Evidence against a Direct Role for the Induction of c-jun Expression in the Mediation of Drug-induced Apoptosis in Human Acute Leukemia Cells

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ABSTRACT

Previous reports have demonstrated that a variety of anticancer drugs, e.g., 1-β-D-arabinofuranosylcytosine (ara-C), mitoxantrone, etoposide, camptothecin, and cisplatin, induce the expression of c-jun oncogene in leukemic cells prior to producing internucleosomal DNA fragmentation and the morphological features of apoptosis. This has led to the impression that the induction of c-jun expression may be directly involved in the molecular signaling of the final common pathway of programmed cell death or apoptosis. In the present study, we examined the role of c-jun expression in three different settings of anticancer drug-induced apoptosis in human leukemic cells. First, exposure of human myeloid leukemia HL-60 cells to high-dose ara-C for 4 h produced internucleosomal DNA fragmentation preceded by c-jun induction. However, pretreatment of HL-60 cells with staurosporine, a protein kinase C inhibitor, repressed c-jun yet enhanced DNA fragmentation and apoptosis due to ara-C. Second, in human pre-B leukemia 697/BCL-2 cells which are transfected with the cDNA of the bcl-2 oncogene and overexpress p26BCL-2, although ara-C or mitoxantrone treatment caused greater c-jun induction than in the 697/neo cells, significantly reduced endonucleolytic DNA fragmentation and apoptosis was observed in 697/BCL-2 cells. Finally, taxol-induced internucleosomal DNA fragmentation and morphological features of apoptosis in HL-60 cells were not associated with the induction of c-jun expression. These lines of evidence indicate that the induction of c-jun expression may not have a direct role in the molecular signaling of anticancer drug-induced apoptosis, and that the anticancer drug-induced apoptosis can occur by a mechanism that does not involve the induction of c-jun expression.

INTRODUCTION

A variety of anticancer drugs including ara-C, cisplatin, etoposide, camptothecin, and taxol, which interact with diverse intracellular molecular targets, have been shown to engage the final common pathway of programmed cell death, resulting in apoptosis in human leukemic cells (1-5). This is characterized by the biochemical hallmark of endonucleolytic internucleosomal DNA fragmentation and the morphological features of apoptosis (6, 7). Recent studies with cells transfected with the cDNA of bcl-2 gene have indicated that the overexpression of p26BCL-2 protein inhibits anticancer drug-induced apoptosis (8-11). This resistance to apoptosis mediated by p26BCL-2 has been shown to occur despite normal interactions between the antileukemic drugs and their intracellular molecular targets (10, 11). The molecular signaling which results in the activation of the endonuclease and the final steps of apoptosis have been the focus of many studies. Although a common theme has not emerged, these studies have demonstrated that in many cases the drug-induced internucleosomal DNA fragmentation is preceded by the induction of c-jun proto-oncogene (2-4). c-jun is a member of a family of early response genes which encode for sequence-specific, bZIP DNA-binding proteins (12). As a homodimer, or a heterodimer with the c-fos protein, c-jun protein forms the AP-1 transcription factor involved in the transcriptional regulation of genes responsive to phorbol esters or growth factors (13). Therefore, AP-1 can modulate the transcription of a variety of genes which may affect cell proliferation and differentiation (14). Recent reports have implicated PKC activation as a step leading to the induction of c-jun in the molecular cascade leading to apoptosis induced by antileukemic drugs, e.g., cisplatin, camptothecin, and ara-C (2-4, 15). Indeed, in separate reports, the modulation of PKC activity has been shown to affect ara-C-mediated c-jun induction as well as apoptosis (15, 16). Taken together, these results have suggested that c-jun induction may play a direct role in drug-induced apoptosis in leukemic cells.

In this report, utilizing the three separate settings of ara-C-, mitoxantrone-, or taxol-induced apoptosis in human leukemia cells possessing normal or high p26BCL-2 levels, we present evidence indicating that the induction of c-jun does not play a direct role in the mediation of apoptosis due to anticancer drugs.

