Mitoxantrone has been shown in vitro to exhibit a steep dose-response relationship with respect to the clonogenic survival of acute myeloid leukemia cells. In this report, we show that 1-hour exposure of human myeloid leukemia HL-60 and KG-1 cells to mitoxantrone concentrations ranging between 0.1 and 10.0 μmol/L induced internucleosomal DNA fragmentation of approximately 200-bp integer multiples, characteristic of cells undergoing programmed cell death (PCD) or apoptosis. Mitoxantrone-mediated PCD was associated with a steep inhibition of the clonogenic survival of the leukemic cells. In addition, intracellularly, mitoxantrone-induced PCD was associated with a marked induction of c-jun and significant repression of c-myc and BCL-2 oncogenes. Pretreatment with the protein kinase C stimulator phorbol myristate acetate enhanced mitoxantrone-induced internucleosomal DNA fragmentation, whereas protein kinase C inhibitors staurosporine and H7 had no effect. These findings suggest that PCD is a potential mechanism underlying the steep dose-response relationship of mitoxantrone to the inhibition of clonogenic survival of acute myeloid leukemia cells.

MATERIALS AND METHODS

Cells. The human myeloid leukemia cells HL-60 and KG-1 were derived from the original lines. They are maintained in suspension culture as a short intravenous infusion, which produces peak plasma concentrations in the range of 1.0 μmol/L. Recent in vitro data have shown that high concentrations of mitoxantrone (≥1.0 μmol/L) exhibit a steep dose to inhibition of clonogenic survival relationship toward leukemic blasts.

The mechanism underlying this has not been clearly established. Nonetheless, based on these studies, high-dose mitoxantrone-containing regimens are being tested before autologous bone marrow transplantation and in dose-intensive therapy of relapsed and refractory leukemia.

Recently, a variety of antileukemic drugs such as Ara-C, nitrogen mustard, cisplatin, and topoisomerase II inhibitors, including etoposide and amacrine, have been shown to induce leukemic cell death by the alternative mechanism of programmed cell death (PCD) or apoptosis. In contrast to cell necrosis, apoptosis or PCD is an active, energy-dependent process of gene-directed cellular self-destruction that involves fragmentation of genomic DNA into integer multiples of 200 bp units by the activation of a nuclear endonuclease. Apoptosis is morphologically characterized by early cell shrinkage, chromatin condensation, nuclear disintegration, cell surface blebbing, and membrane bound apoptotic bodies. Some studies have shown that the modulation of the activity of protein kinase C (PKC) by a variety of agents affects the induction of PCD. In addition, the protein encoded by the oncogene BCL-2 has been shown in vivo to block PCD. High levels of expression of BCL-2 protects against apoptosis due to antileukemic drugs, including Ara-C and nitrogen mustard. Also, Ara-C-induced PCD is associated with altered expression of several genes, including c-jun, c-myc, and BCL-2. Because high-dose mitoxantrone is highly active against AML cells, the purpose of the present studies was to determine whether mitoxantrone, like Ara-C, can induce PCD in human myeloid leukemia cells HL-60 and KG-1, and how the modulation of intracellular PKC activity affects this process. An additional aim was to examine the effect of mitoxantrone on c-jun, c-myc, and BCL-2 expression in human myeloid leukemia cells.

Drugs. Mitoxantrone was kindly provided by Lederle Laboratories (Pearl River, NY). Phorbol myristate acetate (PMA), H7, and staurosporine were purchased from Sigma Chemicals (St Louis, MO).

Quantitative and qualitative analyses of internucleosomal DNA fragmentation. The internucleosomal DNA fragmentation was assayed by a modification of previously described methods. Cells were treated with mitoxantrone for 1 hour. Alternatively, cells were preincubated with PMA (10 ng/mL for 10 minutes), staurosporine (50 ng/mL for 3 hours), or H7 (50 μmol/L for 3 hours) before mitoxantrone treatment. Staurosporine and H7, but not PMA, were kept in the culture medium during mitoxantrone treatment. After these incubations, cells were washed and incubated in drug-free media for an additional 4 hours. At the end of this incubation, cells were pelleted and washed with phosphate-buffered saline (PBS) at 4°C and disrupted by suspension for 20 minutes at 4°C in

