Activation of the AP-1 Transcription Factor by Arabinofuranosylcytosine in Myeloid Leukemia Cells

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Previous studies have shown that 1β-D-arabinofuranosylcytosine (ara-C) induces transcription of the c-jun immediate early response gene in human myeloid leukemia cells. The present work has examined the mechanisms responsible for this effect. Deleted forms of the c-jun promoter were linked to the chloramphenicol acetyltransferase (CAT) gene and transfected into KG-1 cells. The results demonstrate that ara-C-induced c-jun transcription is mediated by an element between positions −74 and −20 upstream to the start site. Electrophoretic mobility shift assays with the fragment f(−74/−20) showed an increase in binding with nuclear proteins from ara-C-treated cells as compared with untreated cells. Competition with an oligonucleotide containing the AP-1 consensus sequence indicated that ara-C stimulates binding of nuclear proteins at the AP-1 site in the c-jun promoter. These findings were confirmed in other gel shift studies with the collagenase enhancer AP-1 consensus sequence and with a DNA fragment containing an altered AP-1 site. The binding of JUN/AP-1 was maximal at 1 hour of ara-C treatment and decreased to baseline levels at 12 hours. The finding that ara-C induces AP-1 binding in the absence of protein synthesis indicated that this agent activates already synthesized JUN/AP-1. To confirm these findings, the AP-1 consensus sequence was introduced 5′ to the heterologous SV40 promoter. The results show that AP-1 enhances SV40 promoter activity in ara-C-treated cells. Taken together, these findings indicate that: (1) enhancement of JUN/AP-1 activity in ara-C-treated cells involves a posttranslational modification of JUN/AP-1; and (2) binding of activated JUN/AP-1 to the AP-1 site in the c-jun promoter confers ara-C inducibility of this gene.

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extract, 0.025 μCi [14C]chloramphenicol (Amersham, Arlington Heights, IL; 57 mCi/mmol), 275 mmol/L Tris-HCl, pH 7.8, and 0.4 mmol/L acetyloenzyme A for 2 hours at 37°C. The acetylated products were separated from unacetylated chloramphenicol by thin-layer chromatography. Both unacetylated and acetylated forms of [14C]chloramphenicol were cut from the thin-layer chromatography plate and radioactivity determined by scintillation counting. Protein concentration as determined by the Bradford protein assay[2] was used to normalize conversion of chloramphenicol to its acetylated forms.

**Electrophoretic mobility shift assays.** Nuclear proteins were prepared according to described methods. In certain experiments, the nuclear proteins were incubated for 30 minutes at 37°C in the presence of a monoclonal antibody (MAb) directed against the binding epitope of JUN/AP-1 (Oncogene Science, Inc, Manhasset, NY). The 54-bp SphI/SacI fragment of the c-jun promoter (positions -74 and -20) was end-labeled with [α-32P]dATP using DNA polymerase I. This fragment was either purified in a 12% polyacrylamide gel or digested with HincII to generate a 30-bp SphI/HincII fragment (GGGTGACATCATGGGCTATTTTTAGGGGT) which was also purified by gel electrophoresis. The end-labeled DNA (1 ng; ~2 x 10^6 cpm) was incubated with 10 μg nuclear protein for 20 minutes at 20°C in a buffer containing 25 mmol/L Tris-HCl, pH 7.6, 5 mmol/L MgCl₂, 0.5 mmol/L EDTA, 1 mmol/L DTT, and 10% (vol/vol) glycerol. Competition studies with oligonucleotides corresponding to NF-1, SphI, AP-1, AP-2, and AP-3 binding sites (Stratagene, La Jolla, CA) were performed by adding up to a 200-fold molar excess compared with the labeled fragment. The reaction products were analyzed by 5% polyacrylamide gel electrophoresis and autoradiography.

