Camptothecin and Its Derivatives Induce Expression of the c-jun Protooncogene in Human Myeloid Leukemia Cells

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ABSTRACT

We have recently demonstrated that certain camptothecin derivatives are effective agents in the treatment of human tumor xenografts in nude mice. While camptothecin and its derivatives are recognized as inhibitors of topoisomerase I, little is known about the effects of these agents on specific gene expression, particularly genes involved in growth control. The c-jun early response gene codes for a leucine zipper transcription factor. The present studies demonstrate that 20(S)-camptothecin, 9-amino-20(S)-camptothecin, and 9-nitro-20(S)-camptothecin inhibit the growth of human U-937 myeloid leukemia cells and induce expression of the c-jun gene. c-jun transcripts were increased at 3 h and reached a maximum at 6 h of drug exposure. We also demonstrate that the induction of c-jun gene expression by these agents occurs at the transcriptional level. H7, a nonselective inhibitor of protein kinase C, completely blocked c-jun expression in 20(S)-camptothecin-treated cells, while another protein kinase inhibitor, HA1004, had no detectable effect. Similar findings were obtained for other leucine zipper encoding genes, including jun-B. These results suggest that 20(S)-camptothecin, 9-amino-20(S)-camptothecin, and 9-nitro-20(S)-camptothecin activate a cellular response involving the induction of early response genes. Finally, we demonstrate that induction of c-jun expression occurs in association with internucleosomal DNA fragmentation, a characteristic of programmed cell death.

INTRODUCTION

The antitumor activities of the plant alkaloid camptothecin were reported as early as 1966 (1). Although water-soluble sodium CAM² was used to treat cancer patients in the 1970s (2–5), unacceptable toxicity resulted in discontinuation of this therapy. However, development of semisynthetic CAM derivatives and alternate methods of drug administration in vivo (6–10) have renewed interest in the use of these antitumor agents. CAM is a potent inhibitor of RNA and DNA, but not protein, synthesis (11). Early studies showed that CAM prevents cells from progressing through S phase (12–15) and induces degradation of DNA (13, 16) which is maximal in late S and early G2 phases (12). The sensitivity of malignant cells to CAM has been correlated positively with topoisomerase I levels in these cells (6, 17, 18). CAM does not bind to DNA alone or topoisomerase alone, but interferes with the breakage-reunion process of topoisomerase I by stabilizing the enzyme-DNA “cleavable-complex” (19). This effect results in fragmentation of DNA (19). While CAM and its derivatives are known inhibitors of topoisomerase I, the basis for their selective antitumor activity is unclear.

Recent studies have demonstrated that certain classes of antitumor agents activate transcription of the c-jun immediate early response gene (20–22). This gene is rapidly activated in cells treated with tumor-promoting phorbol esters and growth factors (23–26). The c-jun gene product, which includes a basic DNA-binding domain and a leucine zipper motif, is the major component of the transcription factor AP-1 (27, 28). The c-jun gene is a member of a multigene family of transcription factors which includes Jun-B, jun-D, c-fos, fos-B, and fra-1 (reviewed in Ref. 29). These transcription factors bind as dimers to the consensus sequence TGA^CTCA which has been identified in the promoter regions of several genes (27, 28). The protein product of the c-jun gene, Jun/AP-1, also increases its own transcription by an autoregulatory mechanism (30).

In the present study, we demonstrate that CAM and CAM derivatives induce expression of the c-jun gene. The increase in c-jun expression is dependent on drug concentration and duration of exposure. This effect is regulated in part at the transcriptional level. Similar findings have been obtained for the jun-B gene. Moreover, we demonstrate that induction of these genes is temporally associated with internucleosomal DNA cleavage.

MATERIALS AND METHODS

Cell Culture. U-937 cells were cultured in RPMI 1640 medium supplemented with 10% heat-activated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Experiments were performed with exponentially growing cells at 3–6 × 10^5/ml. Viability was determined by trypan blue exclusion.

Reagents. Semipurified CAM obtained from Sigma (St. Louis, MO) and the Institute of Materia Medica, Academia Sinica, Shanghai, China, was further purified to analytical standards (31). The CAM derivatives, 9-amino-20(S)-camptothecin, 9-nitro-20(S)-camptothecin, and 12-nitro-20(S)-camptothecin were synthesized as described (31). The drugs were used as a fine suspension in polyethylene glycol 400 (Aldrich, Milwaukee, WI) at a concentration of 5 μg/ml, distributed in small aliquots, and stored at −70°C. The protein kinase inhibitors H7 and HA1004 were obtained from Seikagaku America, Inc.