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5 mmol/L Tris-HCl buffer containing 0.5% (vol/vol) Triton X-100 and 20 mmol/L EDTA. The cellular lysates were centrifuged at 27,000g for 20 minutes to separate low molecular weight DNA from intact chromatin. The pellet was resuspended in the lysis buffer (0.5 mL), and 50 μL of bovine serum albumin (2 mg/mL) was added to this as well as to the supernatant. After adding 1 mL of 10% 12-0-tetradecanoylphorbol-13-acetate (TCA), the microfuge tubes were centrifuged for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of 100% ethanol at 4°C and incubated at −20°C. After another centrifugation, the pellet was treated with 500 μL of 1 N sodium hydroxide to hydrolyze RNA and incubated at 37°C for 1 hour, or until it was resuspended. The reaction mixture was incubated for 15 minutes on ice, treated with 1.5 mL of 20% TCA for 30 minutes at 4°C, and then centrifuged at 27,000g for 20 minutes to separate low molecular weight DNA from intact chromatin. Purified DNA fragments from the supernatant of lysed cells were electrophoresed in agarose gel (see text). DNA in lane M is the 123-bp marker DNA ladder (GIBCO, BRL, Grand Island, NY). DNA in the five lanes of the two panels are from cells treated with mitoxantrone as follows: lanes 1, untreated control cells; lanes 2, 0.01 μmol/L mitoxantrone; lanes 3, 0.1 μmol/L mitoxantrone; lanes 4, 1.0 μmol/L mitoxantrone; and lanes 5, 10.0 μmol/L mitoxantrone.

Fig 1. HL-60 (left panel) and KG-1 (right panel) cells were exposed to 0.01 to 10.0 μmol/L of mitoxantrone for 1 hour. Subsequently, cells were pelleted and washed once and resuspended in drug-free media for 4 hours. After these incubations, cells were pelleted and lysed and cellular lysates were centrifuged to separate low molecular weight DNA from intact chromatin. Purified DNA fragments from the supernatant of lysed cells were electrophoresed in agarose gel (see text). DNA in lane M is the 123-bp marker DNA ladder (GIBCO, BRL, Grand Island, NY). DNA in the five lanes of the two panels are from cells treated with mitoxantrone as follows: lanes 1, untreated control cells; lanes 2, 0.01 μmol/L mitoxantrone; lanes 3, 0.1 μmol/L mitoxantrone; lanes 4, 1.0 μmol/L mitoxantrone; and lanes 5, 10.0 μmol/L mitoxantrone.

Fig 3. Quantitative analyses of mitoxantrone (0.01 to 10 μmol/L) induced internucleosomal DNA fragmentation in (□) HL-60 and (■) KG-1 cells. After treatment of cells with different concentrations of mitoxantrone for 1 hour, cells were washed and incubated in drug-free media for an additional 4 hours. Subsequently, cells were pelleted, lysed, and centrifuged and the fragmented DNA was purified and quantitated in the supernatant and pellet by diphenylamine reaction (see text). The quantity of fragmented DNA in the supernatant was expressed as a percentage of total DNA from 2 × 10⁷ cells.
fuged at 4°C for 10 minutes at 1,500 rpm. The pellet was vortexed or sonicated and DNA was hydrolyzed with 1 mL of 5% perchloric acid (PCA) for 15 minutes at 90°C with frequent vortexing to break up the pellet and release DNA from the pellet. Subsequently, the reaction mixture was centrifuged at 3,000 rpm and the supernatant was treated overnight at 37°C with 2 mL of Burton’s reagent that uses a diphenylamine reaction. The absorbance at 600 nm was compared with that of 0 to 250 μg/mL of DNA as standard, using linear regression. The quantity of fragmented DNA in the supernatants was expressed as a percentage of total DNA from 2 × 10⁶ cells.

