a critical effector of apoptosis. Indeed, caspase-3 is required for the activation of caspase-activated DNase, an endonu-

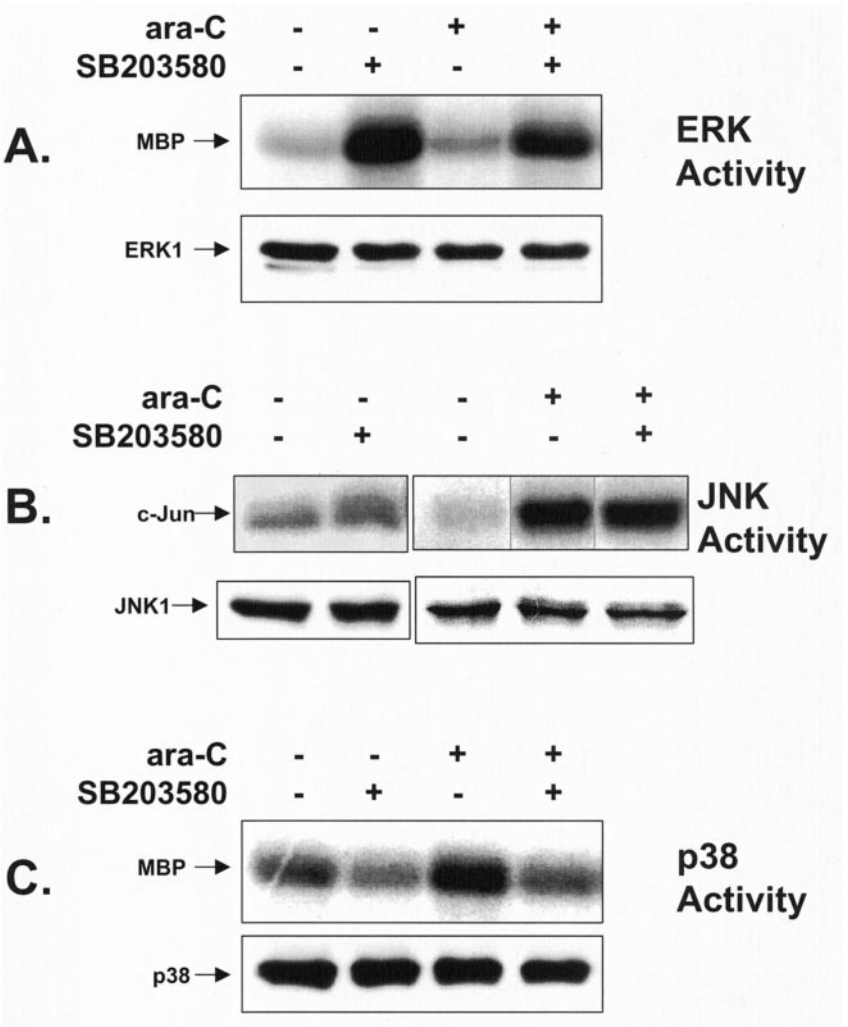

FIG. 4. Differential effects of SB203580 on ERK, JNK/SAPK, and p38 activity. HL-60 cells were treated with 0.1% DMSO (drug vehicle) or 20 μM SB203580 for 30 min followed by exposure to vehicle PBS or 1 μM ara-C for 3 hr. Whole cell lysates were prepared and immunoprecipitated with (A) anti-ERK2, (B) anti-JNK1, or (C) anti-p38 followed by an *in vitro* immunocomplex kinase assay using MBP (for ERK2 and p38), and GST-c-Jun (1–79) (for JNK1) as substrate (upper panel). The substrates subsequently were heated at 95° for 5 min and then separated by 12% SDS–PAGE. The level of phosphorylated substrate was determined by PhosphorImager analysis. (A) ERK2, (B) JNK1, or (C) p38 protein expression was evaluated by western blotting of whole cell lysates with anti-ERK1/2 (1:1000), anti-JNK1 (1:2000), and anti-p38 (1:2000), respectively; blots were exposed to anti-rabbit horseradish peroxidase conjugate and developed by enhanced chemiluminescence (lower panel). The experiments shown are from the same whole cell lysates and are representative of two additional experiments with similar results.

clease believed to cause apoptotic DNA fragmentation [32]. Ara-C at 1 μM activated caspase-3-like activity, and this activation was attenuated in cells pretreated with 20 μM SB203580 (Fig. 3B). This result demonstrated that ara-C-mediated caspase-3-like activity was p38-dependent.

