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Activation of the Transcription Factor κB in Human KG-1 Myeloid Leukemia Cells Treated with 1-β-D-Arabinofuranosylcytosine

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SUMMARY

The present studies have examined the effects of 1-β-D-arabinofuranosylcytosine (ara-C) on activation of the transcription factor κB (NF-κB). The results demonstrate that treatment of human KG-1 myeloid leukemia cells with ara-C is associated with induction of protein binding to the NF-κB consensus sequence. NF-κB binding was activated at 30 min and reached maximal levels of binding at 1–2 hr of ara-C treatment. The NF-κB consensus sequence was ligated to the heterologous thymidine kinase (TK) promoter and the human growth hormone (GH) reporter gene to determine whether ara-C-induced NF-κB activity includes an enhancer function. Ara-C treatment had little effect on transient expression of pTKGH in KG-1 cells but increased transcription of the p (NF-κB) TKGH vector by 8-fold. The results also demonstrate that ara-C transiently increases NF-κB mRNA levels. However, the finding that ara-C-induced binding of NF-κB to DNA occurs in the presence of cycloheximide indicates that this agent activates preexisting NF-κB protein. These results suggest that ara-C induces a cytoplasmic pathway that transduces signals to the nucleus by activation of NF-κB.

Ara-C is one of the most effective agents in the treatment of acute myelogenous leukemia (1, 2). This agent incorporates into cellular DNA and slows chain elongation by altering reactivity of the 3′ terminus (3–5). The extent of ara-C-DNA formation correlates with inhibition of DNA synthesis and loss of clonogenic survival (3, 4). Other studies have demonstrated that the effects of ara-C are related to incorporation at specific sequences in the DNA template (6, 7). However, the precise mechanisms responsible for ara-C-induced cell lethality remain unclear. Recent findings have demonstrated that treatment of myeloid leukemia cells with ara-C is associated with the induction of specific gene expression. In particular, ara-C induces the *jun* family of early response genes (8–10). These genes code for DNA-binding proteins that regulate transcription through the AP-1 consensus sequence (11).

The present studies were performed to determine whether ara-C also activates other signaling pathways involved in transcriptional regulation. In this context, the NF-κB complex is a heterodimer or heterotetramer consisting of 50-kDa (p50) and 65-kDa (p65) polypeptides (12). Activation of NF-κB is accompanied by translocation of the cytoplasmic complex to the nucleus, where it binds to an 11-bp consensus DNA sequence that functions as an enhancer for a variety of mammalian genes (13–15). Although NF-κB was initially identified as a factor that enhances transcription of the κ-chain immunoglobin gene, NF-κB binding sites have been found in the promoters of a variety of cellular and viral genes (13). The present results demonstrate that ara-C activates NF-κB binding to DNA and that this effect is associated with induction of an NF-κB enhancer function.

Materials and Methods

Cell culture. Human KG-1 myeloid leukemia cells (American Type Culture Collection, Bethesda, MD) were grown in Iscove's modified Dulbecco's medium supplemented with 1.5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 400 μg/ml streptomycin.

Drug treatment. The cells were treated with ara-C (Sigma Chemical Co., St. Louis, MO) at concentrations ranging from 10^{-7} to 10^{-3} M. In certain experiments, cells were treated with 10 μg/ml CHX (Sigma) alone or before addition of ara-C.

EMSA. Nuclear proteins were prepared according to previously described methods (16). Purified NF-κB protein from human placenta was kindly provided by P. Bauerle, University of Munich, FRG (17). A 22-bp synthetic oligonucleotide (5′-GATCGAGGGGACTTTCCCT-AGC-3′) containing the 11-bp NF-κB consensus sequence (GGGGACTTTCC) was end-labeled with [α-^{32}P]dATP and DNA polymerase I. The labeled DNA (1 ng) was purified from a 12% polyacrylamide gel and incubated with 10 μg of nuclear protein for 20 min at 20° in 25 mM Tris·HCl, pH 7.6, 250 ng/ml poly(dI/dC), 5 mM $MgCl_2$,

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ABBREVIATIONS: ara-C, 1-β-D-arabinofuranosylcytosine; NF-κB, nuclear factor κB; TK, thymidine kinase; GH, growth hormone; CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; kb, kilobase(s); bp, base pair(s).

