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Role of c-*jun* in Human Myeloid Leukemia Cell Apoptosis Induced by Pharmacological Inhibitors of Protein Kinase C

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SUMMARY

Recent study results suggest that protein kinase C [PKC (EC 3.1.4.3)]-dependent up-regulation of c-*jun* may be involved in leukemic cell programmed cell death, or apoptosis, occurring in response to various chemotherapeutic agents. The current study was undertaken to further evaluate the contribution of c-*jun* in apoptosis with the use of two highly specific pharmacological inhibitors of PKC (calphostin C and chelerythrine). To address this issue, two human leukemic cell lines, HL-60 and U937, and a U937 subline stably expressing a dominant negative c-*jun* mutant (TAM67) were exposed to calphostin C and chelerythrine, and c-*jun* expression was monitored at both the mRNA and protein levels. Both PKC inhibitors induced the classic morphological features of apoptosis as well as internucleosomal DNA degradation in a concentration- and schedule-dependent manner. Concomitant with these changes, unequivocal increases were observed in c-*jun* mRNA (U937 and HL-60) and protein (U937). In contrast, up-regulation of c-*jun* mRNA and protein in TAM67-expressing cells exposed to both PKC inhibitors was markedly attenuated relative to effects observed in parental U937 cells. Importantly, despite impaired up-regulation of c-*jun* at both the message and protein levels, TAM67-expressing cells were equally susceptible to PKC inhibitor-induced apoptosis as parental and empty vector U937 cells. Collectively, these findings raise the possibility that c-*jun* up-regulation in human myeloid leukemia cells undergoing PKC inhibitor-associated apoptosis represents a response to, rather than a cause of, apoptotic events. They further suggest that this phenomenon involves pathways that do not require PKC activation.

The proto-oncogene c-*jun* is a member of the leucine-zipper family of transcription factors (1). Other members of this gene family include *jun*-B, *jun*-D, c-*fos*, *fos*-B, and *fra*-1. Proteins encoded by these oncogenes combine to form homodimers and heterodimers, which then bind to upstream DNA promoter sequences regulating rates of transcription (1, 2). These genes, collectively referred to as IER genes, are strongly but transiently induced by the addition of serum or phorbol esters to quiescent cells (3–5). The c-*jun* gene product JUN exerts its influence as a major component of the AP-1 transcription factor, which recognizes and binds to the DNA consensus sequence $TGA^{G}/_{C}TCA$ (also known as an 12-*O*-tetradecanoylphorbol-13-acetate-response element) (6–8). Increased levels of AP-1, in turn, alter gene expression in response to stimuli. Thus, c-*jun* plays an important role in regulating gene expression in response to numerous mitogenic stimuli and to agents that induce differentiation (9–12).

The role of c-*jun* and other IER genes has been examined in programmed cell death. Several studies have demonstrated a temporal correlation among c-*jun* mRNA up-regulation, increased AP-1 binding, and the induction of apoptosis. For example, Rubin *et al.* (13) showed an increase in the level of c-*jun* mRNA, mediated by an increase in the rate of c-*jun* transcription, concomitant with the induction of oligonucleosomal DNA cleavage in HL-60 cells treated with the topoisomerase II inhibitor etoposide. Also, Kim *et al.* (14) showed that induction of c-*jun* mRNA in multidrug-resistant CEM cells was progressively attenuated in proportion to the degree of resistance of the cells to teniposide, another topoisomerase II inhibitor. Others have shown an increase in c-*jun* mRNA after the administration of various anticancer drugs to human myeloid leukemia cells (15–17). However, a direct causal relationship between c-*jun* up-regulation and

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ABBREVIATIONS: IER, immediate early response; PKC, protein kinase C; ara-C, 1-β-D-arabinofuranosylcytosine; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CP, calphostin C; CE, chelerythrine; DMSO, dimethylsulfoxide; NGF, nerve growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis, AP-1, activator protein 1.

drug-induced apoptosis has not been established. In contrast, Colotta *et al.* (18) demonstrated that the induction of c-*jun* mRNA in malignant lymphoid cells after growth factor withdrawal was abrogated by the addition of antisense oligonucleotides directed against c-*fos* and c-*jun*. Furthermore, they showed that such antisense oligonucleotides protected cells from growth factor deprivation-induced apoptosis. Similarly, Estus *et al.* (19) showed that antibodies to c-*jun* protein protected murine neurons from NGF deprivation-induced apoptosis, and Ham *et al.* (20) showed that a c-*jun* dominant negative mutant, when microinjected into sympathetic neurons, protected cells from NGF withdrawal-induced apoptosis. Thus, in lymphoid and neuronal cells, there is direct evidence that c-*jun* may play a regulatory role in apoptosis associated with growth factor deprivation. Finally, in human myeloid leukemia cells, it has been reported that certain agents (i.e., taxol) induce apoptosis without prior up-regulation of c-*jun* (21). Together, these observations suggest that the relationship between up-regulation of c-*jun* and the induction of apoptosis is cell line and stimulus dependent.

