Increased gadd153 Messenger RNA Level Is Associated with Apoptosis in Human Leukemic Cells Treated with Etoposide¹

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ABSTRACT

Treatment of leukemic cells with topoisomerase inhibitors can lead to growth arrest and subsequent apoptotic cell death. The relationships between cell cycle regulation and apoptosis triggering remain poorly understood. The gadd153 gene encodes the nuclear protein CHOP 10 that acts as a negative modulator of C/EBP enhancer binding protein transcriptional factors and inhibits cell cycle progression. We have investigated the relationships between gadd153 gene expression and apoptosis induction in four human leukemic cell lines with different sensitivities to apoptosis induced by etoposide (VP-16), a topoisomerase II inhibitor. The gadd153 gene was constitutively expressed in the four studied cell lines. In U937 and HL-60 cells that were very sensitive to apoptosis induction by the drug, VP-16 induced a time- and dose-dependent increase of gadd153 gene mRNA expression. Using agarose gel electrophoresis and a quantitative filter elution assay, apoptotic DNA fragmentation was observed to begin when gadd153 gene expression increased. Equitoxic doses of VP-16 (as defined using a 96-h 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide assay) did not increase the gadd153 mRNA level in K562 and KCL22 cell lines that were more resistant to apoptosis induction by the drug. Nuclear run-on and mRNA stability experiments demonstrated that VP-16 treatment increased gadd153 gene transcription in the sensitive U937 cells. Cycloheximide did not prevent gadd153 expression increase. Both gadd153 mRNA level increase and internucleosomal DNA fragmentation were inhibited by N-tosyl-l-phenylalanine chloromethylketone, a serine threonine protease inhibitor, N-acetyl-leucyl-leucyl-norleucine, an inhibitor of calpain, N-acetylcycteine, an inhibitor of oxidative metabolism, and overexpression of Bel-2. Z-DEVD peptides that inhibit interleukin 1β-converting enzyme-like proteases suppressed DNA fragmentation without preventing gadd153 mRNA increase in VP-16-treated U937 cells. These results indicate that gadd153 gene expression increase occurs downstream of events sensitive to N-tosyl-l-phenylalanine chloromethylketone, calpain inhibitor I, and Bel-2 and upstream of interleukin 1β-converting enzyme-related proteases activation in leukemic cells in which treatment with VP-16 induces rapid apoptosis.

INTRODUCTION

VP-16¹ is a topoisomerase II inhibitor which stabilizes an intermediate of the topoisomerase reaction, the cleavable complex, thereby inducing a series of events that can lead to cell cycle arrest and apoptotic cell death (1, 2). Several recent evidences suggest a relationship between cell cycle regulation and apoptosis triggering in response to VP-16 and other DNA-damaging agents (3, 4). The p53 tumor suppressor gene, which triggers G1 arrest in response to DNA damage, plays a crucial role in the execution of apoptosis following cell treatment with topoisomerase inhibitors (5). In p53-negative cells, a DNA damage-responsive G2 checkpoint appears to determine the sensitivity of cells to topoisomerase inhibitors (2). Moreover, unscheduled activation of p34cdc2/cyclin B1 kinase was observed in leukemic cells undergoing apoptosis after treatment with topoisomerase poisons (6), and several other cyclins or kinases involved in cell cycle regulation were shown to play a role in the apoptotic response to these cytotoxic drugs (7-9).

Genes involved in cell differentiation and growth arrest in response to various stimuli are also potential candidates for apoptosis regulation (10-12). The gadd genes (growth arrest and DNA damage) were originally isolated on the basis of rapid induction by UV radiation in Chinese hamster ovary cells, and their expression was subsequently found to be induced by a wide variety of DNA-damaging agents and growth arrest treatments (13, 14). One of these genes, the human gadd153 gene, encodes the nuclear protein CHOP 10 (C/EBP homologous protein) that acts as a negative modulator of C/EBP transcriptional factors (15) and inhibits cell cycle progression. C/EBP genes encode transcriptional factors that participate in the process of terminal differentiation and growth arrest in adipose tissues (15). CHOP 10 is unique among the C/EBP-related proteins in that it cannot bind to DNA. It can, however, form stable heterodimers with other C/EBP proteins and either prevent their binding to DNA (16) or promote their binding to nonclassical CCAAT sites (16). Recently, it was suggested that CHOP 10 could be a positively acting transcription factor as well (17). Microinjection of CHOP 10 into NIH-3T3 fibroblasts induces G1 arrest (16). Similarly, transient expression of CHOP 10 into several different human tumor cell lines leads to growth arrest (18). CHOP 10 was also suggested to contribute to chemotherapy-induced apoptosis in myeloid cells (19). gadd153 mRNA expression is very low in growing cells and is markedly increased following treatment with a wide variety of DNA-damaging agents, including the alkylating agent EMS (14) and the topoisomerase II inhibitor VP-16 (20). gadd153 mRNA expression is also increased in response to a number of different growth arrest conditions including serum starvation, medium depletion, and exposure to prostaglandin A2 (14, 21).