0.5 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol. Competition studies with unlabeled NF-κB oligonucleotide were performed by addition of a 25-fold excess, compared with the end-labeled fragment. The reaction products were analyzed by 5% polyacrylamide gel electrophoresis and autoradiography.

Reporter assays. The NF-κB consensus sequence was inserted into the restriction site of a plasmid (pTKGH) containing the TK promoter linked to the human GH gene. Plasmids used for transfection were purified twice by cesium chloride gradient centrifugation. KG-1 cells (2×10^7) were transfected with 20 μg of pTKGH or p (NF-κB) TKGH by the DEAE-dextran technique (18). Twenty-four hours after transfection, one aliquot of the cells was treated with 10^{-6} M ara-C for 24 hr, while the other aliquot was maintained in medium without drug. Culture supernatant was analyzed for GH production by enzyme-linked immunosorbent assay (DRG Instruments, Marburg, FRG).

Northern blot analysis. Poly (A)-enriched RNA (15 μg) was separated in a 1% agarose/2.2 M formaldehyde gel, transferred to a nitrocellulose filter, and hybridized (8) to the following ^{32}P-labeled DNA probes: 1) the 3.9-kb *Not*I fragment isolated from the NF-κB cDNA (19) and 2) the 2.0-kb *Pst*I fragment of a chicken β-actin DNA purified from the pA1 plasmid (20).

Results and Discussion

In untreated KG-1 cells, there was a low level of nuclear protein binding to the NF-κB consensus sequence, as determined by EMSA (Fig. 1A). In contrast, treatment of these cells with 10^{-6} M ara-C was associated with increases in intensity of the retarded fragment. This effect was detectable at 30 min and was maximal at 1–2 hr of ara-C treatment (Fig. 1A). Longer exposures to this agent were associated with a decline in NF-κB binding to that in control cells (Fig. 1A). In contrast, there was no detectable increase in binding of ara-C-induced nuclear proteins when a *Sac*II/*Dde*I fragment (positions −166 to −103) of the c-*jun* gene promoter that contains the SP1 consensus sequence was used (data not shown). Furthermore, addition of the unlabeled NF-κB oligonucleotide at a 25-fold excess, compared with the labeled fragment, resulted in complete inhibition of protein binding (data not shown). Other studies with purified NF-κB demonstrated a gel retardation pattern identical to that obtained with ara-C-induced nuclear proteins (Fig. 1B). Taken together, these findings indicated that ara-C specifically activates NF-κB and the binding of this complex to DNA.

Similar studies were performed with nuclear proteins from KG-1 cells exposed to varying concentrations of ara-C for 1 hr. Treatment with 10^{-7} M ara-C was associated with detectable increases in nuclear protein binding to the NF-κB oligonucleotide (Fig. 2). The signal intensity obtained with nuclear extracts from cells treated with 10^{-7} M ara-C was similar to that observed with preparations from cells exposed to 10^{-6} M drug (Fig. 2). Treatment with higher ara-C concentrations, particularly 10^{-4} M and 10^{-3} M, was associated with further increases in signal intensity (Fig. 2). Moreover, at least two major retarded bands were more clearly apparent after exposure to the higher ara-C concentrations. The presence of an additional band may be related to the size of the NF-κB complex binding to the oligonucleotide. In this context, DNA binding specificity in EMSAs appears to be related to the p50, and not the p65, component of the complex (12). Moreover, recent data have demonstrated that the DNA binding subunits of NF-κB and KBF1 are probably identical and are members of a family that includes the c-*rel* proteins (19, 21). Thus, the NF-κB consensus may be capable of binding several related factors.