The goal of the following experiments was to investigate whether or not basal p38 activity regulates ERK and JNK/SAPK signaling. It has been reported previously that SB203580 binds to p38 in a selective manner *in vitro* [31]. Treatment with SB203580 for 3.5 hr potently activated ERK in serum-fed HL-60 cells, whereas the addition of ara-C attenuated this activation (Fig. 4A). ERK protein analysis demonstrated that SB203580-induced ERK activation was mediated by existing protein levels (Fig. 4A, bottom panel). Furthermore, we analyzed JNK1 activity from SB203580-treated cell lysates and observed an increase (~2-fold) in basal activity (Fig. 4B). However, ara-C-induced JNK1 activity was not affected reproducibly by pretreatment with 20 μM SB203580. It also was determined that pretreatment with SB203580 inhibited ara-C-induced p38 activity (Fig. 4C) as well as p38 activity in untreated HL-60 cells. Thus, these experiments demonstrated that exposure of intact cells to SB203580 stimulated ERK activity and blocked ara-C-mediated p38 signaling.

Next, we specifically tested the role of JNK/SAPK in ara-C-induced apoptosis by direct inhibition of the JNK/SAPK signaling pathway. We stably transfected HL-60 cells with a pcDNA/SEK-AL expression construct and isolated selection-resistant clones by subcloning (data not shown, manuscript submitted for publication). Subclones