MATERIALS AND METHODS

Cells. Human pre-B leukemia 697/neo cells and 697/BCL-2 cells, which had been transfected with the cDNA of the bcl-2 gene and possess high levels of p26BCL-2, were main-
tained in culture in RPMI 1640 medium with 10% fetal bovine serum as previously described (8). The human myeloid leukemia HL-60 cells were derived from the original lines (17). They are maintained in suspension culture as previously described (5). Logarithmically growing cells were utilized for all experiments.

**Drugs and Chemicals.** Taxol was kindly provided free of cost by Dr. Patricia A. Pilia of NaPro Bio Therapeutics, Inc. (Boulder, CO). Taxol was made fresh for each experiment. It was dissolved in 100% DMSO to make a stock solution of 10.0 mg/ml. The human myeloid leukemia HL-60 cells were purchased from either Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific Co. (Springfield, NJ). Drugs were stored as dry powders at -20°C and reconstituted in sterile medium prior to use. Mitoxantrone was kindly provided by Lederle Laboratories (Pearl River, NY). Staurosporine was purchased from Sigma Chemical.

**Analyses of Drug-induced Internucleosomal DNA Fragmentation.** Internucleosomal DNA fragmentation was assayed by a modification of a previously described method (5). Cells were treated with the designated concentrations and schedule of drugs. At the end of the incubations, cells were washed and resuspended in drug-free media for varying intervals. Following this, 2 × 10⁶ cells from each condition were pelleted and resuspended in lysis buffer as previously described (5). The suspension was centrifuged at 27,000 × g for 20 min to separate low molecular weight DNA from intact chromatin (5). DNA was extracted from the supernatant by treatment with phenol:chloroform:isoamyl alcohol (25:24:1). To the upper aqueous phase, centrifuged, and the pellet dried. DNA samples were resuspended in lysis buffer and incubated for 30-60 min at 70°C. Following a cold centrifugation for 30 min at 10,000 rpm, the supernatant was discarded and the pellet was resuspended in 0.5 ml Tris-EDTA buffer at pH 8.0. After adding 10 μl DNase-free RNase A to the suspension, it was mixed by gentle agitation and incubated for 30 min at 37°C. To this, 50 μl 5 M NaCl was added and the phenol/chloroform/isoamyl alcohol extraction was repeated. DNA was precipitated with ethanol as above, centrifuged, and the pellet dried. DNA samples were mixed with 16 μl (5 × 10⁶ cells) Tris-EDTA (pH 8.0) loading buffer and 2 μl 5X 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 1X Tris-acetate-EDTA running buffer containing ethidium bromide. DNA was separated over 90 min at 60 V and the bands were visualized by UV illumination.

**RNA Extraction and Northern Blot Analysis.** Total cellular RNA was extracted by the guanidine thiocyanate-phenol-chloroform method (18). Following exposure to the designated concentrations and schedule of drugs, cells were washed with PBS and homogenized in GIT buffer (4 M guanidine isothiocyanate, 2 M sodium acetate, pH 4, 0.1 M 2-mercaptoethanol). The homogenate was then treated with 10% Sarkosyl, 2 M sodium acetate, and the total cellular RNA extracted through a phenol:chloroform:isoamyl alcohol suspension (25:24:1) and subsequent centrifugation. The resulting DNA 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 a A260:A280 ratio of 1.95. The levels of c-jun and β-actin RNA are determined by Northern blot analysis as previously described (5, 19) using the following ³²P-labeled probes: 1.8-kilobase BamHI/EcoRI insert of a human c-jun cDNA purified from a pBluescript SK(+) plasmid and the human-actin probe was a 600-base pair EcoRI/BamHI fragment from the KSII(+) plasmid. The probes were kindly provided as follows: Dr. Donald Kufe of the Dana-Farber Cancer Center (Boston, MA), c-jun, and Dr. James S. Norris of the Medical University of South Carolina (Charleston, SC), β-actin.