The finding that ara-C activates NF-κB binding prompted

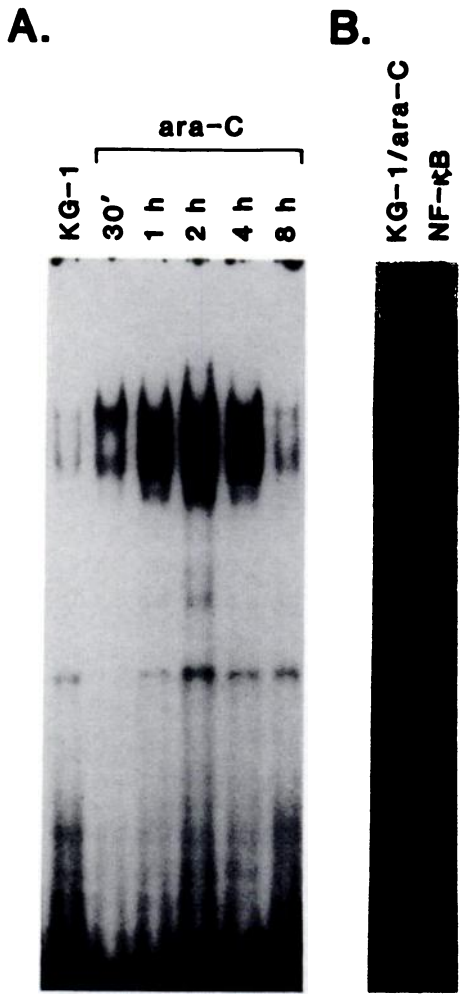

Fig. 1. Effects of ara-C on the kinetics of NF-κB-binding activity. A, KG-1 cells were treated with 10^{-6} M ara-C, and nuclear extracts were prepared at the indicated times. B, KG-1 cells were treated with 10^{-6} M ara-C for 30 min. EMSAs were performed using 10 μg of nuclear protein or 20 pg of purified NF-κB (17) and 1 ng of ^{32}p-labeled oligonucleotide containing the NF-κB consensus sequence. Retardation of the labeled DNA was analyzed by 5% polyacrylamide gel electrophoresis and autoradiography.

further studies on whether this event is functional in gene activation. In order to address the potential functional role of ara-C-induced NF-κB binding, we ligated the NF-κB oligonucleotide to the heterologous TK promoter and the human GH reporter gene. Transfection of the enhancer-less reporter construct (pTKGH without NF-κB sequences) into KG-1 cells was associated with low but detectable levels of GH production (Fig. 3). Furthermore, there was no significant change in GH levels when this construct was introduced into cells that were subsequently treated with ara-C (Fig. 3). GH production was also relatively low when the p (NF-κB) TKGH vector was introduced into untreated cells. In contrast, transient expression of p (NF-κB) TKGH was 8-fold higher in cells exposed to 10^{-6} M ara-C (Fig. 3). Similar increases in GH production obtained in three separate experiments were significantly different from production detected for untreated cells ($p < 0.007$, by Student's *t* test). These findings indicated that induction of NF-κB binding by ara-C is associated with *trans*-activation of gene expression.