For qualitative DNA analysis, 1 × 10⁶ cells were treated with mitoxantrone, as described above. At the end of the incubations, cells were washed in PBS and resuspended in the lysis buffer, as above. The suspension was centrifuged and DNA was extracted from the supernatant by treatment with phenol, and chloroform/isoamyl alcohol (25:24:1). To the upper aqueous layer placed in a fresh tube, 50 μL of 3 mol/L sodium acetate and 1 mL of 100% ethanol at 4°C was added to precipitate the DNA. The mixture was vortexed and incubated for 30 to 60 minutes at −70°C. After a cold centrifugation for 30 minutes at 10,000 rpm, the supernatant was discarded and the pellet was resuspended in 0.5 mL of Tris EDTA (TE) buffer at pH 8.0. After adding 10 μL of DNase-free RNase A to the suspension, it was vortexed and incubated for 30 minutes at 37°C. To this, 50 μL of 5 mol/L NaCl was added and the phenol/chloroform/isoamyl alcohol extraction was repeated. DNA was precipitated with ethanol as above and centrifuged, and the pellet was dried. DNA samples were mixed with 16 μL (per 5 × 10⁶ cells) of TE (pH 8.0) loading buffer and 2 μL of 5× tracking dye containing glycerol, bromophenol blue, and xylene cyanol. The reaction mix was loaded into wells of a 1% agarose gel, and electrophoresed in a 1× Tris-acetate-EDTA (TAE) running buffer containing ethidium bromide. DNA was separated over 90 minutes at 60 V and the bands were visualized by UV illumination.

Southern blot method for the evaluation of DNA fragmentation. To improve the sensitivity of detecting DNA fragmentation associated with apoptosis, a slightly modified version of a previously described Southern blot method was used. DNA from the leukemic cells treated with the designated concentration of the drugs was isolated and purified by phenol/chloroform/isoamyl alcohol extraction and precipitation with ethanol, and electrophoresed in 1% agarose with 1× TAE runner buffer containing ethidium bromide. The electrophoresed DNA was transferred overnight to nylon membranes by standard capillary methods. Blots were UV cross-linked and hybridized at 55°C overnight in 50% formamide/10% dextran sulfate/1% sodium dodecyl sulfate (SDS)/5.6% sodium chloride containing denatured salmon sperm DNA. Separately, fragmented DNA from drug-treated leukemic cells was used to make a cDNA probe labeled by the random hexamer priming system by Prime-a-Gene. Blots were washed for 30 minutes at 27°C in 2× SSC/0.1% SDS and for 30 minutes at 65°C in 0.1× SSC/0.1% SDS, and autoradiographed for 10 minutes to 5 hours at −70°C.

RNA extraction. Total cellular RNA was extracted by the guanidine thiocyanate-phenoI-chloroform method. After exposure to the designated concentrations and schedule of mitoxantrone (see above), cells were washed with PBS and homogenized in GIT buffer (4 mol/L guanidine isothiocyanate, 2 mol/L sodium acetate, pH 4, 0.1 mol/L, 2-mercaptoethanol). The homogenate was then treated with 10% Sarkosyl and 2 mol/L sodium acetate, and the total cellular RNA extracted through a phenol/chloroform/isoamyl alcohol suspension (25:24:1) and subsequent centrifugation. The resulting
RNA pellet was washed with absolute ethanol, dissolved in diethyl pyrocarbonate-treated water, and stored at −20°C until used. Total RNA extracted by this procedure has an A260/A280 ratio of 1.95. The level of BCL-2, c-jun, c-myc, and β-actin RNA is determined by Northern blot analysis (vide infra) using the following P-labeled probes: (1) the 1.8-kb BamHI/EcoRI insert of a human c-jun complementary DNA purified from a pBluescript SK(+) plasmid; (2) the human c-myc probe was a 1.3-kb EcoRI/ClaI fragment from the PMC 445 plasmid; (3) the human β-actin probe was a 600-bp EcoRI/BamHI fragment from the KSII(+) plasmid; and (4) murine BCL-2 probe was an 830-bp EcoRI-HindIII fragment from pBluescript plasmid. The probes were kindly provided as follows: Dr Donald Kufe (Dana Farber Cancer Center, Boston, MA) provided the c-jun; Dr R. Dalla-Favera (Columbia University, New York, NY) provided the c-myc; Dr Stanley Korsmeyer (Washington University, St Louis, MO) provided the BCL-2; and Dr James S. Norris (Medical University of South Carolina, Charleston, SC) provided the β-actin.