**Assessment of the Morphology of Apoptosis and Cell Viability of Leukemic Cells.** Briefly, following incubations with the designated concentrations and schedules of drugs, cells were cytospun onto glass slides and stained with Wright stain. Cell morphology was determined by light microscopy. Five different fields were randomly selected for counting at least 500 cells. Percentage of apoptotic cells was calculated for each experiment. Cells designated as apoptotic were those which displayed the characteristic morphological features of apoptosis including cell volume shrinkage, chromatin condensation, and the presence of membrane-bound apoptotic bodies (7). The assessment of the percentage of apoptotic cells was confirmed by an additional independent observer who was blinded to the results of the first observer. Alternatively, cells were stained with trypan blue to assess the percentage of dead cells unable to exclude the dye (5).

**Statistical Analysis.** Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions were determined by paired t test analyses.

**RESULTS**

Since cotreatment with staurosporine has been previously shown to inhibit ara-C-mediated c-jun RNA induction (15), the effect of staurosporine on ara-C-induced internucleosomal DNA fragmentation and apoptosis was examined in human myeloid leukemia HL-60 cells (Fig. 1). The top pane! of Fig. 1 is a Northern blot of RNAs hybridized with c-jun cDNA probe. As previously reported (1), it demonstrates that compared to the untreated HL-60 cells (Lane 1) exposure to 10 μmol/liter ara-C for 4 h results in a significant induction of c-jun in HL-60 cells (Lane 2). Cotreatment with 5 ng/ml staurosporine markedly inhibited c-jun induction due to ara-C (Lane 4 versus Lane 2), while treatment with staurosporine alone had no significant affect on c-jun RNA levels in untreated HL-60 cells (Lane 3). The bottom panel! of Fig. 1 shows that treatment with 5 or 50 ng/ml staurosporine for 4 h did not produce internucleosomal DNA fragmentation in HL-60 cells, while exposure to 10 μmol/liter ara-C for 4 h produced the characteristic ladder of oligonucleosomal DNA fragments of 200-base pair integer multiples in size. The Fig. 1 also shows that the combined treatment with 5 or 50 ng/ml staurosporine and ara-C versus ara-C alone significantly increased the intensity of the DNA fragmentation ladder (bottom panel), as well as produced a 2-fold increase (mean of three separate experiments) in the percentage of cells showing the morphological features of apoptosis. These results indicate that despite a significant inhibition of c-jun expression,
staurosporine augments ara-C-induced internucleosomal DNA fragmentation and apoptosis, thereby making it very unlikely that c-jun expression plays a direct role in ara-C-induced apoptosis.

ara-C and mitoxantrone-induced c-jun RNA expression and internucleosomal DNA fragmentation was also determined in the human pre-B leukemia 697/neo and 697/BCL-2 cells. In contrast to HL-60 cells, treatment of 697/neo cells with 10 μmol/liter ara-C produced only a slight increase in c-jun RNA expression. But, as seen in HL-60 cells, exposure of 697/neo cells to 1.0 μmol/liter mitoxantrone (Fig. 3, Lanes 2 and 3) for either 4 or 24 h had no significant effect on c-jun RNA expression. However, as previously reported, these concentrations and schedules of taxol showed features of apoptosis revealed that following treatment with ara-C and mitoxantrone, a significantly lower percentage of 697/BCL-2 (2.0 + 1.2 and 2.5 + 1.5%, respectively) versus 697/neo (18.5 + 2.4 and 21.7 + 1.5%, respectively) cells showed morphological features of apoptosis (P < 0.01). These results indicate that although ara-C or mitoxantrone treatment produces greater induction of c-jun expression in 697/BCL-2 cells, this did not result in an increase in the percentage of cells expressing features of apoptosis or overcome the inhibitory effect of high p26BCL-2 levels on ara-C-induced apoptosis in 697/BCL-2 cells. This again suggests the lack of a direct role for c-jun in mediating apoptosis.