The induction of NF-κB binding to DNA could be related to

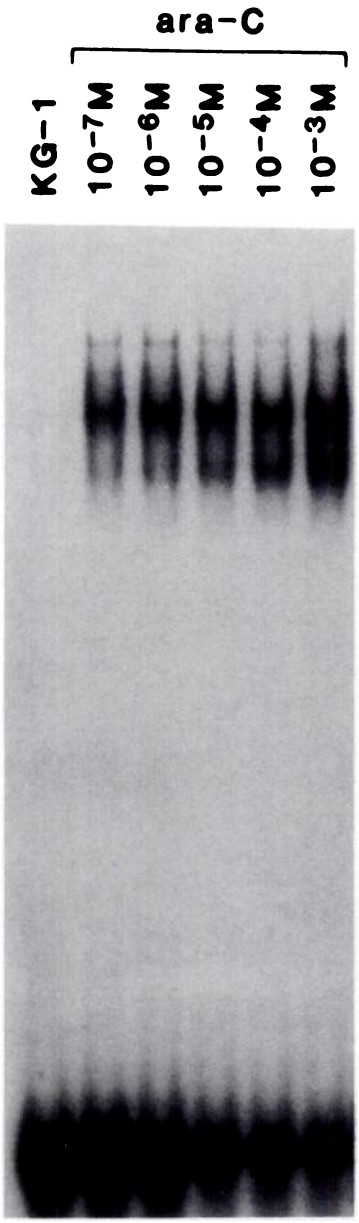

Fig. 2. Activation of NF-κB-binding activity in cells exposed to varying ara-C concentrations. Nuclear extracts were prepared from KG-1 cells treated with the indicated concentrations of ara-C for 1 hr. EMSAs were performed as described in the legend to Fig. 1.

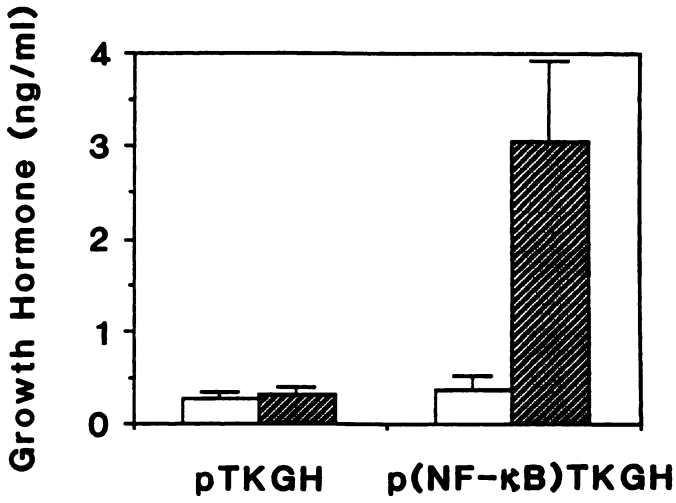

Fig. 3. Ara-C induces the enhancing activity of NF-κB sequences linked to the pTKGH promoter-reporter construct. KG-1 cells (2×10^7) were transfected with 20 μg of pTKGH or p (NF-κB) TKGH. After 24 hr, the cells were incubated in the absence (□) or presence (■) of 10^{-6} M ara-C. After incubation for another 24 hr, the culture supernatants were analyzed for GH levels by enzyme-linked immunosorbent assay. The results represent the mean ± standard deviation of three separate experiments. There was no significant effect of ara-C on hormone production by cells transfected with pTKGH ($p = 0.6$; Student's t test), whereas ara-C significantly increased GH levels in cells transfected with p (NF-κB) TKGH ($p < 0.007$).

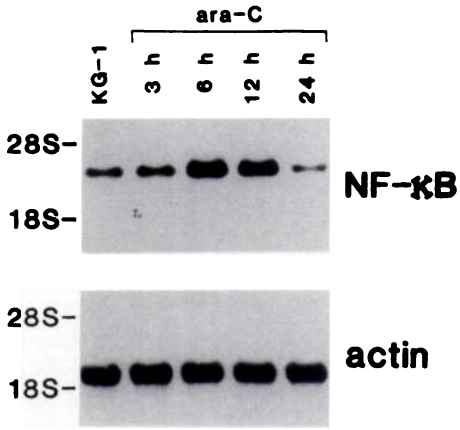

Fig. 4. Induction of NF-κB mRNA levels by ara-C. KG-1 cells were treated with 10^{-6} M ara-C. Poly(A)-enriched RNA (1.5 μg/lane) was isolated at the indicated times and hybridized to ^{32}P-labeled NF-κB or actin DNA probes. *KG-1*, RNA from untreated cells.

an increase in expression of this gene or to activation of already synthesized protein. Studies were, therefore, performed to determine whether treatment with ara-C is associated with changes in NF-κB mRNA levels. Levels of 4.0-kb NF-κB transcripts were detectable in untreated KG-1 cells (Fig. 4). Whereas exposure to 10^{-6} M ara-C for 3 hr had little effect on NF-κB expression, increases in these transcripts were apparent at 6 and 12 hr (Fig. 4). This effect was transient, and NF-κB expression at 24 hr of ara-C treatment returned to levels comparable to that in control cells. Taken together with an absence of changes in actin transcripts, these findings indicated that ara-C transiently induces NF-κB p50 mRNA expression.