**Northern blot analysis.** RNA hybridization analysis was performed as previously described. Total RNA (10 μg) was obtained as described above, was mixed with electrophoresis sample buffer (100% deionized formamide, 37% formaldehyde, 10× MOPS, glycerc, 4% bromophenol blue, 4% xylene cyanol), was denatured at 65°C for 15 minutes, and was run on a 1.0% agarose gel in 1× MOPS buffer. The RNA in the gel was denatured with 0.05 N NaOH/0.15 mol/L NaCl for 30 minutes and neutralized with 0.1 mol/L Tris (pH 7.5)/0.15 mol/L NaCl for 30 minutes. The gel was then placed in contact with nitrocellulose filter and the RNA was transferred to the solid support on a PosiBlot Pressure Blotter (Stratagene) at 80 psi for 2 hours. The RNA was cross-linked to the filter by a UV cross-linker (Stratagene). Prehybridization and hybridization of the RNA was performed in low stringency conditions. The nitrocellulose membrane was prehybridized overnight at 55°C in the following solution containing deionized formamide, 50% dextran sulfate, 10% SDS, and 100 μg/mL salmon sperm DNA. Random-primed, 32P-labeled cDNA probes were heat-denatured, cooled, and added to the buffer and hybridized for 18 to 72 hours at 55°C. The hybridized blots were washed twice in 2× SSC (sodium chloride and sodium citrate), 0.1% SDS at 65°C for 15 minutes per washing, and twice in 0.1× SSC, 0.1% SDS at 65°C, again for 15 minutes per washing. The blots were then exposed to Kodak X-Omat AR films with intensifying screens (Eastman Kodak, Rochester, NY). The developed films were scanned by densitometry.

**Colony culture of leukemic cells.** The colony growth of the leu-kemic cells was determined by a minor modification of a previously described method. Briefly, after incubation of log growth phase cells with the different concentrations of mitoxantrone for 1 hour, cells were washed twice in RPMI 1640 medium and incubated at 37°C in a 5% CO2 fully humidified incubator for an additional 4 hours. Subsequently, serial dilutions of cells were plated at cell densities ranging from 1 to 1 × 106 cells/well in 96-well U-bottomed microplates (Baxter) in 16 replicates in a plating medium consisting of RPMI 1640, 20% fetal bovine serum (FBS), and 0.15% agarose. The plates were incubated at 37°C in 5% CO2 atmosphere for 10 days. At the end of this incubation, colony-forming efficiency was determined in each well. Maximum likelihood analysis was performed by using the formula described by Tasswell.

**Statistical analysis.** Significant differences between values obtained in a population of leukemic cells (HL-60 or KG-1) treated with different experimental conditions were determined by paired t-test analyses.

**RESULTS**

The effect of treatment with mitoxantrone (0.01 to 10.0 μmol/L) for 1 hour on internucleosomal DNA fragmentation in HL-60 and KG-1 cells is shown in Fig 1. Exposure to concentrations of mitoxantrone ≥ 1.0 μmol/L produced the characteristic ladder of oligonucleosomal DNA fragments that were 200-bp integer multiples in size (Fig 1, lanes 4 and 5 in both panels). The intensity of the bands in the DNA fragmentation ladder was not different in HL-60 cells treated with 1.0 or 10.0 μmol/L, whereas the intensity was greater in KG-1 cells treated with 10.0 compared with those treated with 1.0 μmol/L mitoxantrone (Fig 1). However, it should be noted that the intensity of the bands in the ladder provides only a semiquantitative assessment for compari...
Fig 7. HL-60 cells were exposed to mitoxantrone (1.0 μmol/L), staurosporine (50 ng/mL for 4 hours), or H7 (50 μmol/L for 4 hours) alone. Alternatively, cells were exposed to staurosporine or H7 for 3 hours, followed by concurrent treatment with mitoxantrone for 1 hour. After treatment with mitoxantrone, cells were washed and kept in drug-free medium for 4 hours. Subsequently, cells were pelleted and total cellular RNA was extracted. Northern blots of the electrophoresed RNA (20 μg) were hybridized with 32P-CTP–labeled random-primed cDNA probes of c-myc, c-jun, and β-actin genes. Lane 1 has RNA from untreated, control cells. Lanes 2, 3, and 5 have RNA from cells treated with mitoxantrone, staurosporine, or H7 alone, respectively. RNA from cells pretreated with staurosporine or H7 followed by concurrent treatment with mitoxantrone are in lane 4 (staurosporine) and lane 6 (H7).