The role that PKC plays in the up-regulation of c-*jun* after treatment of myeloid cells with phorbol esters (22), chemotherapeutic drugs [i.e., ara-C (15)], and UV irradiation (23) remains the subject of debate. Previous reports have shown that up-regulation of c-*jun* in human leukemia cells exposed to ara-C (24) and *cis*-diamminedichloroplatinum(II) (17) can be blocked by nonspecific PKC inhibitors such as H7 and staurosporine. However, these studies did not specifically address whether interference with c-*jun* up-regulation antagonizes drug-induced apoptosis. In fact, we (25) and others (26, 27) have shown that PKC inhibitors, by themselves, are potent inducers of leukemic cell apoptosis in hematopoietic cells. Moreover, PKC inhibitors may, under some circumstances, potentiate drug-induced apoptosis (21, 28) and associated c-*jun* up-regulation (28). Such results raise the possibility of a PKC activation-independent pathway of gene regulation and programmed cell death.

Currently, little direct evidence exists linking c-*jun* expression and apoptosis in human myeloid leukemia cells. The first aim of this study was to characterize the relationship between c-*jun* expression and apoptosis in HL-60 and U937 cells induced by the PKC inhibitors CP and CE. The second aim of this study was to gain further insights into the functional role of c-*jun* through the use of a dominant negative c-*jun* construct. To this end, a U937 cell line stably transfected and expressing a c-*jun* dominant negative transactivation domain-deficient protein (TAM67) was used to determine whether dysregulation of c-*jun* might prevent or attenuate PKC inhibitor-induced apoptosis. Our results indicate that leukemic cells undergoing apoptosis in response to specific PKC inhibitors display up-regulation of c-*jun* message and, in some cases, protein. However, in contrast to lymphoid and neuronal cells subjected to growth factor deprivation (18–20), dysregulation of c-*jun* does not protect human myeloid leukemia cells from PKC inhibitor-induced apoptosis.

Materials and Methods

Drugs and reagents. CP (Sigma Chemical Corp., St. Louis, MO) [EC_{50} (PKC) = 50 nM; EC_{50} (PKA) ≥ 50 μM] (29) was diluted in sterile water. CE (LC Laboratories, Woburn, MA) [EC_{50} (PKC) = 0.66 μM; EC_{50} (PKA) = 170 μM] (30) was diluted in sterile DMSO. All stock reagents were stored at −20° under light-free conditions and diluted to appropriate final concentrations in the media. Vehicle controls of water and DMSO (≤0.01%) were included in all experiments and consistently found to be equivalent to drug-free controls in gene expression, protein expression, and occurrence of apoptosis.

Cell culture and drug exposure. The human promyelocytic leukemia cell line HL-60 was derived from a cell line originally described by Gallegher *et al.* (31). The human monocytic cell line U937 was obtained from American Type Culture Collection (Rockville, MD) and was isolated as previously described (32). U937 cell lines expressing TAM67 (c-*jun* dominant negative mutant lacking most of the transactivation domain, amino acids 3–122) were established by introducing pMexMet-Neo-TAM67 into U937 cells via cationic liposome transfection (lipofectin; Life Technologies, Gaithersburg, MD) and selection in 200 μg/ml G418 (33). The pMexMet-Neo-TAM67 plasmid is a eukaryotic expression vector in which the TAM67 deletion mutant is under the control of the mouse metallothionein I promoter (33, 34). All cells were grown in RPMI 1640 (phenol red-free formulation; Sigma) supplemented with 0.2% sodium bicarbonate, 1.0% sodium pyruvate, nonessential amino acids, L-glutamine, penicillin/streptomycin/neomycin antibiotic mix (Sigma), and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT); TAM67 cells were also supplemented with 400 μg/ml geneticin G418 (Life Technologies). All cultures were maintained under a fully humidified atmosphere of 95% air/5% CO_2 at 37°. Cultures were routinely screened for mycoplasma contamination with a rapid hybridization assay for mycoplasma RNA (Gen-Probe, San Diego, CA) and consistently found to be mycoplasma free. Cell densities were determined with a Coulter counter, and cell viability was assessed with a hematocytometer and trypan blue exclusion. For experimental incubations, cells in log-phase growth were suspended at a density of 4×10^5 cells/ml containing the appropriate concentrations of inhibitor and maintained as described above. Incubations containing CP were constantly exposed to direct light to promote photoactivation (35). Experimental incubations were terminated through pelleting of the cells at 400 × *g* for 10 min and aspiration of the media. Cell pellets were subsequently prepared for procedures described below.