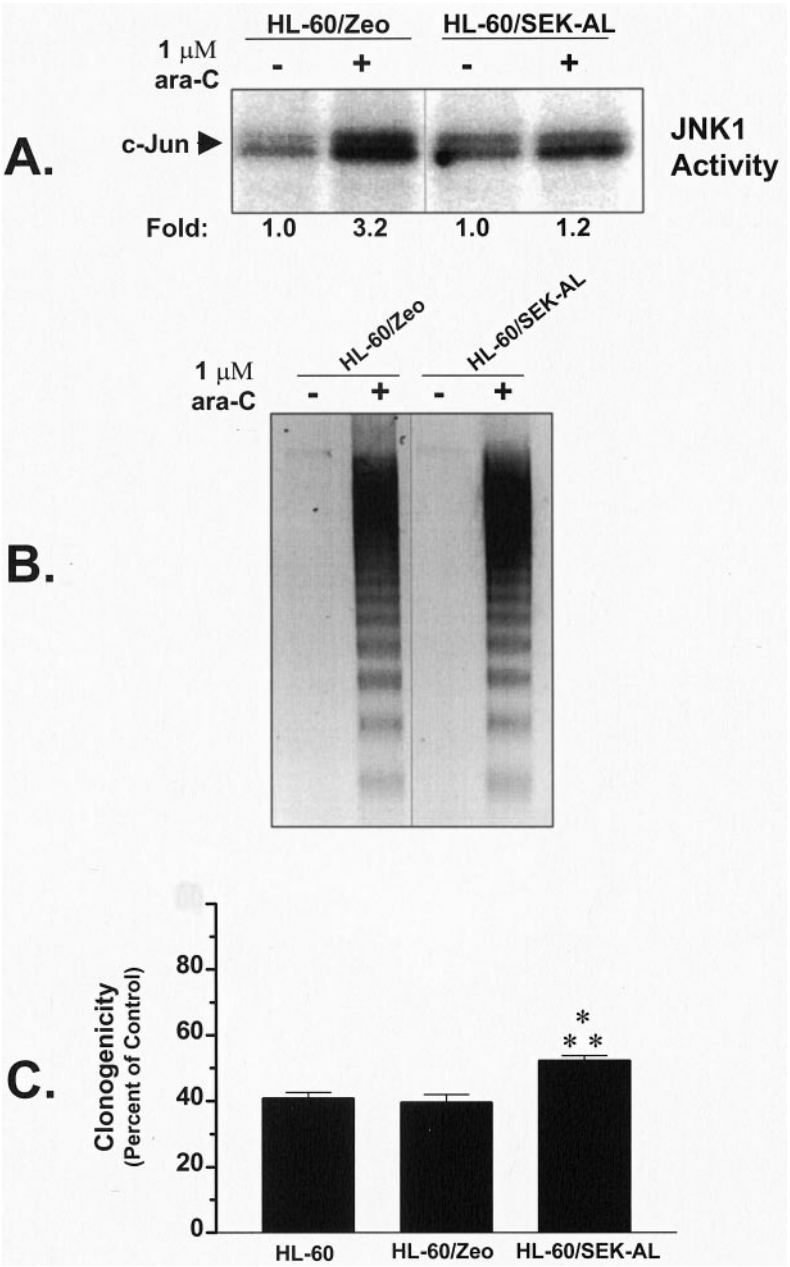

FIG. 5. Effects of dominant-negative SEK-AL on ara-C-induced internucleosomal DNA fragmentation. HL-60 cells were electroporated with pcDNA3.1/Zeo(+) (empty vector) or pcDNA3.1/SEK-AL and selected for resistance to Zeocin. Resistant HL-60/SEK-AL cells were subsequently subcloned and assayed for expression of the mutant SEK-AL protein. (A) HL-60/Zeo and HL-60/SEK-AL cells were left untreated or exposed to 1 μM ara-C for 3 hr and then assayed for JNK1 activity in a kinase assay using GST-c-Jun (1–79) as a substrate. The fold JNK1 activation depicted represents the average of two independent experiments. (B) Apoptotic DNA fragmentation was measured in HL-60/Zeo and HL-60/SEK-AL cells that were either untreated or exposed to 1 μM ara-C for 4 hr. The results shown are representative of three independent determinations with similar results. (C) HL-60 cell lines were stimulated with 1 μM ara-C for 4-hr, plated in 0.3% soft agar medium, and scored for colonies (>50 cells) after 7–10 days. The percent decrease in clonogenicity was based on an untreated control group from each cell line. The data represent the means ± SEM for three independent experiments performed in quadruplicate. Key: (*) indicates a significant difference between the HL-60 and HL-60/SEK-AL groups ($P < 0.05$); and (**) denotes a significant difference between the HL-60/Zeo and HL-60/SEK-AL groups ($P < 0.01$). Control plating efficiency averaged 50.6 ± 5.9%, HL-60/Zeo control plating efficiency averaged 63.1 ± 12.9%, and HL-60/SEK-AL control plating efficiency averaged 60.7 ± 13.6%. Forty-five hundred cells were plated in each experiment.

(hereafter termed HL-60/SEK-AL) then were analyzed for expression of the SEK-AL protein, which acts in a dominant-negative capacity to inhibit endogenous MKK4/SEK1 activation, by immunoprecipitation and western blotting with an antibody to the hemagglutinin fusion tag. The transfected cell lines displayed wild-type responses to both TPA-induced ERK activation and sorbitol-mediated p38 induction (data not shown, manuscript submitted for pub-