We have demonstrated previously that VP-16 was able to induce apoptosis in various human leukemic cell lines. However, the kinetics of induction of apoptosis in response to equitoxic concentrations of VP-16, as defined using a 96-h MTT assay, varied widely from one cell line to another. VP-16-induced apoptosis was observed within a few hours in U937 and HL-60 cell lines and was detected only after 48-72 h of treatment in K562 and KCL22 cell lines. Delayed apoptosis in the later cell lines was associated with better inhibition of cell cycle progression in response to DNA damage. The reported effects of CHOP 10 on cell cycle regulation prompted us to investigate the expression of gadd153 mRNA in these four cell lines. Results of this study suggest that gadd153 mRNA expression could be related to apoptotic DNA fragmentation rather than cell cycle arrest in human leukemic cells treated with VP-16.

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³ The abbreviations used are: VP-16, etoposide; C/EBP, CCAAT/enhancer binding protein; CPI, calpain inhibitor I; ICE, interleukin 1β-converting enzyme; MMS, methylmethanesulfonate; NAC, N-acetylcysteine; TPCK, N-tosyl-l-phenylalanine chloromethylketone; Glc30, 50% inhibition of cell growth; MTT, 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide. 
MATERIALS AND METHODS

Cell Lines and Culture. The four human leukemic cell lines, HL-60 (promyeloeytic), U937 (monocytes), KCL22 (chronic myelogenous leukemia in lymphoid blast crisis), and K562 (chronic myelogenous leukemia in erythroid blasts crisis) were grown in suspension in RPMI 1640 (BioWhittaker, Fontenay-sous-Bois, France) supplemented with 10% (v/v) heat-inactivated FCS and 2 mM L-glutamine in an atmosphere of 95% air and 5% CO2 at 37°C. HL-60/Neo and HL-60/Bcl-2 were derived from HL-60 cells infected with the control neomycin or Bcl-2 retroviruses, respectively (22). HL-60/1C2 was subcloned by limiting dilution from the HL-60/Bcl-2 cells. Cell viability was determined using the trypan blue exclusion test. To ensure an exponential growth, cells were resuspended in fresh medium 24 h before each treatment. Doubling time was determined by counting cells each day until 72 h as described previously (2). The percentage of cells in the S-phase was determined by flow cytometry after propidium iodide staining (2).

Drugs and Chemical Reagents. VP-16, camptothecin, and daunorubicin were purchased from Sigma (Saint Quentin Fallavier, France). Drug stock solutions were made in DMSO at 20 mM and stored in small aliquots at −20°C. Further dilutions were made in culture medium just before use. Actinomycin D (5 mg/ml) and cycloheximide (20 mg/ml) were dissolved in DMSO, stored in small aliquots at −20°C, and added 30 min before VP-16 treatment in the culture medium at the indicated concentrations. TPCK and NAC were obtained from Sigma. TPCK was dissolved in DMSO at 50 mM and stored in small aliquots at −20°C. N-acetylcysteine solutions were made just before use in fresh medium and adjusted to pH 7.4. The CPT N-acetyl-lescyl-lescyl-norleucin was obtained from Boehringer Mannheim (Meylan, France) and dissolved at 50 mM in DMSO. CPI, NAC, and TPCK were added to culture medium at 0.05 mM, 25 mM, and 0.2 mM respectively. In all experiments, the final concentration of DMSO did not exceed 1% (v/v), a concentration which was nontoxic to the cells. Z-VAL-CHF2 and Z-DEV-D-CHF2 were purchased from Enzyme Systems Products (Dublin, CA) and dissolved in DMSO at 50 mM. [2-14C]thymidine (50 mCi/ml), [α-32P]dCTP (3000 Ci/mmol), and [α-32P]dUTP (3000 Ci/mmol) were obtained from Amersham (Les Ulis, France). All other chemicals were of reagent grade and purchased from local sources. Cell survival studies were performed using a 96-h MTT assay, and the percentage of cells in the S-phase was determined by flow cytometry as described previously (2).