Son of DNA fragmentation under different conditions of exposure. Ethidium bromide staining of the electrophoresed DNA from the supernatant of lysed control (untreated) cells (Fig 1, lane 1 in both panels) or from the cells treated with 0.01 and 0.1 μmol/L mitoxantrone in lanes 2 and 3 of Fig 1, respectively, did not show any significant DNA fragmentation. However, as shown in Fig 2, by using the Southern blot method with a significantly greater sensitivity (50- to 100-fold),59 oligonucleosomal DNA fragmentation could be detected after treatment with ≥0.1 μmol/L (Fig 2, lanes 2 and 3) but not after treatment with 0.01 μmol/L mitoxantrone, whereas high mitoxantrone concentrations, 20 μmol/L and above, produced random DNA fragmentation characterized by a smear (Fig 2, lane 5) on the blot. Quantitative analyses of the fragmented DNA in the supernatant of the lysed HL-60 and KG-1 cells expressed as the mean percent of the total starting DNA are shown in Fig 3. Figure 3 shows that a marked increase in the DNA fragmentation was observed when HL-60 and KG-1 cells were treated with greater than 0.1 μmol/L mitoxantrone, and a maximal increase in the amount of internucleosomal DNA fragmentation occurred when the cells were exposed to 10 μmol/L mitoxantrone. Higher concentrations of mitoxantrone did not further increase the DNA fragmentation (data not shown). Plasma mitoxantrone concentrations in the range of 1 to 10 μmol/L are achievable with drug regimens containing high-dose mitoxantrone.7,33 These concentrations of mitoxantrone produced a several log decline in clonogenic survival of HL-60 cells, as shown in Fig 4. These concentrations have also been previously demonstrated to have a steep dose to the inhibition of clonogenic survival relationship for fresh AML blasts.8

Figure 5 shows the results of the Northern blot analysis of RNA from cells treated with different concentrations of mitoxantrone and hybridized to c-myc, c-jun, BCL-2, and β-actin c-DNA probes. Control HL-60 cells exhibited low BCL-2 and high c-myc expression, whereas c-jun expression in these cells was low to undetectable (Fig 5, lane 1). Figure 5 also shows that 1 hour of exposure to 1.0 (lane 3) but not 0.1 μmol/L (lane 2) of mitoxantrone resulted in a marked induction of c-jun and a decline in c-myc and BCL-2 expression. Treatment with 10.0 μmol/L mitoxantrone produced a lesser induction of c-jun, but resulted in a marked repression in BCL-2 and c-myc expression. This concentration of mitoxantrone also decreased the expression of the presumed housekeeping β-actin gene.