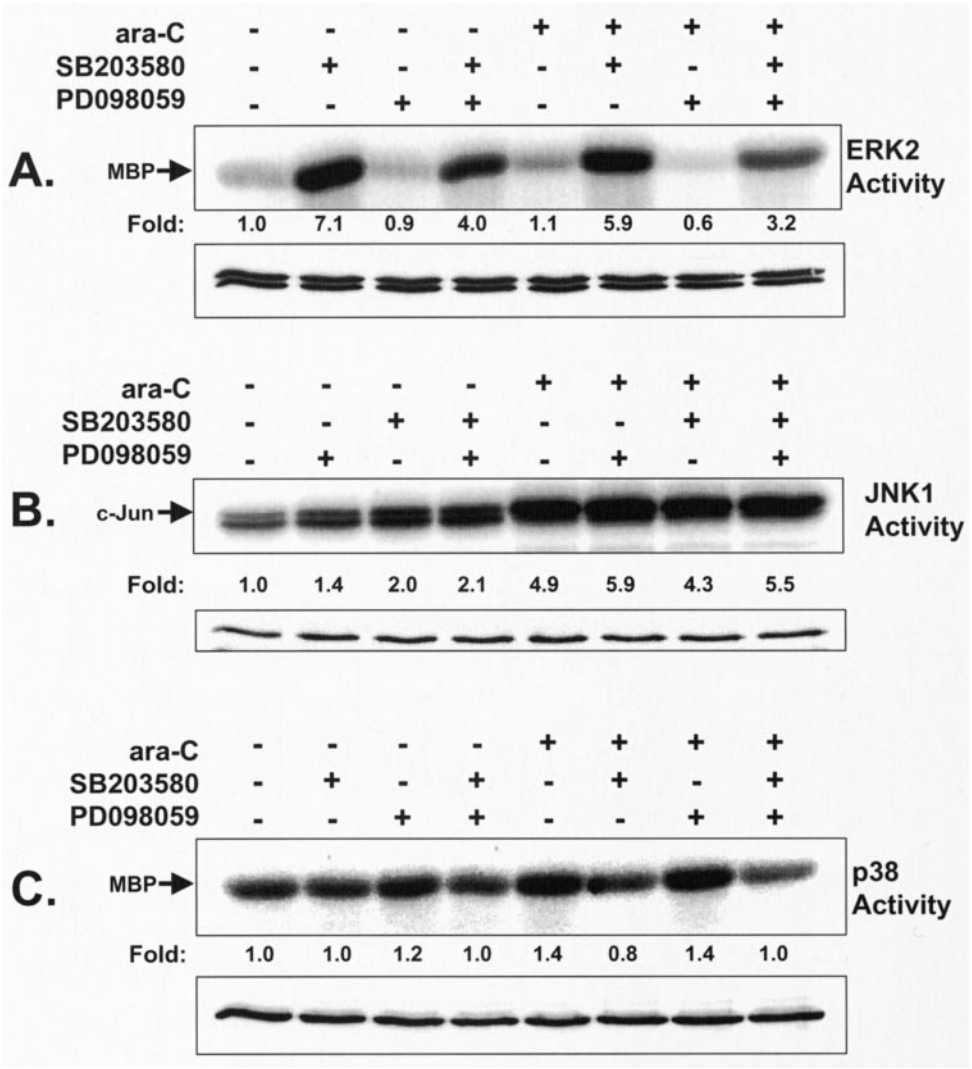

FIG. 6. Effect of PD098059 on SB203580-mediated ERK induction. HL-60 cells were treated with 0.1% DMSO or 25 μM PD098059 for 15 min followed by 0.1% DMSO or 20 μM SB203580 for 30 min. Then the cells were exposed to PBS or 1 μM ara-C for 3 hr, followed by lysis and immunocomplex assays. (A) ERK2, (B) JNK1, or (C) p38 activity (top panels) was assessed in an *in vitro* immunocomplex kinase assay using MBP (for ERK2 and p38) or GST-c-Jun (1–79) (for JNK1) as substrate. Kinase reactions were stopped with sample buffer, heated at 95° for 5 min, and separated by 12% SDS–PAGE. Phosphorylation of substrate proteins was evaluated by PhosphorImager analysis. The results shown are representative of at least two independent experiments with similar results. The expression levels of ERK2, JNK1, and p38 were determined by immunoblot analysis (lower panels).

lication). Figure 5A demonstrates that HL-60 cells transfected with the pcDNA3.1/Zeo(+) vector (empty vector control, termed HL-60/Zeo) exhibited an increase in JNK1 activation in response to a 3-hr incubation with 1 μM ara-C (2.3-fold, average of two experiments). On the other hand, HL-60/SEK-AL cells appeared to be refractory to the effects of ara-C on JNK1 activation (1.2-fold, average of two experiments). However, the HL-60/SEK-AL cell line was as sensitive as the HL-60/Zeo cells to the induction of apoptosis (Fig. 5B). Furthermore, we performed soft agar clonogenicity assays in the presence of ara-C with HL-60 wild-type, HL-60/Zeo, and HL-60/SEK-AL cell lines. We observed that HL-60/SEK-AL cells were slightly more resistant to the anti-clonogenic effects of a 4-hr incubation with 1 μM ara-C (Fig. 5C, HL-60 vs HL-60/SEK-AL, $P < 0.05$; HL-60/Zeo vs HL-60/SEK-AL, $P < 0.01$).