We have previously reported that the non-DNA interactive, antimicrotubule drug taxol produces intracellular microtubular bundling and cell cycle growth arrest in G2-M phase in HL-60 cells, which results in internucleosomal DNA fragmentation and apoptosis (5). Fig. 3 demonstrates the effects of 4 (left panels) and 24 h of treatment (right panels) with various concentrations of taxol on c-jun RNA expression (top panels) and internucleosomal DNA fragmentation in HL-60 cells. As shown in the top panels of Fig. 3, the exposure to 0.1 and 1.0 μmol/liter taxol (Fig. 3, Lanes 2 and 3) for either 4 or 24 h had no significant effect on c-jun RNA expression. However, as previously reported, these concentrations and schedules of taxol produced the

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Fig. 1. Top and middle panels, Northern blots of electrophoresed RNA from HL-60 cells hybridized to 32P-labeled cDNA probes of c-jun and β-actin genes. RNA in each lane was extracted from cells treated as follows: Lane 1, untreated control cells; Lane 2, 10.0 μM ara-C; Lane 3, 5 ng/ml staurosponine; and Lane 4, cotreated with 5 ng/ml staurosponine and 10 μM ara-C. Lanes in the bottom panel, ethidium bromide-stained, electrophoresed, fragmented DNA purified from the supernatant of untreated control cells (C), cells treated with staurosponine (ST) alone, 10.0 μM ara-C alone (Ara-C), or cotreatment with ara-C and ST. Lane M, 123-base pair marker DNA ladder.

Fig. 2. Top and middle panels, Northern blots of electrophoresed RNA; bottom panel, ethidium bromide-stained, electrophoresed, fragmented DNA purified from the supernatant of 697/neo (Lanes 1–3) and 697/BCL-2 cells (Lanes 4–6) hybridized to 32P-labeled cDNA probes of c-jun and β-actin genes. RNA (top and middle panels) or DNA was extracted from cells treated as follows: Lanes 1 and 4, untreated control cells; Lanes 2 and 5, 10.0 μM ara-C; and Lanes 3 and 6, 1.0 μM mitoxantrone (see text).
characteristic internucleosomal DNA fragmentation of apoptosis in HL-60 cells (5). Although not shown, shorter exposures to these and higher concentrations of taxol (e.g., 10 μmol/liter) for 0.5, 1.0, and 2 h also had no significant effect on c-jun RNA expression in HL-60 cells.

**DISCUSSION**

Because PCD is an active process of gene-directed cellular self-destruction (7), the molecular signals which control this process leading to apoptosis would be important targets to modulate to selectively promote apoptosis of cancer cells. Previous reports have highlighted the fact that several anticancer drugs including ara-C, etoposide, and cisplatin, which cause DNA damage, induce c-jun expression preceding internucleosomal DNA fragmentation and apoptosis (1–3). Also, the induction of a continuous c-fos expression has been shown to precede etoposide-induced apoptosis (20). In contrast, suppression of c-fos induction in UV-irradiated epidermis by c-fos antisense oligodeoxynucleotides did not affect UV-induced apoptosis of epidermal cells (21). Furthermore, dexamethasone- or γ-irradiation-induced overexpression of c-jun mRNA during apoptosis of CEM C7 cells was not found to be accompanied by either increased expression of c-jun protein or increased AP-1 binding activity (22). These conflicting data raise concerns whether the induction of c-fos and c-jun directly controls drug-induced apoptotic cell death or is involved in the repair of drug-induced DNA damage (1–3, 20, 21). The results presented here demonstrate that early c-jun induction following treatment of human leukemic cells with DNA-interactive drug ara-C is unlikely to have a direct role in ara-C-induced apoptosis.