Recent studies have indicated that a modulation of intracellular PKC activity may affect the internucleosomal DNA fragmentation and apoptosis mediated by a variety of drugs.17,18 Based on previous studies that have shown that a short exposure to PMA (≤15 minutes) produces PKC stimulation but longer exposures and that long-term treatment inhibits PKC activity, we selected PMA pretreatment at 10 ng/mL concentrations for 10 minutes as the optimal PKC stimulatory dose for our experiments.34 Figure 6 shows that the treatment of HL-60 cells with PMA repressed c-myc expression by approximately threefold, but produced a 1.5-fold increase in c-jun expression (lane 3). Figure 6 also shows that pretreatment with PMA reproducibly further enhanced mitoxantrone- (1.0 μmol/L) mediated induction of c-jun by approximately twofold and the inhibition of c-myc expression by approximately eightfold (lane 4 vs lane 2). However, PMA pretreatment did not affect BCL-2 repression due to mitoxantrone (data not shown). Nonetheless, prior exposure to PMA (Fig 6, lane 4) produced increased intensity of the bands on the DNA fragmentation ladder induced by 1.0 μmol/L mitoxantrone alone (Fig 6, lane 2). This was confirmed by a significant quantitative increase in the mean DNA fragmentation with mitoxantrone alone from 30.5% ± 1.2% to 39.4% ± 1.5% (mean ± SEM of 3 experiments, P < .05) observed after treatment with PMA followed by mitoxantrone. These findings do not necessarily establish a direct causal relationship between the PMA-induced enhancement in mitoxantrone-mediated c-jun in-
duction and the increase in the DNA fragmentation. PMA pretreatment also produced a 1.6±0.1-fold increase in the mitoxantrone-mediated inhibition of clonogenic survival of HL-60 cells. However, the viability of cells, as detected by trypan blue exclusion method, just before DNA extraction for the DNA fragmentation studies, was ≥95%. This is consistent with the previously published observations that internucleosomal DNA fragmentation precedes loss of membrane integrity and viability.11,14,16 We also examined the effect of PKC inhibitors staurosporine and H7 on mitoxantrone-induced alterations in c-jun, c-myc, and BCL-2 expression as well as the internucleosomal DNA fragmentation. Although both compounds inhibit PKC, H7 is also an equally potent inhibitor of cAMP- and cGMP-dependent protein kinases.35 Figure 7 shows that treatment with staurosporine (lane 3) or H7 (lane 5) alone for 4 hours significantly inhibited c-jun expression, whereas c-myc expression was downregulated by staurosporine (lane 3) but not by H7 (lane 5). Exposure to 1.0 μmol/L mitoxantrone for 1 hour (Fig 7, lane 2), compared with untreated cells (Fig 7, lane 1), again showed induction of c-jun and repression of c-myc. Prior (3 hours) as well as concurrent (1 hour) treatment with staurosporine (Fig 7, lane 4) or H7 (Fig 7, lane 6) had no significant effect on mitoxantrone-induced alterations in c-jun expression, although c-myc repression was further enhanced by staurosporine but not by H7 pretreatment. Stau-

rosporine or H7 alone had no significant effect on BCL-2 expression, and they also did not affect BCL-2 repression due to mitoxantrone (data not shown). Importantly, these PKC inhibitors had no effect on mitoxantrone-induced internucleosomal DNA fragmentation (data not shown). These results suggest that, whereas the PKC stimulator PMA enhanced mitoxantrone-induced c-jun expression and internucleosomal DNA fragmentation, PKC inhibitor staurosporine did not inhibit these effects of mitoxantrone. It should also be noted that in both Figs 6 and 7 c-jun RNA expression in untreated HL-60 cells was greater than those used in the experiments whose results have been presented in Fig 5. This discrepancy may be due to the differences in cell density and differentiation status, or due to slight differences in the duration of washing of control HL-60 cells with serum-free medium, in different experiments, before exposure to the various treatment conditions.

**DISCUSSION**

Previous in vitro studies have shown that mitoxantrone concentrations between 0.1 and 10 μmol/L exhibit a steep dose-response relationship with respect to the clonogenic survival of AML blasts as well as for their self-renewal capacity.8 These concentrations are clinically achievable with the regimens containing high-dose mitoxantrone that have been shown to have significant clinical activity in leukemias.1,2,7,35 The cytotoxic effect of mitoxantrone is thought to be related to its binding to DNA as well as to its inhibition of DNA topoisomerase II by trapping it in a cleavable, covalent, topoisomerase-DNA ternary complex.36 The accumulation of mitoxantrone-stabilized cleavable complexes that sequester DNA strand breaks is also associated with the inhibition of DNA synthesis and the arrest of cells in G2 phase of the cell cycle.37,38 Although other mechanisms for mitoxantrone-induced DNA damage involving free radical production and the inhibition of DNA helicase II activity have been proposed,39,40 the mechanisms underlying its steep dose-response relationship for the inhibition of clonogenic survival of AML blasts has not been elucidated. Recently, a variety of DNA interactive drugs have been shown to induce leukemic cell death by the alternative process of PCD.11,12,21,23 The biochemical hallmark of this gene-directed, active form of cell death is the nuclear endonucleasc-mediated internucleosomal DNA fragmentation.14,15