**RESULTS**

The c-jun gene is transcriptionally activated during treatment of human myeloid leukemia cells with ara-C. Because previous studies have shown that the c-jun gene is auto-induced by its product JUN/AP-1,[2] the present work first examined whether the effects of ara-C involve activation of the AP-1 consensus sequence between positions -72 and -63 in the c-jun promoter. To address this issue, we prepared promoter fragments that included -74 bp and -20 bp upstream to the transcription start site. These fragments were ligated to the CAT gene (Fig 1A) and transfected into KG-1 cells. Transfection of the p-74CAT vector into unstimulated cells showed a basal level of CAT activity that was increased from 2.5- to 4-fold after ara-C treatment (Fig 2). Taken together, these findings provided further evidence that activation of the c-jun gene by ara-C is mediated by the AP-1 element.

The results of the CAT assays prompted on analysis of the interaction between ara-C-induced nuclear proteins and potential cis-regulatory elements. The region of the c-jun promoter between positions -74 and -20 contains the AP-1 consensus sequence, as well as two TATA-like elements.[2] To identify the DNA binding factor(s) mediating ara-C inducibility, we performed electrophoretic mobility shift assays (EMSAs) using the fragment f(-74/-20). Incubation of end-labeled f(-74/-20) with nuclear proteins from untreated KG-1 cells showed two faint but detectable retarded bands (Fig 3). In contrast, the intensity of these bands was increased when using nuclear extracts from ara-C-treated cells (Fig 3). KG-1 cells were also treated with TNF, an agent previously shown in myeloid cells to activate JUN/AP-1 binding to its consensus sequence. A similar retardation pattern was obtained with nuclear proteins from TNF-treated KG-1 cells, although there appeared to be slight differences in the mobility of the retarded bands compared to that obtained with ara-C (Fig...
These results suggested that ara-C and TNF activate similar, but perhaps not identical, DNA binding complexes.

While the EMSA findings suggested that ara-C treatment is associated with an increase in DNA binding of JUN/AP-1, competition studies were performed with an oligonucleotide containing the AP-1 binding site. The AP-1 oligonucleotide decreased binding of nuclear proteins from both untreated and ara-C-treated cells (Fig 4A). This competitive effect was concentration-dependent and nearly complete for both retarded bands when using a 200-fold excess of oligonucleotide (Fig 4B). In contrast, there was no detectable effect when using a similar excess of oligonucleotides containing sites for NF-1, Sp1, AP-2, or AP-3 (data not shown). Taken together, these findings suggested that ara-C stimulates binding of KG-1 nuclear proteins at the AP-1 site.

The effects of ara-C on binding of nuclear proteins to f(−74/−44) were detectable by 30 minutes and maximal by 1 hour (Fig 5A). Longer exposures of this agent were associated with a progressive decrease in intensity of the retarded bands to a level at 12 hours comparable with that in control cells. Moreover, our previous studies showed that induction of c-jun gene transcription by ara-C occurs in the absence of protein synthesis. In this regard, activation of protein binding to f(−74/−44) was identical at 1 hour in the absence or presence of cycloheximide (Fig 5B). Furthermore, treatment with cycloheximide alone had no detectable effect on nuclear protein binding to f(−74/−44) (data not shown). These results suggested that ara-C activates already synthesized JUN/AP-1 and that this activation process stimulates binding to the c-jun promoter.

We also performed EMSAs with a sequence from the collagenase gene enhancer that contains another known AP-1 binding site (GGAGCTTGATGAGTCAGCGGACTC). Incubation of this labeled oligonucleotide with nuclear proteins from untreated KG-1 cells showed a retarded band (Fig 6A). The size of this band was increased when using nuclear extracts from ara-C-treated cells (Fig 6A). Moreover, a similar pattern was obtained when cells were treated with TNF (Fig 6A). The binding of these nuclear proteins was completely blocked by adding a 25-molar excess of unlabeled f(−74/−44) from the c-jun promoter (data not shown). In contrast, there was no detectable retardation observed when these protein preparations were incubated with the StyII/HincII fragment of the c-jun promoter (positions −74 to −44) that contains the altered AP-1 site (TGGGATCAT) (Fig 6A). Other studies were performed with nuclear proteins from nonconfluent FH 109 lung fibroblasts that have been shown to constitutively express JUN/AP-1. Incubation of these nuclear proteins with ara-C and TNF was associated with a decrease in intensity of the retarded band (Fig 6A).
proteins with the collagenase enhancer AP-1 site resulted in a pattern similar to that obtained with untreated KG-1 cells (Fig 6B). Furthermore, stimulation of FH 109 cells with TNF resulted in the same pattern of increased retardation observed with ara-C-treated KG-1 cells. Finally, there was no detectable binding of FH 109 nuclear proteins to the StyI/HincII fragment with the altered AP-1 site (Fig 6B).