Microscopy. Untreated and drug-treated cells were harvested by gentle centrifugation. Cell pellets were suspended in phosphate-buffered saline containing 20% fetal calf serum at 3 × 10^6 cells/ml. Cell smears on glass slides were fixed and stained with Wright-Giemsa or methylene blue. Dry preparations of stained cells were covered with immersion oil (type A, Cargille Laboratories, Cedar Grove, NJ) and coverglass. The slides were examined under a Zeiss microscope, and photomicrographs were taken on Gold 100 Kodak film.

Cell Growth Studies. Cells were harvested by gentle centrifugation and then resuspended in fresh media at a concentration of 3 × 10^6 cells/ml. The cell suspension was divided into equal volumes, and the cultures received various drugs at a concentration of 1 ng/ml in PEG. Control cell cultures received an equal volume of PEG alone or received no treatment. The cultures were agitated gently to assure distribution of the drug and PEG, and then incubated at 37°C. Aliquots were removed from the cultures at 24-h intervals for determination of viable cells.

RNA Isolation and Northern Blot Hybridization. Total cellular RNA (20 μg/lane) was isolated by a modification of the guanidine isothiocyanate-cesium chloride method (20), analyzed by gel electrophoresis in 1% agarose-formaldehyde gels, and transferred to nitrocellulose
filters. The hybridization conditions have been described previously (20). The filters were hybridized to the following 32P-labeled probes: (a) the 1.8-kilobase BamHI/EcoRI insert of a human c-jun DNA probe containing 1.0 kilobase complementary DNA and 0.8 kilobase 3'-untranslated sequences purified from a pBluescript SK(+) plasmid (27); (b) the 1.8-kilobase EcoRI fragment of a murine jun-B complementary DNA purified from the p465.20 plasmid (32); (c) the 0.9-kilobase NcoI/SacI fragment of the human c-fos gene purified from the pc-fos-1 plasmid (33); and (d) the 2.0-kilobase PstI insert of a chicken β-actin DNA in the pAl plasmid (34). The filters were washed and exposed to Kodak X-Omat XAR film with the use of an intensifying screen.

Nuclear Run-on Assays. Labeled nuclear RNA was prepared from 10⁶ U-937 cells as described previously (20) and hybridized to the following digested DNAs: (a) the 2.0-kilobase PstI fragment of the chicken β-actin gene (34); and (b) the 1.8-kilobase BamHI/EcoRI fragment of the human c-jun DNA (27). The digested DNAs were run in 1% agarose gels and transferred to nitrocellulose filters by the method of Southern. Prehybridizations and hybridization to the 32P-labeled nuclear RNA were performed as described (20).

Analysis of DNA Fragmentation. Cells were washed twice in phosphate-buffered saline and resuspended in 20 μl of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.5 μg/ml proteinase K (Sigma Chemical Co.). After incubation at 50°C for 3 h, 10 μl of 0.5 μg/ml RNase A were added for an additional 3 h. The samples were mixed with 10 μl of 10 mM EDTA (pH 8.0) containing 1% (w/v) low melting point agarose, 0.25% bromophenol blue, and 40% sucrose at 70°C. The DNA was separated in 2% agarose gels and visualized by UV illumination after ethidium bromide staining.

RESULTS

Growth Inhibition of U-937 Cells. The growth curves of U-937 cells treated with CAM derivatives are shown in Fig. 1. Exposure to 9NC and 9AC at concentrations of 1 ng/ml completely inhibited cell growth by day 3 of treatment. At the same concentration, CAM resulted in a similar inhibition of cell growth (data not shown). In contrast, 1 ng/ml of 12NC had no detectable effect on cell growth (Fig. 1). U-937 cells remained viable up to 6 days of treatment with 1 ng/ml of CAM, 9NC, or 9AC. However, treatment of cells with 5 ng/ml CAM and 9AC resulted in complete inhibition of cell growth at day 2 and the number of viable cells began to decrease at day 3. On the basis of these results, further experimentation was conducted with drug concentrations of 5 ng/ml or less.