Quantification of DNA Fragmentation Using a Filter Elution Assay. DNA fragmentation was measured using an alkaline elution filter method as described elsewhere (23). Briefly, at specified times after drug treatment, 1×10^6 14C-labeled cells were loaded onto protein-absorbing filters (polyvinylidene difluoride filters, 20 μm pore size, 25 mm in diameter, Durapore membrane; Millipore, St. Quentin Fallavier, France) and washed in ice-cold PBS. Lysis was subsequently performed with 5 ml of LS10 buffer (0.2% sarkosyl sodium, 2 mM NaCl, and 0.04 mM EDTA, pH 10). Filters were washed with 7 ml of 0.02 mM EDTA (pH 10), heated for 45 min at 65°C in the presence of 1 N HCl, and then treated for 45 min with 2.5 mM of NaOH. DNA fragmentation was determined as the percentage of dpm in the lysis + washed fractions (fragmented DNA) relative to total intracellular dpm (total DNA).

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. Cellular DNA from 5×10^6 controls or treated cells was extracted by a salting-out procedure (24) using the Stratagene kit (LaJolla, CA). Briefly, control and treated cells were washed once in ice-cold PBS. Lysis was performed in 10 mM Tris-HCl, 400 mM NaCl, 2 mM EDTA (pH 8.2), 10% SDS, and 100 μg/ml proteinase K overnight at 37°C. After subsequent proteins precipitation, DNA was purified and resuspended in TE buffer. Electrophoresis of 15 μg of DNA was performed in a 1.8% agarose gel in Tris-borate buffer (pH 8) with 0.4 μg/ml ethidium bromide at 20 V for 14 h. After electrophoresis, DNA was visualized under UV examination.

Total RNA Extraction and Northern Blot Analysis. Total RNA was extracted using the RNA Plus kit (Bioprobe System, Montreuil, France) according to the method of Chomczynski and Sacchi (25). Twenty μg of total RNA were fractionated by electrophoresis in 1% agarose-formaldehyde gels and transferred one night by capillarity with 20× SSC onto nitrocellulose membranes (Hybond C extra; Amersham). The 1 kb human gadd153 probe was the cDNA clone pHU 175-2A (26, 27) excised from the PCD plasmid by BamHI (kindly provided by Dr. Nikki Holbrook, National Institute on Aging, Baltimore, MD). The α-actin probe was a 513-bp EcoRI fragment from pHμα-A1 (28). Fifty to 80 ng of purified RNA, previously denatured at 100°C for 10 min, were labeled with [α-32P]dCTP (3000 Ci/mmol) using the random primed DNA labeling kit (Boehringer Mannheim). High specific activity (2×10^6 dpm/μg) was obtained. The membranes were prehybridized and hybridized in 50% formamide, 5× Denhardt’s solution, 50 mM phosphate buffer (pH 6.5), 5× SSC, 0.1% SDS, and 2.5 μg/ml salmon sperm DNA for 42 h at 42°C. Activity in final hybridization buffer was at least 3×10^6 dpm/ml. The most stringent final wash was for 20 min at 65°C in 0.2× SSC and 0.1% SDS, and membranes were exposed for autoradiography at −80°C for at least 3 days. The probes were removed by washing water 1 h at 80°C, and membranes were rehybridized with an α-actin probe to normalize RNA amounts. The Northern blot analysis experiments were quantitated by densitometric scanning using the Phoretix 1D software (Phoretix International, Newcastle, England). Results were expressed as the ratio of gadd153 to α-actin mRNA. Then the control lane was arbitrarily assigned the value 1, signals in the treated lanes being expressed relative to this control.