In two previous reports, similar to this, Gunji et al. (1) and Kharbanda et al. (15) have presented the evidence that ara-C-mediated increase in c-jun expression was due to the activation of PKC, and the inhibition or down-regulation of PKC was associated with the inhibition of ara-C-induced c-jun expression. Besides being one of the most potent inhibitors of PKC with an 50% inhibitory concentration value between 2 and 10 nm (23), at higher concentrations staurosporine can inhibit other protein kinases including p34cdc-2 kinase (24). The present report demonstrates that although staurosporine suppresses ara-C-induced c-jun expression, it enhances ara-C-mediated internucleosomal DNA fragmentation and apoptosis. Jarvis et al. (16) have also shown that PKC inhibitors enhance ara-C-induced apoptosis. They have also reported that the ability of bryostatin-1 to potentiate ara-C-mediated apoptosis is related to the down-regulation of total PKC activity by bryostatin-1 (16). These, and our observations, are consistent with a previous report demonstrating that the phorbol ester phorbol dibutyrate, which activates PKC and results in the induction of c-jun expression, blocks glucocorticoid-mediated apoptosis in immature thymocytes (25). Collectively, these reports demonstrate that an increase or decrease in drug-induced apoptosis by modulators of PKC activity is associated with just the opposite effect on c-jun expression. The cytotoxic effects of another DNA damaging agent cisplatin, which induces c-jun, have also been shown to be synergistically enhanced by PKC inhibitors (26, 27). However, the authors speculated that this may be due to the interference in the function of p21ras-controlled signaling cascade, ultimately attenuating the repair of platinated DNA (23, 27). Following growth factor deprivation that results in apoptosis of lymphoid cell lines, although c-jun was induced and its inhibition by antisense oligonucleotides was shown to improve the survival of these growth factor-deprived lymphoid cells, it was not clearly demonstrated that this occurred due to suppression of apoptosis (28). More important, recent evidence has demonstrated that in a dominant negative U937 transactivation mutant for c-jun (TAM-67) exposure to ara-C induces apoptosis but not c-jun expression, while both c-jun and apoptosis are induced in the parental U937 cells (29). These data also cast a doubt on a direct role of c-jun induction in mediating apoptosis.

Our results in 697/neo and 697/BCL-2 cells also highlight the absence of a direct correlation between high-dose ara-C or mitoxantrone-induced c-jun expression and internucleosomal DNA fragmentation or apoptosis. In 697/BCL-2 cells which contain high p26BCL-2 levels, the induction of significantly greater c-jun expression by these drugs was not associated with apoptosis. Although the precise mechanism(s) by which high intracellular levels of p26BCL-2 retard apoptosis due to a variety of drugs has not been elucidated, p26BCL-2 has recently been shown to reduce the net cellular generation of reactive oxygen species and lipid peroxidation (30, 31). However, this is not known to be a mechanism underlying the cytotoxic effects of high-dose ara-C. If the activation of PKC and c-jun induction had a direct role in apoptosis, a markedly greater c-jun induction by ara-C and mitoxantrone would have resulted in appreciable...
apoptosis in at least a small percentage of 697/BCL-2 cells. In the absence of this, it is difficult to assign a significant or direct role to c-jun induction in mediating apoptosis. This is further supported by our finding that the non-DNA-interactive drug taxol, although an effective mediator of apoptosis does not induce c-jun expression in HL-60 cells. However, the possibility remains that multiple distinct pathways to apoptosis may exist and the one engaged by taxol may not involve c-jun induction (32).

Proliferating cells have been shown to express higher levels of c-jun mRNA, and amplified expression of c-jun has been shown to be associated with the inhibition of differentiation (14, 33). In contrast, inhibition of c-jun expression causes entry into G0 and growth arrest (34, 35). These observations highlight c-jun as a growth-regulatory gene, which makes it unlikely that increased c-jun expression would also play a direct role in mediating internucleosomal DNA fragmentation and apoptosis. The results presented in this report as well as other recent data clarify this issue by demonstrating that anticancer drug-induced apoptosis occurs in the absence of c-jun RNA induction (32). However, the potential role of c-jun and c-fos induction through the AP-1 transcription factor in regulating the genes involved in the repair of drug-induced DNA damage remains a valid hypothesis worthy of further investigation.

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