![Fig. 1. CAM derivatives inhibit growth of U-937 cells. Exponentially growing cultures of U-937 cells (3 × 10⁵/ml) were exposed to CAM derivatives diluted directly from stock solutions. Cells were counted every 24 h. The cell cultures contained no additive (O); 0.5% PEG (■); 1 ng/ml 9AC-0.1% PEG ( △); 1 ng/ml 9NC-0.1% PEG (△); and 5 ng/ml 12NC-0.5% PEG (△). Cell numbers represent the average of three separate experiments each counted in triplicate.](image)

Microscopy. A comparison of untreated U-937 cells (Fig. 2a) with those exposed to 1 ng/ml 9AC for 3 h (Fig. 2b) revealed an increase in cell size and the appearance of more multinucleate cells. Further increases in size were observed in cells treated with 9AC for 6 h (Fig. 2c), 24 h (Fig. 2d), 48 h (Fig. 2e), and 72 h (Fig. 2f). An increase in the amount of nucleolar material was also observed in the cells treated with 9AC. The finding that these enlarged cells excluded trypan blue dye indicated that their membranes were intact. Treatment of U-937 cells with 1 ng/ml CAM for 72 h had less pronounced effects on cell size (data not shown), whereas exposure to 1 ng/ml 9NC (Fig. 2g) yielded effects similar to those of 9AC. Finally, concentrations of 12NC at up to 10 ng/ml had no apparent effect on the size and morphology of these cells (Fig. 2h).

Expression of the c-jun Gene. Total cellular RNA was prepared from U-937 cells treated with 0.5 and 5 ng/ml 9NC for various periods of time (Fig. 3A). Low levels of c-jun mRNA were detectable in untreated cells. However, exposure to 0.5 ng/ml 9NC for 1 h resulted in an increase in c-jun gene expression. Longer exposures resulted in higher levels of c-jun mRNA, with maximum expression observed at 6 h and then partial down-regulation at 12 h. Similar kinetics of c-jun induction were observed in cells treated with 5 ng/ml 9NC but the hybridization signals at 1, 3, and 6 h were more intense than those obtained with the lower concentrations of this agent. Similar results were obtained when U-937 cells were exposed to 0.5 and 5 ng/ml 9AC (Fig. 3B).
INDUCTION OF c-jun BY CAMPTOTHECIN

A.

Fig. 3. Camptothecin derivatives induced expression of c-jun gene. U-937 cells were treated with the indicated concentrations of 9NC (A) or 9AC (B) and then harvested at the indicated times. Total cellular RNA (20 μg) was isolated for Northern blot analysis with hybridization to a 32P-labeled c-jun DNA probe. The lane labeled U-937 represents RNA from untreated cells. Hybridization to a labeled actin probe revealed equal loading of the lanes.

B.

Fig. 4. Camptothecin derivatives induce jun-B expression. U-937 cells were treated with the indicated concentrations of 9NC (A) or 9AC (B) for the indicated times. Total cellular RNA (20 μg) was hybridized to the labeled jun-B probe. Hybridization to an actin probe demonstrated equal loading of the lanes.

jun-B and c-fos mRNA Levels. We also investigated whether CAM derivatives regulate expression of other members of the jun gene family. Low levels of jun-B mRNA were detected in untreated U-937 cells, while treatment with 0.5 ng/ml 9NC resulted in increased expression of this gene (Fig. 4A). jun-B transcripts increased progressively through 12 h of exposure to this concentration, while a more rapid and intense induction of jun-B expression was apparent in cells treated with 5 ng/ml 9NC (Fig. 4A). Similar findings were obtained in cells treated with 9AC (Fig. 4B) and CAM (data not shown). Maximal expression of the jun-B gene was detected at 6 and 3 h of exposure to 0.5 and 5 ng/ml 9AC, respectively (Fig. 4B).