Northern blot analysis. Total cellular RNA was isolated from 1×10^7 cells with the use of RNA STAT-60 and protocol (Tel-Test, Inc., Friendswood, TX). Total RNA (15 μg) was then separated on a 1.2% agarose/formaldehyde gel as previously described (36). The RNA was blotted onto nylon (Schleicher and Schuell, Keene, MD) by upward capillary transfer for 16 hr and then cross-linked to the nylon by baking at 80° for 2 hr. The blots were then hybridized as previously described (25, 36) with a c-*jun* cDNA probe (provided by M. Karin, University of California, San Diego). To ensure equal loading, the blots were either simultaneously or subsequently hybridized with a GAPDH cDNA probe. Probes were nick-translated with [α-^{32}P]dCTP (3000 Ci/mM) (New England Nuclear, Boston, MA) with use of a kit and protocol from Life Technologies. The blots were then washed extensively in 0.2× standard saline citrate/0.1% SDS (1× standard saline citrate = 150 mM NaCl, 15 mM sodium citrate) at 65°, placed on Fuji RX film (Fuji Photo Film Co.) with intensifying screens, and exposed at −90°. Autoradiographs were quantified via laser densitometry (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis. Whole-cell pellets (1×10^7) were washed twice in PBS. Cell pellets were then resuspended in 50 μl PBS and lysed by the addition of 50 μl 2× loading buffer (1× = 30 mM Tris-base, pH 6.8, 2% SDS, 2.88 mM β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Lysates were boiled for 10 min, centrifuged at 12,800 × *g* for 5 min, and quantified with Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (100 μg) were separated by SDS-PAGE (5% stacker and 12% resolving) and electroblotted to nitrocellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure transfer and equal loading. The blots were then blocked in PBS-Tween (0.05%) and 5% nonfat dry milk for 1 hr at 22°. The blots were

incubated in fresh blocking solution with a 1:250 dilution of c-*jun* primary antibody, Ab 1 (Oncogene Science, Uniondale, NY), for 4 hr at 22°. Blots were washed for 5 min three times in PBS-T and then incubated with a 1:2000 dilution horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) for 1 hr at 22°. Blots were again washed for 5 min three times in PBS-T and then developed with the use of enhanced chemiluminesence (Amersham Life Sciences, Arlington Heights, IL). Autoradiographs were quantified via laser densitometry (Molecular Dynamics).

DNA fragmentation. Quantitative spectrofluorophotometry of apoptotic DNA fragments was performed as previously described (28). Briefly, cell pellets (3×10^6) were lysed overnight at 4° in 300 ml of 0.1% Triton X-100, 5 mM Tris·HCl, pH 8.0, and 20 mM EDTA. The lysates were centrifuged at 48,000 × *g* for 40 min at 4°, and the pellets were discarded. The presence of low molecular weight DNA fragments in all lysates was determined by diluting 20-μl samples in 980 μl assay buffer (3 M NaCl, 10 mM Tris·HCl, 1 mM EDTA, 1 μg/ml bisbenzimide trihydrochloride) and monitoring net fluorescence (λex = 365 nm, λem = 460 nm). DNA values were calculated against a calf thymus DNA standard and expressed as ng DNA/10^6 cells. Qualitative analysis of DNA fragmentation was performed with agarose gel electrophoresis as previously described (28). Briefly, cell pellets (2×10^7) were lysed overnight at 56° in 500 ml 0.1% Nonidet P-40, 10 mM Tris·HCl, pH 7.4, 25 mM EDTA, and 100 μg/ml proteinase K. The lysates were centrifuged at 48,000 × *g* for 40 min at 4°, and the pellets were discarded. The supernatants were treated with Ribonuclease A (100 μg/ml) for 4 hr at 37°. Equal volumes (40 μl) from each DNA sample were then separated on a 2% LMP agarose (Sigma) gel containing 200 ng/ml ethidium bromide at 4 V/cm for 2 hr.

Cell morphology. After inhibitor treatment, cytocentrifuge slides were prepared containing 8×10^4 cells/slide. Slides were stained with 20% Wright-Giemsa, and photomicrographs taken at 1000× showed typical cell morphology (Polaroid Microcam and 331 film, Polaroid, Atlanta, GA).