The nature of SB203580-mediated ERK activation was investigated further with the specific MEK1/2 inhibitor PD098059. HL-60 cells were incubated with 25 μM PD098059 for 15 min prior to the addition of 20 μM SB203580 for 30 min. This was followed by a 3-hr exposure to drug vehicle (PBS) or 1 μM ara-C. Figure 6 illustrates the effect of this schedule on ERK, JNK/SAPK, and p38 MAPK kinase activities. Similar to the effects of ara-C, PD098059 partially blocked the robust activation of ERK by SB203580 (Fig. 6A), and PD098059 and ara-C in combination were additive with respect to ERK inhibition. Thus, the experiment with PD098059 and SB203580 in combination suggests that SB203580-induced ERK activity is mediated partially by signaling events at the level of or upstream from MEK1/2.

Measurements of JNK1 activity indicated that PD098059

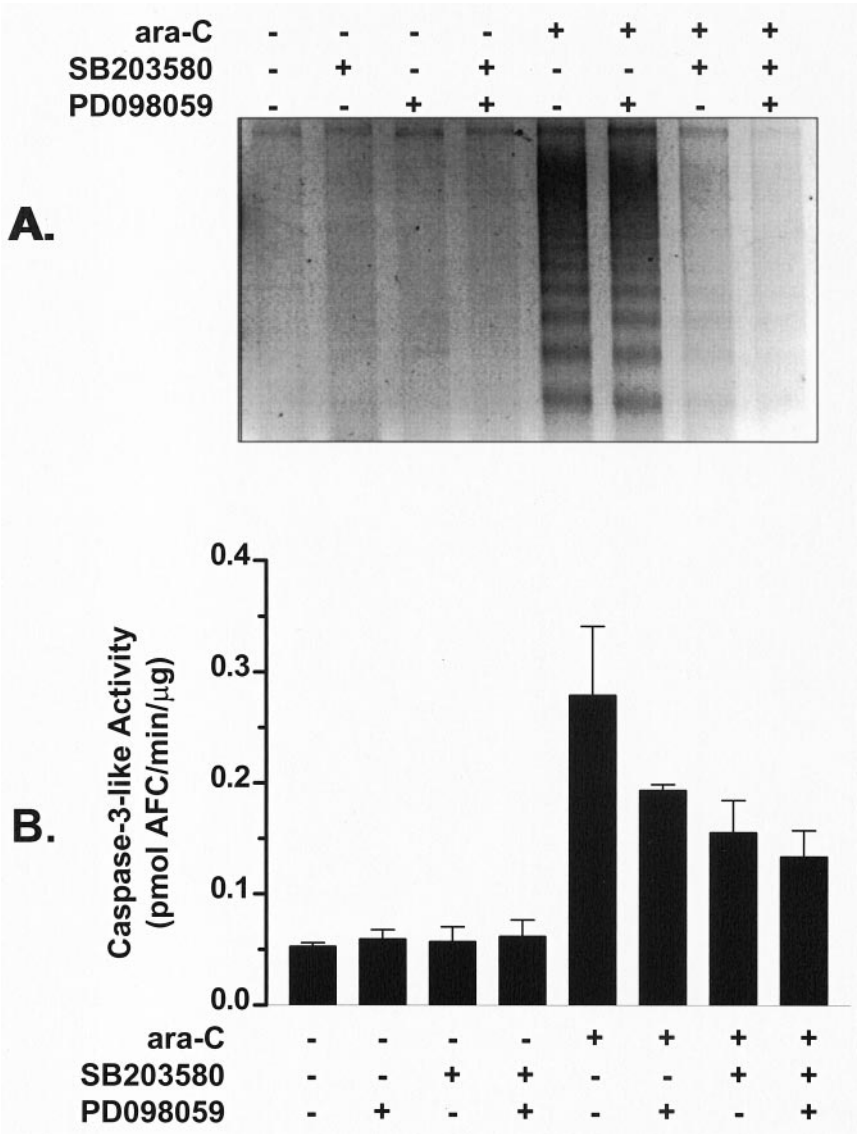

FIG. 7. Differential effects of PD098059 on ara-C-induced apoptotic DNA fragmentation and caspase-3-like activity. HL-60 cells were treated with 0.1% DMSO or 25 μM PD098059 for 15 min followed by 0.1% DMSO or 20 μM SB203580 for 30 min. Then the cells were exposed to PBS or 1 μM ara-C for 4 hr followed by lysis. (A) Apoptotic DNA fragmentation was assessed by separation of purified DNA fragments by electrophoresis in a 2% agarose gel at 125 V for 40 min. DNA fragmentation was observed by ethidium bromide staining and UV illumination. The experiment shown was repeated two times with similar results. (B) Whole cell lysates were diluted in assay buffer with *z*-DEVD-AFC, and the fluorescence of liberated AFC was measured over 15 min. Total protein was determined by Bradford analysis. The results are depicted as caspase-3-like activity and represent the means ± SEM of three independent experiments.