Since the products of the c-jun and jun-B genes form heterodimers with the c-Fos protein, we also studied the effects of these drugs on expression of c-fos mRNA levels. Low levels of c-fos transcripts were detected in untreated U-937 cells, while treatment with 0.5 ng/ml 9NC resulted in an increase in c-fos mRNA levels that was apparent at 6 h (Fig. 5A). Moreover, treatment with 5 ng/ml 9NC was associated with increases in c-fos expression which were detectable at 1 h and increased through 6 h of drug exposure (Fig. 5A). Taken together, these findings indicated that 9AC and 9NC induce the expression of at least three genes (c-jun, jun-B, and c-fos) which code for components of the AP-1 transcription factor. In contrast, no detectable increase in c-jun mRNA was observed in cells treated with 0.5, 5, and 10 ng/ml 12NC (Fig. 5B; data not shown). There was also no effect of 10 ng/ml 12NC on jun-B and c-fos expression (data not shown). This lack of activity with 12NC is in concert with previous studies which have demonstrated that substitutions at position 12 of camptothecin result in analogues which are inactive against topoisomerase I (31, 35).

Activation of c-jun Gene Transcription. Nuclear run-on assays were performed to determine whether CAM and CAM derivatives regulate c-jun mRNA levels by a transcriptional mechanism. While a low level of c-jun gene transcription was detectable in untreated U-937 cells, exposure to 0.5 ng/ml CAM or 9AC for 6 h resulted in a 4- or 6-fold increase in transcription, respectively (Fig. 6). These changes were accompanied by little if any change in the transcription rate of the actin gene.
INDUCTION OF c-jun BY CAMPTOTHECIN

Fig. 5. Effects of 9NC and 12NC on c-fos and c-jun mRNA levels. U-937 cells were treated with 0.5 and 5 ng/ml of 9NC or 12NC for the indicated times. Total cellular RNA (20 ¿ig) was hybridized to the labeled c-fos (A) or c-jun (B) probes. Hybridization to an actin probe demonstrated equal loading of the lanes.

Regulation of c-jun mRNA Levels. Previous studies have demonstrated that the induction of c-jun expression by phorbol ester is increased by concurrent inhibition of protein synthesis (26). Similar studies were conducted in 9AC-treated U-937 cells. Treatment of cells with cycloheximide alone for 3 and 6 h resulted in increased levels of c-jun mRNA (Fig. 7). Moreover, treatment with both cycloheximide and 9AC resulted in levels of c-jun transcripts similar to those obtained with cycloheximide alone, but less than that in cells treated with 9AC alone (Fig. 7). These findings indicated that induction of c-jun expression by 9AC involves, at least in part, new protein synthesis.

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Previous studies have also demonstrated that activation of protein kinase C is associated with induction of c-jun expression (26). In order to address the role of protein kinases in camptothecin-induced expression of the c-jun gene, U-937 cells were treated with the isoquinolinesulfonamide derivative, H7, which inhibits protein kinase C (36). H7 decreased constitutive c-jun expression and blocked induction of this gene by 9AC (Fig. 8).

The present studies demonstrate that treatment of human U-937 cells with camptothecin or its derivatives is associated with induction of early response gene expression. This effect is not limited to U-937 cells since similar findings have been obtained in other human tumor cell lines treated with these agents. Camptothecin is a specific inhibitor of topoisomerase I (1). Furthermore, the cytotoxic effects of this agent in yeast require expression of this enzyme (47, 48). Taken together, these findings have led to the conclusion that topoisomerase I is required for gene transcription.

DISCUSSION

Previous studies have demonstrated that DNA topoisomerase I is involved in the transcription of ribosomal RNA and heat shock genes in HeLa cells (39, 40) and in the transcription of adenoviral genes (40). Transcriptional elongation requires the movement of RNA polymerase through double helical DNA and topoisomerase I appears to relieve the torsional consequences of this process (41). In this context, topoisomerase I is localized in regions of active transcription (42-45). Moreover, microinjection of antibodies against topoisomerase I into nuclei is associated with a block in elongation of transcripts (46). Taken together, these findings have led to the conclusion that topoisomerase I is required for gene transcription.

Fig. 6. Regulation of c-jun gene transcription by CAM and 9AC. U-937 cells were treated with 0.5 ng/ml CAM or 9AC for 6 h. Nuclei were isolated and the newly synthesized 32P-labeled RNA was hybridized to actin and c-jun inserts subjected to restriction enzyme digestion and Southern blotting. The solid lines in the schematic indicate the positions of the DNA inserts. Similar findings were obtained in two separate experiments. Ordinate, molecular weight in thousands.
zyme by camptothecin can result in both down- and up-regulation of specific genes. This differential effect on gene expression could be dependent on drug concentration. For example, we have used concentrations of camptothecin that were 2-5 x 10^3-fold lower than that used by others. Thus, lower concentrations that partially inhibit topoisomerase I may permit induction of certain genes, while transcription of all genes is inhibited at high drug concentrations.