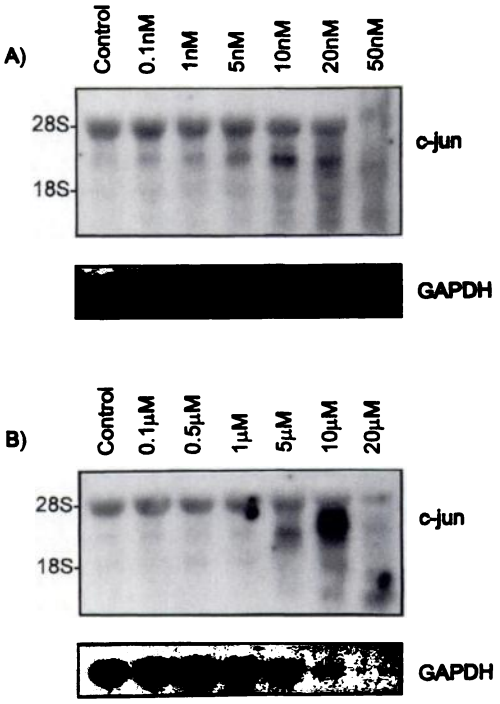


Fig. 1. Effects of increasing pharmacological PKC inhibitor concentrations on c-*jun* and GAPDH mRNA levels in HL-60 cells. HL-60 cells were treated with the indicated concentrations of CP (A) and CE (B) for 6 hr. *Control lanes*, RNA from untreated HL-60 cells. Total cellular RNA was isolated, and 15 μg/lane was separated and transferred to nylon as described in Materials and Methods. The blots were first hybridized to a c-*jun* proto-oncogene cDNA. The blots were then stripped and reprobed with a GAPDH cDNA (house-keeping gene) to monitor cellular integrity and loading disparities. *Left*, positions of the 28S and 18S rRNA bands. Two additional experiments yielded similar results.

Results

HL-60 cells were treated with increasing concentrations of two pharmacological inhibitors of PKC to determine their effects on c-*jun* expression. CP and CE are highly specific inhibitors of PKC that interact with the regulatory and catalytic domains of PKC, respectively (29, 30), and have been previously shown to induce apoptosis in HL-60 cells (25). Treatment with CP (0.1–50 nM; Fig. 1A) and CE (0.1–20 μM; Fig. 1B) for 6 hr induced a clear concentration-dependent increase in steady state levels of c-*jun* mRNA. Notably, there were low to undetectable levels of c-*jun* mRNA in untreated controls, whereas maximal up-regulation of c-*jun* was observed at concentrations of 10 nM CP and 10 μM CE. At the highest inhibitor concentrations evaluated (i.e., 50 nM CP and 20 μM CE), up-regulation of c-*jun* was not readily apparent because these concentrations were associated with extensive cellular disintegration and RNA degradation, accompanied by the virtual loss of expression of the house-keeping gene GAPDH. However, examination of earlier time points (i.e., 2–3 hr) at the highest inhibitor concentrations revealed maximal up-regulation of c-*jun* mRNA (not shown). The proportion of cells exhibiting classic apoptotic morphology after drug treatment increased progressively with inhibitor concentrations such that the majority of cells were apoptotic at concentrations of ≥10 nM CP and ≥20 μM CE (data not shown), as we previously reported (25). Vehicle controls of water (CP) and DMSO (≤0.01%; CE) were included in all experiments and consistently found to exert no effect on gene or protein expression or the induction of apoptosis.

In additional experiments conducted in HL-60 cells, we examined the time course of c-*jun* up-regulation induced by 10 nM CP and 10 μM CE. Again, levels of c-*jun* mRNA in drug-free controls were low to undetectable, whereas maximal up-regulation of c-*jun* was observed at 6 hr in CP-treated cells (Fig. 2A) and at 3–6 hr in CE-treated cells (Fig. 2B). In both cases, steady state mRNA levels of GAPDH had markedly declined by 6 hr. Extended time points of 12 and 24 hr showed decreased levels of c-*jun* and GAPDH mRNAs concomitant with decreased recovery of total RNA and increased RNA degradation (not shown).

The concentration response and time course of c-*jun* mRNA up-regulation induced by CP and CE was also examined in U937 cells. Treatment with CP (10 nM) for 3 hr and with CE (10 μM) for 6 hr induced a 3.6- and 2.4-fold increase in steady state c-*jun* mRNA levels, respectively, compared with drug-free controls and normalized to GAPDH (Fig. 3A). In contrast to HL-60 cells, U937 cells displayed considerably higher endogenous c-*jun* mRNA levels and greater up-regulation of c-*jun* in response to CP than to CE. However, as in the case of HL-60 cells, there was a progressive decline in steady state GAPDH mRNA levels in cells treated with increasing concentrations of both inhibitors, particularly CE. Also, extended time points of 12 and 24 hr showed decreased levels of c-*jun* and GAPDH mRNAs concomitant with decreased re-