While the results of EMSAs provided evidence for protein binding to the AP-1 site, two major complexes perhaps each composed of sub-bands were detectable with the ara-C–induced nuclear preparations. For example, ara-C treatment was predominantly associated with an increase in the upper complex when using the c-jun promoter f(−74/−20) probe (Figs 3 through 5), while the lower complex was increased to a greater extent with the collagenase enhancer AP-1 probe (Fig 6). To determine whether JUN/AP-1 binding activity is increased by ara-C, nuclear proteins from ara-C–treated cells were treated with an anti-JUN MAb before adding the end-labeled f(−74/−20). Exposure to ara-C was associated with an increase in

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**Fig 4.** Effects of an AP-1 oligonucleotide on binding of nuclear proteins to f(−74/−20). (A) End-labeled f(−74/−20) was incubated with nuclear proteins from control and ara-C–treated cells in the presence of a 200-fold molar excess of an oligonucleotide (Stratagene) containing the AP-1 consensus sequence. (B) Nuclear proteins from ara-C–treated cells were incubated with end-labeled f(−74/−20) in the presence of a 50-, 100-, and 200-fold molar excess of the AP-1 oligonucleotide. Signal intensity as determined by densitometric scanning was compared with that obtained in the absence of competitive AP-1 oligonucleotide.

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**Fig 5.** Kinetics and effects of cycloheximide on ara-C–induced nuclear protein binding to f(−74/−44). (A) KG-1 cells were treated with 10−4 mol/L ara-C for the indicated times. (B) KG-1 cells were treated with 10−4 mol/L ara-C for 6 hours in the presence and absence of cycloheximide (CHX). Nuclear proteins were isolated and incubated with end-labeled f(−74/−44).

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**Fig 6.** Nuclear protein binding to the collagenase enhancer AP-1 site and a fragment of the c-jun promoter with a partially deleted AP-1 site. (A) An end-labeled oligonucleotide for the collagenase enhancer AP-1 site (col AP-1: GGAGCTTGATGAGTCAGCCGACTC) was incubated with nuclear proteins from control, ara-C–treated, and TNF–treated KG-1 cells. The same nuclear proteins were incubated with a StyI/HincII fragment of the c-jun promoter (positions −74 to −44) but with a partially deleted AP-1 site (del AP-1: ATCAT). (B) Nuclear proteins from control and TNF–treated FH 109 lung fibroblasts were incubated with end-labeled col AP-1 and del AP-1.
both the upper and lower complexes (Fig 7). In contrast, treatment of the ara-C-induced nuclear proteins with the anti-JUN antibody completely inhibited formation of the upper complexes and resulted in only a partial decrease in intensity of the lower band (Fig 7). These findings indicated that the increase in AP-1 binding by ara-C is at least in part due to JUN/AP-1 and that other AP-1 proteins may contribute to this activity.

To further confirm the functional capacity of the AP-1 site in mediating ara-C inducibility, we ligated fragments with and without the AP-1 consensus sequence to a heterologous SV40 promoter-CAT construct. These vectors (Fig 8A) were transfected into unstimulated and ara-C-treated cells. Ara-C treatment was associated with an increase in CAT activity when using the AP-1-containing construct. In contrast, there was no difference in CAT activity between unstimulated and ara-C-treated cells transfected with the AP-1-negative vector (Fig 8B). The finding that AP-1 mediates ara-C inducibility with a heterologous promoter provides further support for the involvement of this element in signaling events activated by this agent.