Previous work has shown that camptothecin results in a reversible fragmentation of DNA (13, 16). Inhibition of topoisomerase I by this agent stabilizes the covalent enzyme-DNA intermediate and results in strand breaks (19, 49, 50). This induction of DNA damage may function in the activation of a signaling cascade that includes early response gene expression. In this context, other studies have demonstrated that agents, such as ionizing radiation, 1-β-D-arabinofuranosylcytosine, UV light, and etoposide, which damage DNA by distinct mechanisms, also activate c-jun gene transcription (20-22, 37, 51).

Recent work has suggested that H_2O_2-induced ROI can contribute to the induction of c-jun expression (51). While ROI could activate AP-1 directly, these intermediates can also damage DNA (52). Moreover, 1-β-D-arabinofuranosylcytosine, etoposide, and camptothecin are not recognized as agents which induce ROI. Thus, given our present understanding, it would appear that different types of DNA damage induce a common signaling pathway which activates genes regulated by the cis-acting AP-1 element. Indeed, DNA damage in bacteria is associated with the SOS response and induction of specific genes (53). A similar process exists in mammalian cells and may involve activation of genes that regulate DNA repair or the cell cycle.

The present results also demonstrate that camptothecin induces cleavage of DNA into multiples of nucleosome-sized fragments. This process of endonucleolytic DNA cleavage is one of the characteristic of apoptosis or programmed cell death (54). The finding that novel mRNAs are induced during apoptosis (55) has suggested that activation of certain transcription factors may contribute to the control of events in this program. The results of the present study indicate that c-jun expression is associated at least temporally with internucleosomal DNA fragmentation. However, the relationship between these two events remains unclear. Moreover, other characteristics such as

![Fig. 7. Expression of c-jun and actin genes in U-937 cells treated with 9AC and cycloheximide. U-937 cells were treated with 0.5 ng/ml 9AC alone, 10 µg/ml cycloheximide (CHX) alone, and 9AC plus CHX for the indicated periods of time. Total cellular RNA (20 µg) was analyzed by Northern blot hybridization by using 32P-labeled c-jun and actin DNA probes.](image)

![Fig. 8. Effects of protein kinase inhibitors on expression of c-jun and actin mRNA in cells treated with 9AC. Total RNA was prepared from U-937 cells treated with 0.5 ng/ml 9AC (6 h), 50 µM H7 (2 h), 50 µM HA1004 (2 h), or the indicated combinations of these agents. Total cellular RNA was hybridized to the 32P-labeled c-jun and actin probes.](image)

![Fig. 9. Fragmentation of nuclear DNA. U-937 cells were treated with 0.5 ng/ml CAM or 9AC for the indicated times. Nuclear DNA was isolated and separated in 2% agarose gels. After staining of the gels with ethidium bromide, DNA was visualized by UV illumination.](image)
chromatin condensation and plasma membrane blebbing are needed to determine whether camptothecin induces programmed cell death. Indeed, the cell volume expansion detected in the present studies is in contrast to the retraction usually found in apoptosis. Nonetheless, the activation of endonucleolytic DNA cleavage may contribute to the cell lethality induced by camptothecin and its derivatives. Stabilization of the topoisomerase I-DNA cleavable complex by these agents and the associated strand breaks is probably the initial signal that activates a cellular response. The induction of early response genes as part of this signaling cascade could contribute to the regulation of DNA repair mechanisms and, in the event of irreparable damage, to the initiation of programmed cell death.

Finally, the induction of early response gene expression and DNA fragmentation preceded the marked increases in size of the CAM-treated U-937 cells and their nuclei. Similar increases have been observed in other human tumor cells, including malignant melanoma, treated with CAM derivatives in vitro and in vivo. These morphological changes appear to be specific responses of malignant cells to CAM treatment since they have not been reported for other antineoplastic agents. The mechanism(s) responsible for this increase in size is not yet known, but apparently involves events that contribute to unbalanced growth of cells. Further studies are needed to determine whether these nondividing, enlarged cells express specific mRNAs not present in untreated cells.

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