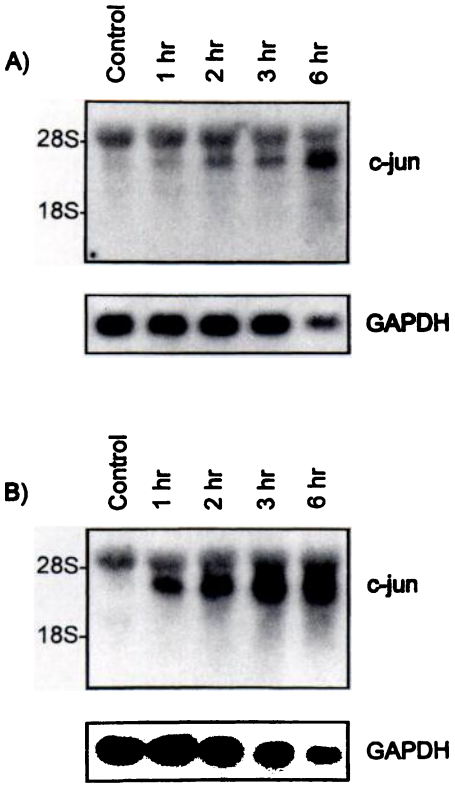


Fig. 2. Effects of increasing exposure time to pharmacological PKC inhibitors on c-*jun* and GAPDH mRNA levels in HL-60 cells. HL-60 cells were treated with 10 nM CP (A) and 10 μM CE (B) for the indicated times. *Control lanes*, RNA from untreated HL-60 cells. Total cellular RNA was isolated and hybridized as described in legend to Fig. 1. *Left*, positions of the 28S and 18S rRNA bands. Two additional experiments yielded similar results.

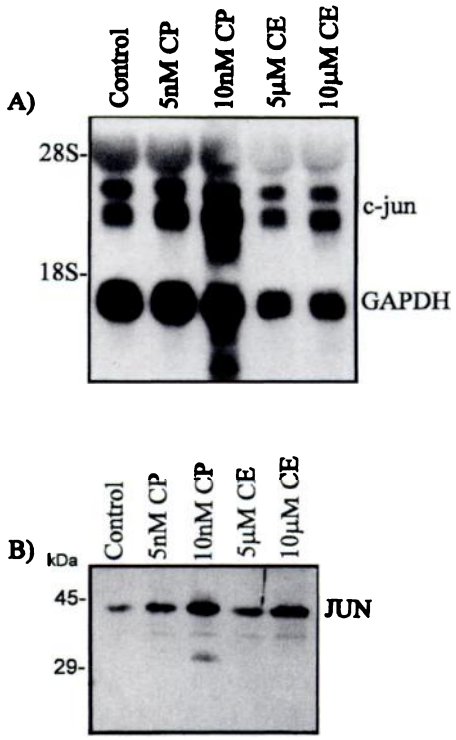


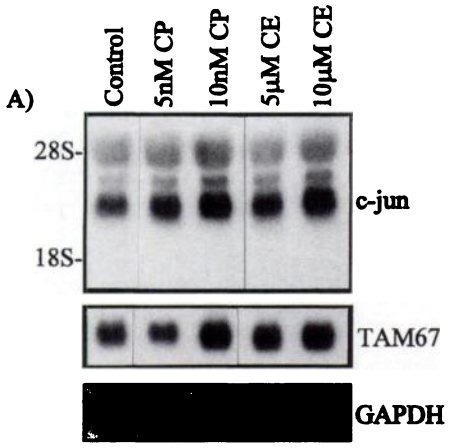
Fig. 3. Effects of increasing pharmacological PKC inhibitor concentrations on c-*jun* and GAPDH mRNA levels (A) and JUN protein levels (B) in U937 cells. Cells were treated with the indicated concentrations of CP and CE for 3 and 6 hr, respectively. *Control lanes*, RNA or protein from untreated cells. A, Total cellular RNA was isolated as described in legend to Fig. 1 and hybridized simultaneously to the c-*jun* and GAPDH cDNA probes. *Left*, positions of the 28S and 18S rRNA bands. B, Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed and developed as described in Materials and Methods. *Left*, migration of prestained molecular weight markers. *Right*, JUN protein. Three additional experiments yielded similar results.

covery of total RNA and increased RNA degradation (not shown).

The observed up-regulation of c-*jun* mRNA in two human leukemia cell lines led us to examine levels of c-*jun* protein (JUN) expressed in response to both PKC inhibitors. The expression of JUN in HL-60 cells under all conditions was essentially below the limits of detection (data not shown), whereas U937 cells expressed easily detectable basal JUN levels. When U937 cells were treated with CP (10 nM) and CE (10 μM) for 6 hr, there was a 5.6- and 5.0-fold increase in JUN expression, respectively, compared with drug-free controls (Fig. 3B). Maximal increases in protein were observed at concentrations associated with maximal stimulation of mRNA levels (i.e., 10 nM CP and 10 μM CE).

Having established a temporal association between the up-regulation of c-*jun* and the induction of apoptosis by pharmacological inhibitors of PKC, we next examined the role of c-*jun* in PKC inhibitor-induced apoptosis through the use of a U937 cell line stably expressing the c-*jun* dominant negative protein, TAM67 (33, 34, 37). We first examined levels of expression of c-*jun* and TAM67 (mRNA and protein) in cells exposed to CP (10 nM) and CE (10 μM). After inhibitor treatment, c-*jun* message levels increased 1.8- and 1.9-fold, respectively, whereas TAM67 message levels increased 1.8- and 1.7-fold, respectively, normalized to GAPDH (Fig. 4A). Similarly, levels of native JUN protein (45 kDa) increased slightly in TAM67-expressing cells (1.4-fold for both CP and CE), but the degree of up-regulation was substantially less than that observed in the parental line. Finally, levels of the truncated TAM67 protein (29 kDa) increased by only 1.2-fold at the higher concentration of both inhibitors (Fig. 4B).