**DISCUSSION**

Certain insights are presently available regarding the mechanism of action of ara-C. The inhibitory effects of ara-C on DNA replication are related to both the extent of incorporation into DNA and sequence of the DNA template. In this regard, DNA strands are progressively terminated by incorporation at sequences containing two, three, and four contiguous cytosine sites. These findings are consistent with the conformational and hydrogen bonding differences of the incorporated arabinose moiety altering reactivity of the 3’ terminus and thereby slowing or terminating DNA chain elongation. The cellular response to this induction of DNA damage by ara-C is unclear. However, recent studies have shown that ara-C induces expression of certain immediate early response genes, including the c-jun gene, in human myeloid leukemia cells. This induction of c-jun gene expression is regulated at the transcriptional level and therefore presumably involves activation of nuclear DNA binding proteins.

Transcription of the c-jun gene is positively autoregulated by its product JUN/AP-1 in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) and serum factors. The enhancement of JUN/AP-1 activity in TPA-stimulated cells is regulated by a posttranslational event acting on preexisting JUN/AP-1 molecules. Binding of activated JUN/AP-1 to a high-affinity AP-1 binding site in the c-jun
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The TPA inducibility of the c-jun gene is similarly activated after promoter is responsible for the induction of the c-jun gene in ara-C–treated cells. The high-affinity AP-1 site that confers TPA inducibility of the c-jun gene is similarly activated after treatment of KG-1 cells with ara-C. Evidence in support of this mechanism includes the results of transient expression assays using fragments of the c-jun promoter that include and exclude this element. Furthermore, we have found that this AP-1 binding site confers ara-C inducibility to the heterologous SV40 promoter. Taken together with the results of EMSAs, which demonstrate increased AP-1 binding of nuclear proteins from ara-C–treated cells, these findings indicate that ara-C stimulates c-jun transcription by activating JUN/AP-1 binding.

Our previous studies showed that ara-C induces transcription of the c-jun gene in the presence of cycloheximide. In the present work, activation of AP-1 binding by ara-C was also detectable in the absence of protein synthesis. These results support a mechanism involving posttranslational modification of preexisting AP-1 protein that results in induction of c-jun transcription. The nature of the modification induced by ara-C is unknown. Treatment of a variety of cell types, including myeloid leukemia cells, with TPA is associated with activation of protein kinase C and induction of c-jun transcription. The nature of the modification induced by ara-C is unknown.

The previous finding that ara-C residues are detectable in DNA strands at 30 minutes of exposure to this agent is in concert with the rapid activation of JUN/AP-1 binding observed in the present studies. The incorporation of ara-C into DNA is associated with both inhibition of DNA synthesis and alteration of chromatin structure. We have similarly treated cells with aphidicolin, an inhibitor of DNA polymerase α that is not incorporated into DNA. While aphidicolin also resulted in complete inhibition of cell growth, the finding that this agent had little if any effect on induction of c-jun expression suggested that inhibition of DNA synthesis is not sufficient for activation of this gene.

Therefore, termination of DNA strand elongation by the incorporated arabinosyl moiety and accumulation of strand breaks may represent the initial signals that stimulate a cascade of events as part of the cellular response to DNA damage. In this context, previous studies have shown that other DNA-damaging agents, such as UV light, induce the transcription of genes, including c-jun, which are also regulated through the activation of AP-1 binding sites. Moreover, we have recently found that ionizing radiation regulates transcription of the c-jun gene and that this effect is independent of protein synthesis. Similar findings have been obtained with etoposide, an inhibitor of topoisomerase II. Thus, a variety of agents that induce DNA damage by distant mechanisms appear to be capable of stimulating a cellular response that includes activation of JUN/AP-1 and induction of c-jun transcription. This response may play a role in the repair of DNA damage or in the initiation of a genetic program associated with cell death.

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