Nuclear Run-On Assay. Nuclei from 5×10^7 untreated and treated U937 cells were isolated by detergent lysis and stored at −80°C in 100 μl of storage buffer containing 50% glycerol. These nuclei were incubated at 30°C for 30 min in reaction buffer [300 mM KCl, 10 mM MgCl2, 10 mM MnCl2, 10 mM Tris-HCl (pH 8), 5 mM DTT, 1 mM ATP, 1 mM GTP, 1 mM CTP, and 100 IU of RNase inhibitor], 20 μl of yeast tRNA (5 mg/ml; Boehringer Mannheim), and 10 μl of [α-32P]dUTP (3000 Ci/mmol). Then 32P-labeled RNA were isolated and hybridized to the following plasmids: (a) the pcDNA plasmid containing the 1-kb BamHI fragment of the human cDNA pHU 175-2A and (b) the pHμα-A1 vector with the 513-bp EcoRI fragment of the human α-actin. The denatured plasmids were resuspended in 0.3 N NaOH, boiled for 10 min, and chilled on ice for 10 min. An equal volume of 5 mM NH4OAc was added, and DNAs were loaded onto nitrocellulose filters. Filters were prehybridized for 48 h at 42°C and hybridized with equivalent amounts (5×10^6 cpm/filters) of 32P-labeled RNA for 48 h at 42°C in phosphate buffer [50 mM NaPO4, 50% formamide, 0.5% SDS, 2 μM EDTA, 0.005× Denhardt’s solution, and 500 μg/ml salmon sperm DNA]. Filters were washed twice at room temperature in 2× SSC, 50 mM NaPO4, 0.5% SDS for 15 min, once at 68°C in 2× SSC, 50 mM NaPO4, 0.5% SDS for 20 min, once at 68°C in 2× SSC, 50 mM NaPO4, 0.1% SDS for 20 min, and once at room temperature in 2× SSC, 50 mM Tris, and 0.1% SDS for 15 min. Final wash was at 37°C in 2× SSC, 50 mM Tris, and 0.1% SDS with 20 μg/ml RNase for 20 min. The radioactivity of every slot was determined using densitometric scanning as described previously.

RESULTS

Characterization of Studied Cell Lines. The gadd153 gene was constitutively expressed in the four studied cell lines. Its basal expression was higher in K562 cells compared to other cell lines (Table 1).
The concentration inducing a GI_{50} was determined using a 96-h MTT assay. These GI_{50} values were used to determine the equitoxic concentrations tested in subsequent experiments and corresponding to about 10-fold or 50-fold the GI_{50}. Differences between cell lines were not related to their doubling time nor their cell cycle distribution.

Modulation of the gadd153 mRNA Level in U937 Cells Treated with VP-16. We used Northern blot analysis to investigate the effects of VP-16 on the gadd153 mRNA level and its relationship with apoptosis. First, we studied the effects of continuous exposure for various times to 30 μM (about 15-fold of the GI_{50}) and 100 μM (about 50-fold of the GI_{50}) VP-16 on gadd153 mRNA expression in these cells. Thirty μM VP-16 induced a 6–7-fold increase of gadd153 mRNA expression that occurred 4 h after the beginning of drug treatment (Fig. 1, A and B). This increase was detected simultaneously with the appearance of internucleosomal DNA fragmentation visualized by agarose gel electrophoresis (Fig. 1C, middle panel) and quantified using the filter elution assay (Fig. 1C, left panel). This increase was transient since the gadd153 mRNA level almost returned to baseline level in 2 h (Fig. 1, A and B). When U937 cells were treated with a higher VP-16 concentration (100 μM), the gadd153 mRNA expression increase was detected earlier, 2.5 h after the beginning of treatment, was higher, reaching 12-fold of the baseline level, and remained high much longer since it was still 10-fold above the baseline level 6 h after the beginning of treatment, when the high level of cell death renders more difficult RNA extraction (Fig. 1, A and B). Again, kinetics of gadd153 mRNA expression increase and apoptotic DNA fragmentation was time related (Fig. 1C, left and right panels). Second, U937 cells were treated with increasing concentrations of VP-16 for 4 h. The gadd153 mRNA level increased with drug concentration until 30 μM, reaching 7-fold of the basal level, then remaining 6-fold above the basal level following a 4-h exposure to 100 or 300 μM. Simultaneously, we observed a dose-dependent increase of internucleosomal DNA fragmentation visualized by agarose gel electrophoresis (data not shown). Thus, a dose-dependent and a time-dependent relationship exists between gadd153 mRNA expression and apoptotic internucleosomal DNA fragmentation in U937 cells treated with VP-16.