had little effect on either basal or ara-C-induced JNK1 activity, whereas SB203580 activated only basal JNK1 activity (Fig. 6B). Although ara-C-induced p38 activity was abrogated by SB203580, PD098059 had no effect on this inhibition (Fig. 6C). Moreover, ERK inhibition by PD098059 had a modest effect on basal p38 activity (1.2-fold). Thus, negative regulation of p38 activity appeared to affect ERK activity positively, and this interaction was reciprocated in that ERK inhibition positively regulated basal p38 activity.

We previously reported that TPA inhibits apoptotic DNA fragmentation in HL-60 cells treated with anisomycin in a PD098059-reversible manner, suggesting that ERK may be mediating the anti-apoptotic effects of TPA [24]. This finding agrees with the present study in that SB203580-induced ERK activation was correlated with its ability to abrogate ara-C-induced apoptotic DNA fragmentation. Therefore, we measured the effect of PD098059 on the inhibition of ara-C-induced apoptosis by SB203580. The results of Fig. 7A demonstrated that, in addition to being non-apoptotic in itself, PD098059 failed to antagonize the anti-apoptotic effects of SB203580. Additionally, we measured caspase-3-like activity in HL-60 cell lysates and found that not only was PD098059 ineffec-

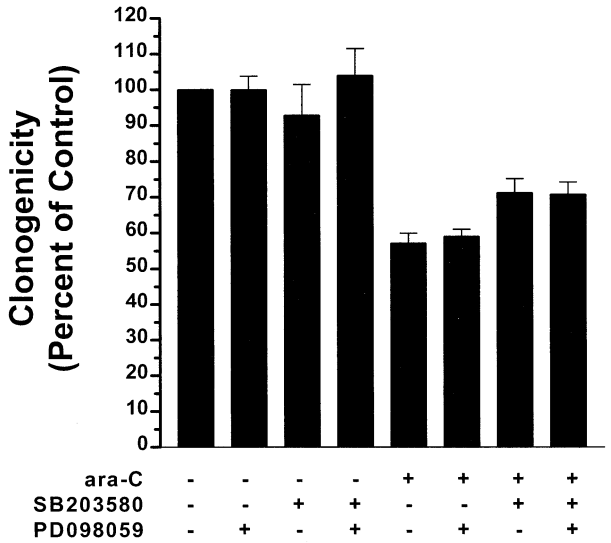

FIG. 8. Clonogenic survival in ara-C-treated HL-60 cells in the presence of p38 and ERK inhibitors. HL-60 cells were treated with 0.1% DMSO or 25 μM PD098059 for 15 min, followed by 0.1% DMSO or 20 μM SB203580 for 30 min. Then the cells were exposed to PBS or 1 μM ara-C for 4 hr and subsequently plated in 0.3% soft agar. After 7–10 days in a humidified incubator 37°, colonies (>50 cells) were scored. The data are reported as clonogenicity compared with an untreated control (100%) and represent the means ± SEM of three independent determinations assayed in quadruplicate. Control plating efficiency averaged 80.7 ± 10.5% of 4500 plated cells.

tive at reversing the protective effects of SB203580, but PD098059 also inhibited ara-C-induced caspase-3-like activity (Fig. 7B).