Studies were subsequently conducted to determine whether dysregulation of c-*jun* in TAM67-expressing cells as demonstrated above would have an impact on PKC inhibitor-induced apoptosis, as observed in the case of other cell types subjected to growth factor deprivation. After 6-hr treatment with 10 nM CP and 10 μM CE, the formation of oligonucleosomal fragments was determined qualitatively and quantitatively. For each inhibitor, the degree of internucleosomal DNA fragmentation was essentially identical in each of the three cell lines (Fig. 5). Thus, drug-free controls yielded little or no fragmentation, 10 μM CE produced a definite oligonucleosomal DNA ladder, and 10 nM CP produced an intense oligonucleosomal DNA ladder. These results were confirmed through quantitative spectrofluorophotometric analysis of DNA, which revealed essentially equivalent amounts of low molecular weight DNA fragmentation in all three cell lines for each condition (Table 1). Consistent with the results of agarose gel electrophoresis, 10 nM CP produced the greatest amount of DNA damage, whereas cells treated with 10 μM CE showed a lesser degree of fragmentation. These results also correlated with cellular morphology after inhibitor treatment (Fig. 6). The large majority of U937- (Fig. 6B) and



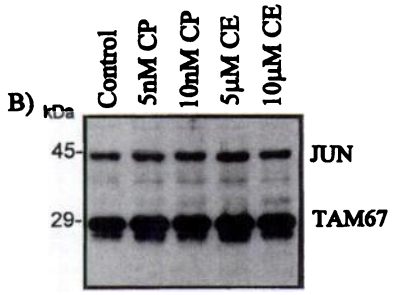


Fig. 4. Effects of increasing pharmacological PKC inhibitor concentrations on c-*jun*, TAM67, and GAPDH mRNA levels (A) and JUN and TAM67 protein levels (B) in TAM67-expressing cells. Cells were treated with the indicated concentrations of CP and CE for 3 and 6 hr, respectively. *Control lanes*, RNA or protein from untreated cells. A, Total cellular RNA was isolated as described in legend to Fig. 1 and hybridized sequentially to the c-*jun* and GAPDH cDNA probes. *Left*, positions of the 28S and 18S rRNA bands. B, Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed and developed as described in Materials and Methods. *Left*, migration of prestained molecular mass markers. *Right*, native (*JUN*) and mutant (*TAM67*) JUN proteins. Three additional experiments yielded similar results.

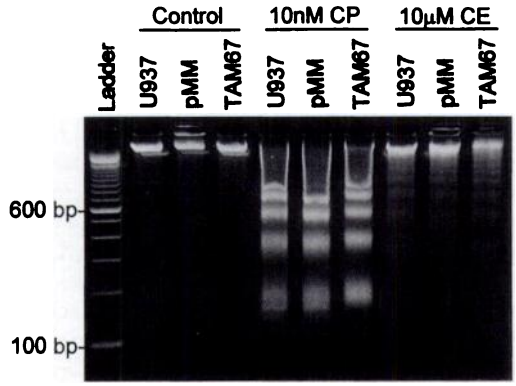


Fig. 5. Induction of internucleosomal DNA fragmentation by pharmacological inhibitors of PKC. Parental U937 cells, pMM cells (vector-alone), and TAM67-expressing cells were untreated (*Control*) or with 10 nM CP or 10 μM CE for 6 hr. The formation of oligonucleosomal DNA fragments was determined by agarose gel electrophoresis as described in Materials and Methods. Each lane was loaded with low molecular weight DNA from 2×10^6 cells. *Left*, a 100-base pair DNA ladder. Four additional experiments yielded comparable results.

TABLE 1

Effect of 10 nM CP and 10 μM CE on oligonucleosomal DNA fragmentation in U937, U937/pMM, and U937/TAM67 cells

Values are expressed as ng DNA/10^6 cells and represent mean ± standard error of triplicate determinations from a representative experiment repeated four times with comparable results.

Cells	Control	10 nM CP	10 μM CE
U937	260 ± 11	2790 ± 15	810 ± 21
U937/pMM	300 ± 8	3310 ± 55	760 ± 19
U937/TAM67	240 ± 7	3040 ± 54	790 ± 18