Modulation of the gadd153 mRNA Level in K562 Cells Treated with VP-16. Continuous treatment of K562 cells with 60 μM VP-16 (about 10-fold of the GI_{50}) induced a progressive decrease of the gadd153 mRNA level until 24 h (Fig. 2, A and B). After 48 h of continuous exposure to 60 μM VP-16, the gadd153 mRNA level remained under the baseline level (Fig. 2, A and B). The filter elution assay demonstrated a very slow and progressive DNA fragmentation, reaching 72% after 48 h of treatment (Fig. 2C, left panel) but apoptotic DNA fragmentation was hardly detectable on agarose gel electrophoresis 48 h after the beginning of VP-16 treatment (Fig. 2C, middle panel). At a higher VP-16 concentration (400 μM, about 50-fold of the GI_{50}), the gadd153 mRNA level remained stable for 24 h (Fig. 2, A and B). At and beyond 24 h of drug exposure, the high percentage of cell death renders difficult total RNA extraction. Accordingly, apoptotic DNA fragmentation was detected at and beyond 24 h of continuous drug treatment by agarose gel electrophoresis (Fig. 2C, left and right panels), and 80% of DNA was fragmented at 48 h when measured using the filter elution assay (Fig. 2C, left panel). Thus, in K562 cells, in which apoptosis is delayed compared to U937 cells, the gadd153 mRNA expression did not increase in response to VP-16 treatment. We conclude that VP-16 modulates differently gadd153 mRNA expression in these two human leukemic cells that dramatically differ in their sensitivity to VP-16-induced apoptosis.

![Figure 1: Time course of gadd153 mRNA expression and DNA electrophoresis in U937 cells treated with VP-16.](image-url)

*Fig. 1. Time course of gadd153 mRNA expression and DNA electrophoresis in U937 cells treated with VP-16. U937 cells were incubated with 30 μM (○) or 100 μM (●) VP-16 for the indicated times. A, Northern blot analysis of gadd153 and α-actin gene expression. B, gadd153 mRNA:α-actin mRNA ratio was calculated after densitometric scanning of Northern blots shown in A. The value 1 was arbitrarily assigned to the ratio measured in untreated cells. C, DNA fragmentation was quantified using a filter elution assay and visualized by agarose gel electrophoresis. Each point of the filter elution assay is the mean of three independent experiments performed in triplicate. Other results shown are representative of one at least three independent experiments. Bars, SD.*
Modulation of the gadd153 mRNA Level in Two Other Human Leukemic Cell Lines Treated with VP-16. We studied gadd153 mRNA expression in two other human leukemic cell lines, HL-60 and KCL22. In HL-60 cells treated with 10 μM VP-16 (about 10-fold of the GI50), the gadd153 mRNA level increased 4 h after the beginning of drug treatment, reaching 5-fold of the baseline level, and remained elevated 20 h, when a majority of cells were apoptotic (Fig. 3, A and B). As in U937 cells, the increase of gadd153 mRNA expression was time related to the appearance of apoptotic DNA fragmentation identified in agarose gel electrophoresis (data not shown) and by filter elution assay (Fig. 3C). In KCL22 cells treated with 100 μM VP-16 (about 10-fold of the GI50), the gadd153 mRNA level progressively decreased, reaching a minimal level at 8 h, then increased again to return to the basal level 24 h after the beginning of treatment. Strong mortality rendered difficult mRNA analysis beyond 24 h but gadd153 mRNA seemed to increase to 3.5-fold of the baseline level after 48 h, when significant apoptotic DNA fragmentation occurred (Fig. 3, A and B). These results confirmed the temporal relationship between gadd153 mRNA expression increase and the appearance of internucleosomal DNA fragmentation, at least in HL-60 cells which were the most sensitive to VP-16-induced apoptosis.

Modulation of the gadd153 mRNA Level in U937 and K562 Cell Lines Treated with Camptothecin and Daunorubicin. We studied the effects of two other cytotoxic drugs on the gadd153 mRNA level in U937 and K562 cell lines. Daunorubicin, a topoisomerase II inhibitor that intercalates in DNA, increases gadd153 expression in U937 cells (Fig. 4A). This increase occurs after 4 h of drug exposure at the tested concentration (1 μM) that was observed to induce apoptosis in these cells (data not shown). Similar results were obtained in HL-60 cells (data not shown). In K562 cells treated with 1 μM daunorubicin, gadd153 expression decreases after 24 h of drug exposure (Fig. 4B) and apoptosis is delayed (data not shown). Similar results were obtained in U937 and K562 cells when treated with 10 μM camptothecin (data not shown).