Previous studies have correlated the induction of internucleosomal DNA fragmentation by the antileukemic drugs with their inhibition of clonogenic survival of leukemic cells.11,22 In these studies, the drug-induced internucleosomal DNA fragmentation was also associated with marked induction of c-jun and downregulation of the expression of BCL-2 and c-myc genes.11,23 In the present report, we have shown that the concentrations of mitoxantrone ranging between 0.1 and 10 μmol/L produce a steep decline (by several logs) in clonogenic survival of myeloid leukemia cells that corresponds with increasing amounts of internucleosomal DNA fragmentation characteristic of PCD. This DNA fragmentation is associated with a marked induction of c-jun but a decrease in BCL-2 and c-myc oncogene expression. However, it should be noted that, with respect to c-jun, only an association and not a cause and effect relationship has been established between its induction and the DNA fragmentation associated with apoptosis.

The gene product of the oncogene BCL-2 is an inner mitochondrial membrane protein that blocks PCD.19 In pre-B-leukemia cells, high levels of BCL-2 expression block glucocorticoid-induced apoptosis.4 Recently, the transfection and stable expression of BCL-2 in thymocytes renders these cells relatively resistant to a variety of drugs known to induce internucleosomal DNA fragmentation associated with apoptosis.21 It has also been previously reported that in the cells expressing low levels of BCL-2 a continuous decline in c-myc expression may be responsible for their growth arrest and apoptosis.42 Our findings show that HL-60 cells express relatively low levels of BCL-2. Also, 1.0 and 10.0 μmol/L mitoxantrone produced a marked inhibition in BCL-2 expression with a concomitant decline in c-myc expression, thereby facilitating the induction of apoptosis. It is also noteworthy that HL-60 cells lack the expression of wild-type tumor suppressor p53 gene, which has been shown to induce apoptosis without differentiation when transfected into myeloid leukemia cells.43,44 Therefore, it is conceivable that the myeloid leukemia cells that lack a functioning wild-type p53 gene and possess low levels of BCL-2 express may be particularly susceptible to the induction of PCD by increasing concentration of DNA interactive drugs such as Ara-C and mitoxantrone.

Because PCD is an active process of gene directed cellular self-destruction, the molecular signals that govern this process and are triggered before the induction of apoptosis by mitoxantrone would be important targets for modulation in an attempt to selectively facilitate PCD in myeloid leukemia cells. Although Ara-C has been shown to activate PKC...
and induce c-jun and AP-1 transcription factors, the molecular signal responsible for mitoxantrone-induced c-jun expression remains to be elucidated. Although the stimulation of PKC has been shown to block glucocorticoids induced PCD in lymphocytes, in sarcoma or leukemic cells it has resulted in the augmentation of doxorubicin-induced PCD.[15,16] Our results show that pretreatment with PKC stimulator PMA enhances mitoxantrone-induced c-jun expression and the internucleosomal DNA fragmentation. However, this occurred without significant effect on BCL-2 repression seen with mitoxantrone alone, which suggests that, in addition to BCL-2, other genes may have an important role in regulating apoptosis in leukemic cells. Unlike what has been observed for Ara-C, PKC inhibitors staurosporine and H7 neither inhibited mitoxantrone-induced c-jun expression nor affected mitoxantrone-mediated internucleosomal DNA fragmentation characteristic of PCD. These discordant results suggest that the intracellular PKC activity and c-jun expression do not directly control or may not be the sole regulators of mitoxantrone-induced internucleosomal DNA fragmentation of apoptosis.

High-dose Ara-C and mitoxantrone combination is an effective regimen for the treatment of AML.[12] The findings reported here highlight a possible mechanism for the anti-leukemic activity and the steep dose response effect of high-dose mitoxantrone against AML. Taken together with similar observations reported for high-dose Ara-C, these results offer an explanation for the effectiveness against AML of the salvage regimen incorporating the two drugs. In addition, our results also underscore a mechanism of cell death mediated by these drugs that can potentially be modulated to augment their antileukemic activity.

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High-dose mitoxantrone induces programmed cell death or apoptosis in human myeloid leukemia cells

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