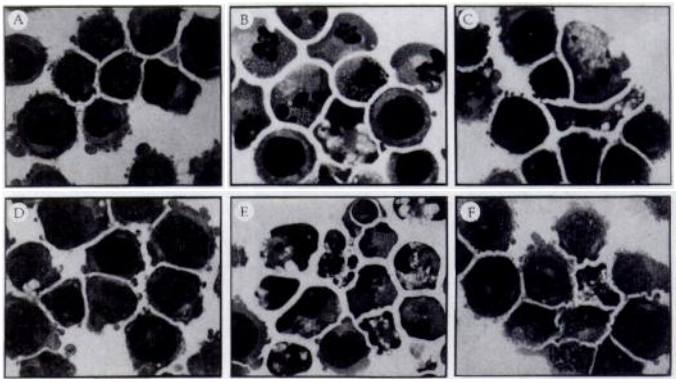


Fig. 6. Effect of pharmacological PKC inhibitors on cell morphology of U937- and TAM67-expressing cells. Cells were treated with 10 nM CP or 10 μM CE or were untreated (control) for 6 hr. Cytospin preparations were made containing 8×10^4 cells/slide and stained with Wright-Giemsa. Photomicrographs were taken at 1000×. A, U937 control. B, U937 10 nM CP. C, U937 10 μM CE. D, TAM67 control. E, TAM67 10 nM CP. F, TAM67 10 μM CE. Four additional experiments yielded comparable results.

TAM67- (Fig. 6E) expressing cells treated with 10 nM CP exhibited apoptotic features, including nuclear condensation, cell shrinkage, and the formation of apoptotic bodies. Although considerably fewer cells treated with 10 μM CE exhibited these features, equivalent percentages of U937- (Fig. 6 C) and TAM67- (Fig. 6F) expressing cells displayed characteristic apoptotic morphology. Moreover, slightly higher concentrations of CE (i.e., 20 μM) induced apoptotic changes equivalent to those observed after treatment with 10 nM CP in both cell lines (data not shown). Spontaneously apoptotic cells were rarely observed in both drug-free control cell lines. Finally, enumeration of apoptotic cells confirmed spectrofluorometric and morphological evidence that both the c-*jun* dominant negative cell line (U937/TAM67) and the parental cell line (U937) were equally sensitive to PKC inhibitor-mediated apoptosis (Table 2). Empty-vector U937 cells demonstrated apoptotic morphology equivalent to U937 parental and TAM67-expressing cells at all drug concentrations. Thus, in the U937 cell line, dysregulation of c-*jun* did not

TABLE 2

Effect of 10 nM CP and 10 μM CE on apoptotic morphology in U937, U937/pMM, and U937/TAM67 cells

Values are expressed as the percentage ± standard deviation of apoptotic cells and represent the mean of at least 500 cells counted from three separate experiments.

Cells	Control	10 nM CP	10 μM CE
U937	1.10 ± .13	72.0 ± 7.6	5.8 ± .91
U937/pMM	1.31 ± .23	66.4 ± 6.9	6.8 ± 1.4
U937/TAM67	0.92 ± .18	78.1 ± 8.5	6.2 ± 1.2

seem to interfere with the capacity of highly specific PKC inhibitors to induce an apoptotic form of cell death.

Discussion

Several previous studies have demonstrated temporal associations between up-regulation of the proto-oncogene c-*jun* and drug-induced apoptosis in myeloid leukemia cells and have suggested that the former event is PKC dependent (13, 15–17). In addition, correlations have been reported between an increase in AP-1 binding and the cytotoxic consequences of antineoplastic drug exposure (14, 38–40). Collectively, these findings have given rise to the hypothesis that activation of c-*jun* may play a pivotal role in triggering apoptotic events in human leukemia cells. Direct evidence implicating c-*jun* in apoptotic events has emerged primarily from studies examining the effects of growth factor deprivation. For example, Colotta *et al*. (18) demonstrated that antisense oligonucleotides to c-*jun* (and *c-fos*) blocked growth factor deprivation-induced apoptosis in the malignant murine lymphoid cell lines 7TD1 and B9. In addition, Estus *et al*. (19) reported that antibodies to c-*jun* protein protected murine neurons from NGF deprivation-induced apoptosis. More recently, Ham *et al*. (20) showed that a dominant negative c-*jun* microinjected into sympathetic neurons protects these cells from NGF deprivation-induced apoptosis. However, to the best of our knowledge, no direct evidence exists implicating c-*jun* in human myeloid leukemia cells undergoing apoptosis triggered by pharmacological agents. In addition, the related question of whether PKC activation is essential to c-*jun* up-regulation, as suggested by studies of leukemic cell differentiation (12, 41, 42), has also been addressed through the use of highly specific PKC inhibitors.