Lastly, we measured the effect of SB203580 and PD098059 on the ara-C-mediated loss of clonogenicity (Fig. 8). Applied to HL-60 cells in the absence of ara-C, PD098059 and SB203580 had little effect on clonogenicity in comparison with the marked decrease in growth caused by ara-C alone. Interestingly, in these experiments, a 4-hr treatment with 1 μM ara-C was not as cytotoxic as the clonogenicity studies performed in Fig. 5C (57.2 ± 2.73 vs 40.9 ± 1.16%, respectively). Although the exact basis for this discrepancy is not known, the fact that the studies performed in Fig. 8 contained 0.1% DMSO as a control vehicle may have altered their sensitivity to the effects of ara-C. Nonetheless, SB203580 and not PD098059 attenuated the anti-clonogenic effects of ara-C. These results demonstrated that apoptotic DNA fragmentation and not caspase-3-like activity correlated with a loss in clonogenicity.

DISCUSSION

The precise mechanisms underlying the biological response to ara-C are still uncertain. Here, we have reported MAPK signaling events mediated by ara-C and how the modulation of the MAPKs affected ara-C-induced apoptosis. Specifically, we provide evidence through direct inhibition of specific MAPK pathways that ara-C-induced apoptosis occurred by a p38-dependent mechanism.

Ara-C is a valuable drug in the treatment and management of acute myelogenous leukemia. Furthermore, it is well documented that the antileukemic effects of ara-C are mediated in part by its ability to induce a form of programmed cell death called apoptosis [33]. Ara-C-induced apoptosis is inhibited by forced expression of the anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-x_L [23], as well as the caspase inhibitors p35 [34] and YVAD-cmk [23]. Bcl-2 and Bcl-x_L are implicated in the upstream regulation of ara-C-induced apoptosis, since their forced expression in HL-60 cells prevents caspase-3 processing [23] as well as mitochondrial cytochrome *c* release [35].

In our studies, ara-C-induced apoptotic DNA fragmentation was preceded by the activation of ERK, JNK/SAPK, and p38 signaling (Figs. 1 and 2). The early and transient activation of ERK was not important for ara-C-induced DNA fragmentation, since the use of the MEK1/2-specific inhibitor PD098059 did not abrogate the appearance of DNA laddering (Fig. 7A), inhibit the anti-clonogenic effects of ara-C (Fig. 8), or alter the effects of ara-C on JNK1 and p38 activity (Fig. 6, B and C, respectively). This is in contrast to the findings of Jarvis *et al.* [36], where the use of PKC inhibitors potentiated ara-C-induced apoptosis and correlated with a loss of ERK activation. Interestingly, however, ERK inhibition by PD098059 attenuated ara-C-induced caspase-3-like activity (Fig. 7B), suggesting that ara-C may depend partially on activated MEK1/2 to optimally activate caspase-3-like activity. In support of this observation, it has been reported previously that *S*-nitrosoglutathione-induced caspase-3-like activity in macrophages can be attenuated by pretreatment with PD098059 [37]. Thus, in cells of myeloid lineage, caspase-3 activation may depend partially on MEK activity.

Ara-C has been reported previously to activate both JNK/SAPK [21] and p38 [22] activity. In Jurkat cells, treatment with the p38 inhibitor SB202190 was sufficient in itself to cause apoptosis [16]. Here, we determined the physiological outcome of inhibition of p38 and JNK/SAPK. Abrogation of p38 activity with SB203580 was non-apoptotic in otherwise unstimulated HL-60 cells (Fig. 3). This was also the case with another p38 inhibitor, SB202190 (data not shown). Inhibition of JNK/SAPK activity with a dominant-negative MKK4/SEK1 mutant appeared to confer a survival advantage in HL-60 cells, since HL-60/SEK-AL mutant cell lines achieved a greater density in cell culture when compared with vector-transfected control cell lines (data not shown). This suggests that the inhibition of JNK/SAPK signaling may attenuate functions controlling cell arrest through differentiation or apoptosis. Evidence supporting the former contention is provided by Grant and coworkers [7], who demonstrated that U937 cells expressing a dominant-negative c-Jun mutant (which lacks the entire AP1 transactivation domain and cannot be phosphorylated by JNK/SAPK) are more resistant to ara-C-induced differentiation than U937 wild-type cells. How-