Because NF-κB is maintained in the cytoplasm in an inactive state through binding to the IκB inhibitor protein (14, 15), activation of NF-κB in ara-C-treated cells could be due to translocation of preexisting cytoplasmic factor to the nucleus. This issue was addressed by pretreating KG-1 cells with CHX to inhibit protein synthesis. Treatment with CHX alone had no detectable effect on NF-κB binding of nuclear proteins from otherwise untreated cells (Fig. 5). Moreover, CHX pretreatment had little if any effect on ara-C-induced activation of NF-κB (Fig. 5). These results indicated that, in addition to increases in NF-κB mRNA levels, ara-C treatment is associated with activation of preexisting NF-κB protein. Although activation of NF-κB protein precedes increases in p50 transcripts, it is not known whether NF-κB contributes to p50 gene transcription or whether ara-C regulates NF-κB expression at the protein and mRNA levels by two distinct mechanisms.

Previous studies have implicated protein kinase C in the activation of NF-κB binding to DNA. IκB, a 36-kDa cytoplasmic

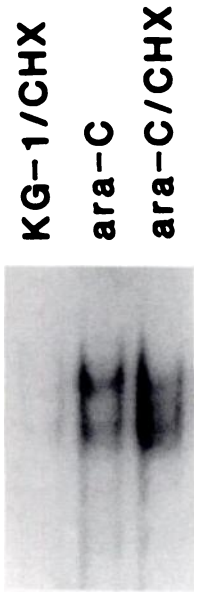

Fig. 5. Activation of NF-κB in the presence of CHX. KG-1 cells were treated with 10 μg/ml CHX for 3 hr (KG-1/CHX) or with 10^{-6} M ara-C for 1 hr (ara-C). Cells were also treated with CHX for 2 hr before addition of ara-C for an additional 1 hr (ara-C/CHX). Nuclear proteins (10 μg) were analyzed in EMSAs for retardation of the labeled NF-κB oligonucleotide.

factor, which prevents uptake of NF-κB into the nucleus, is phosphorylated by protein kinase C and then releases the p50/p65 complex (14). The activation of NF-κB by ara-C could, therefore, be mediated by a protein kinase C-dependent mechanism. In this respect, recent studies have demonstrated that ara-C treatment is associated with the rapid induction of a protein kinase activity (22). The induction of this protein kinase by ara-C is temporally related to the kinetics of NF-κB activation, as shown in the present work. Moreover, this ara-C-induced protein kinase phosphorylates a substrate that has been identified previously as being specific for protein kinase C (22). Although these findings have suggested that ara-C activates a protein kinase C-like enzyme, further studies are needed to characterize this activity and determine whether it is involved in transducing signals that contribute to the regulation of NF-κB.

Because the available evidence indicates that ara-C acts predominantly, if not exclusively, by incorporation into DNA, activation of a protein kinase-dependent pathway would necessitate signaling from the nucleus to the cytoplasm. Indeed, the demonstration that ara-C activates preexisting NF-κB, which is located in the cytoplasm in an inactive form, provides support for the induction of cytoplasmic signaling events by this agent. Thus, ara-C-induced DNA strand breakage or distortion of DNA/nucleoprotein conformation (23, 24) could activate signals that are transduced from the nucleus to the cytoplasm. In this context, activation of NF-κB has recently been described during exposure of mammalian cells to UV irradiation, another DNA-damaging agent (25). Although signaling pathways are generally from the cell membrane to the nucleus, reverse signal transduction from the nucleus to the cytoplasm could represent a response to the effects of DNA-damaging agents. The present findings with NF-κB would support this mechanism in ara-C-treated cells.

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