The present findings indicate that despite previous reports that relatively nonspecific PKC inhibitors such as staurosporine and H7 prevent leukemic cell c-*jun* up-regulation induced by chemotherapeutic agents (16, 17, 21, 24), highly specific PKC inhibitors, administered at concentrations that provoke apoptosis, are by themselves capable of increasing c-*jun* expression. Thus, both CE and CP induced a dose- and time-dependent increase in c-*jun* mRNA in HL-60 and U937 cells; moreover, a parallel increase in JUN protein was noted in the latter cells. Although it was difficult to correlate the extent of apoptosis with the degree of c-*jun* up-regulation due to extensive RNA degradation at higher drug doses, it was clear that increased expression of c-*jun* occurred in association with PKC inhibitor-induced apoptotic events. These results are consistent with those of a previous study in which staurosporine (50 nM) potentiated ara-C-induced apoptosis in HL-60 and U937 cells and led to a further increase in, rather than abrogation of, ara-C-mediated c-*jun* up-regulation (28). Discrepancies between this and other reports, in which PKC inhibitors were found to antagonize c-*jun* up-regulation (21), might reflect differences in the behavior of individual HL-60 sublines, which are known to vary widely in their response to agents acting at the level of PKC (43–45). The current findings suggest that in the case of PKC inhibitor-induced apoptosis, increased leukemic cell c-*jun* expression may occur in the absence of PKC activation. In addition, this process seems to be distinct from that observed after induction of leukemic cell differentiation as, in the latter case, PKC inhibitors prevent both c-*jun* up-regulation and cellular maturation (42).

Despite demonstration of a temporal association between c-*jun* up-regulation and PKC inhibitor-induced apoptosis, it remained to be determined whether a causal relationship existed between these events. To address this issue, cells expressing a dominant negative, transactivation domain-deletion mutant protein (TAM67) were used. Expression of this mutant protein has previously been shown to block transactivation of AP-1-responsive genes in Jurkat cells (46) and to interfere with other putative c-*jun* functions, such as neoplastic transformation in primary rat embryo (34) and PDV (47) cells. Indeed, c-*jun* is itself an AP-1-responsive gene and is up-regulated by its own product through an autoregulatory process (1, 5). The observation that up-regulation of native c-*jun* message and protein is markedly attenuated in TAM67-expressing U937 cells undergoing PKC inhibitor-induced apoptosis compared with the parental line is entirely consistent with a functional c-*jun* dominant negative model. Thus, if up-regulation of c-*jun* represents a critical event in inhibitor-induced apoptosis, then cells expressing TAM67 should be less susceptible to this process than their empty vector and parental counterparts. Conversely, if c-*jun* up-regulation represents a consequence of apoptotic events (i.e., DNA damage), then cells expressing TAM67 should be fully capable of mounting such a response. Based on the current findings, the latter possibility appears to be the case, as U937- and TAM67-expressing cells (as well as the vector-alone cell line) exhibited equivalent degrees of apoptosis, manifested by characteristic morphological changes and internucleosomal DNA fragmentation. In further support of a c-*jun*-independent pathway of apoptosis, we recently reported that 1,2-*bis*-(2-aminophenoxy)ethane-*N*, *N*, *N*, *N*′-tetraacetic acid-acetoxymethyl ester, a permeant Ca^{2+} chelator, potently induces apoptosis in HL-60 cells and that this phenomenon is accompanied by a time- and concentration-dependent down-regulation of c-*jun* transcript levels (36).

How, then, can the present results be interpreted in light of our current understanding of the role of c-*jun* in DNA damage in general and apoptosis in particular? The induction of c-*jun* and other IER genes (including c-*fos*, *p53*, *GADD153*, and *GADD45*) after treatment of cells with phorbol-12-myristate-13-acetate (12), ionizing radiation (23), and anticancer agents such as *cis*-diamminedichloroplatinum(II) (17) and ara-C (23) is well documented. Furthermore, in many of these instances, gene regulation has been shown to be PKC dependent (17, 23, 24), although other signaling cascades (48) may also be involved. Under certain circumstances [i.e., after exposure to PKC inhibitors or to chemotherapeutic agents such as VP-16 (13)] and in certain cell types (i.e., human myeloid leukemia cells), up-regulation of c-*jun* may represent a cellular response to the extensive DNA damage that accompanies apoptotic endonucleolytic DNA degradation and may not play a critical role in mediating this response. However, when other cells are subjected to alternative stresses (i.e., growth factor deprivation), c-*jun* may be required for apoptotic events to proceed (18–20). In this context, with a strategy similar to that used in the current study, we found that normal c-*jun* function does not seem to be necessary for human myeloid leukemia cells to undergo apoptosis after exposure to the antimetabolite ara-C but that it may be essential in tumor necrosis factor-α-induced cell

death.[1] These observations may be analogous to those previously reported for c-*myc*, in which antisense oligonucleotides selectively blocked activation-related, but not steroid-induced apoptosis in T cell hybridomas (49), indicating the presence of c-*myc*-dependent and -independent pathways of apoptosis. Thus, the findings of the current study, combined with those of earlier reports, imply a varied role for the proto-oncogene c-*jun* in apoptosis, which may depend on the specific cell line and the evoking stimulus. Also, the increased expression of c-*jun* in response to PKC inhibitors suggests, albeit indirectly, the presence of a signaling cascade associated with c-*jun* up-regulation that is distinct from pathways involved in cellular differentiation. Collectively, these observations may help to reconcile apparently contradictory reports concerning the role of c-*jun* in apoptosis, as well as the role of PKC in c-*jun* regulation.

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