ever, both the mutant and wild-type U937 cell lines display a similar sensitivity to the pro-apoptotic effects of ara-C [7]. JNK/SAPK phosphorylates transcription factors other than c-Jun, such as ATF-2 and ELK-1. Therefore, activated JNK/SAPK may regulate important cellular effects through substrates in addition to c-Jun. We transfected HL-60 cells with SEK-AL (termed HL-60/SEK-AL) in an attempt to investigate the apoptotic response in HL-60 cells with an impaired MKK4/SEK1→JNK/SAPK signaling pathway. HL-60/SEK-AL cells were resistant to ara-C-induced JNK1 activation (Fig. 5A). Furthermore, we observed in clonogenicity studies that the HL-60/SEK-AL mutant cell line was slightly more resistant to ara-C than either vector control-transfected cells (HL-60/Zeo) or wild-type HL-60 cells (Fig. 5C). In agreement with the studies performed in mutant U937 cells by Grant *et al*. [7], we observed no difference in their capacity to undergo apoptotic DNA fragmentation (Fig. 5B). Thus, the slight resistance to an ara-C-mediated loss in clonogenicity observed in the HL-60/SEK-AL cell line may be due to a loss in the ability to differentiate.

Studies with SB203580 revealed that ara-C-induced apoptotic DNA fragmentation was blocked effectively in the presence of a p38 inhibitor (Fig. 3A). This was corroborated with an associated loss in caspase-3-like activity (Fig. 3B). Furthermore, SB203580 inhibited the anti-clonogenic effects of ara-C, although this protection was incomplete (Fig. 8). Although these results suggest that the blockage of pathways involved in the execution of apoptosis inhibit typical biochemical hallmarks of the apoptotic process, they do not necessarily rescue a given cell population from the deleterious consequences of an apoptotic stimulus. Thus, cells that have sustained a lethal amount of damage may die a passive non-apoptotic death in the presence of apoptotic inhibition. Alternatively, since SB203580 does not inhibit the differentiation of HL-60 cells to the macrophage phenotype in response to TPA [38], the differentiative effects of ara-C may be contributing to the ara-C-induced loss of clonogenicity depicted in Fig. 8. Indeed, morphological analysis of cytospin preparations of drug-treated HL-60 cells with Wright–Giemsa staining suggested that ara-C-induced differentiation of HL-60 cells was unaffected by 20 μM SB203580 (data not shown).

The observation that SB203580 potently activated ERK suggests a possible mechanism for the anti-apoptotic effects of SB203580. It has been shown that the activation of proliferative pathways such as ERK in the presence of an apoptotic stimulus is sufficient to abrogate apoptosis [19], whereas the withdrawal of trophic support activates p38 and induces apoptosis [39]. Moreover, the inhibition of TPA-stimulated ERK activity by PD098059 abrogates the anti-apoptotic effects of TPA on anisomycin-induced apoptosis [24]. Therefore, we investigated whether the inhibition of ERK signaling would abolish the anti-apoptotic effects of SB203580 in ara-C-induced apoptosis. HL-60 cells pretreated with PD098059 displayed an inhibition of SB203580-induced ERK activation, suggesting that SB203580-mediated ERK activation occurred at or upstream from MEK (Fig. 6). However, the effects of ara-C and PD098059 in combination were additive and did not inhibit SB203580-induced ERK activity completely (Fig. 6). Additionally, apoptotic DNA fragmentation was not observed to be induced by pretreatment of ara-C and SB230580-stimulated cells with PD098059 (Fig. 7A). Therefore, the residual ERK activity that is not inhibited by ara-C or PD098059 may still be enough of a protective stimulus to prevent ara-C-induced DNA fragmentation. Equally plausible is the suggestion that the sole inhibition of p38 activity deprives ara-C-treated cells of a stimulus necessary for caspase-3-like activation and apoptotic DNA fragmentation.

In this study we present evidence suggesting that ara-C-induced apoptotic DNA fragmentation occurred by a p38-dependent mechanism, whereas ara-C-mediated caspase-3-like protease activity was both MEK1/2 and p38-dependent. Furthermore, we report the observation that basal ERK activity was regulated by p38 and occurred, in part, by a MEK-dependent mechanism. To our knowledge, this is the first time that these observations have been reported. These data provide additional information in the elucidation of the highly complex nature of ara-C-induced intracellular signaling and apoptosis.

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