Mechanisms of VP-16-induced gadd153 mRNA Level Increase in U937 Cells. We studied the role of transcriptional and posttranscriptional regulation of gadd153 gene expression in VP-16-treated U937 cells. The RNA synthesis inhibitor actinomycin D (5 μg/ml) abolished gadd153 mRNA increase in U937 cells incubated with 30 μM VP-16 for 4 h or 100 μM for 2.5 h (Fig. 5). These results suggested that transcription could be necessary for gadd153 mRNA increase. Nuclear run-on transcription experiments confirmed this hypothesis, demonstrating that gadd153 mRNA transcription was 3-fold increased by a 2.5-h exposure to 100 μM VP-16 (Fig. 6A), whereas the same treatment did not modify α-actin mRNA transcription. To determine whether VP-16 could affect also the half-life of gadd153 mRNA, U937 cells were treated with 100 μM VP-16 for 2.5 h. Then (time 0) VP-16 was either washed out or allowed to remain in the medium and transcriptional activity was suspended by addition of actinomycin D. As shown in Fig. 6B, in the absence of actinomycin D and in the presence of the drug, the gadd153 mRNA level increased during 2 h, then decreased progressively. In the presence of both actinomycin D and the drug, the gadd153 mRNA level still increased during the first hour, then decreased rapidly, suggesting a transient effect of the drug on mRNA stability. This transient increase of gadd153 mRNA expression following actinomycin D addition to the drug-containing medium was observed in three independent experiments. In the absence of the drug and in the presence of actinomycin D, the gadd153 mRNA level decreased slowly. To determine whether induction of gadd153 was a primary response to VP-16 toxicity or a secondary event which required protein synthesis, we examined the effect of cycloheximide on the gadd153 mRNA level increase. Pretreatment of U937 cells with cycloheximide did not prevent the gadd153 mRNA level increase induced by VP-16 (Fig. 5). Instead, cycloheximide alone induced a 2-fold increase of the gadd153 mRNA level, suggesting
that inhibition of protein synthesis could induce \textit{gadd153} expression \textit{per se} (Fig. 5). Cycloheximide, but not actinomycin D, partly prevented DNA fragmentation induced by VP-16 (Fig. 5C). Both actinomycin D and cycloheximide were observed to induce apoptotic DNA fragmentation when tested alone on U937 cells (Fig. 5C).

\textbf{Effects of Protease Inhibitors on \textit{gadd153} mRNA Expression and Apoptotic DNA Fragmentation in U937 Cells Treated with VP-16.} We have observed that various protease inhibitors could prevent VP-16-induced apoptosis in U937 cells (29). To better understand the relationships between the \textit{gadd153} mRNA level and VP-16-induced internucleosomal DNA fragmentation, we analyzed the effects of TPCK, a serine-threonine protease inhibitor, and the CPI N-acetyl-leucyl-leucyl-norleucinal, a cysteine protease inhibitor, on VP-16-induced \textit{gadd153} mRNA level increase. Both protease inhibitors prevented apoptotic DNA fragmentation in U937 cells treated with 100 \textmu M VP-16 for 2.5 h (Fig. 7, lower panel). CPI alone (0.05 mM) and TPCK alone (0.2 mM) did not induce \textit{gadd153} mRNA increase (Fig. 7, upper and middle panels). CPI and TPCK partially prevented \textit{gadd153} mRNA increase and drug-induced DNA fragmentation when associated with 100 \textmu M VP-16 for the treatment of U937 cells. Similar results were obtained in U937 cells treated with 30 \textmu M VP-16 for 4 h in the presence or in the absence of these protease inhibitors (data not shown).

In contrast, two permeant peptide inhibitors of ICE-like proteases that efficiently prevent VP-16-induced apoptotic DNA fragmentation did not prevent \textit{gadd153} mRNA level increase in VP-16-treated U937 cells (Fig. 8A). Moreover, at 50 \textmu M, a concentration that completely inhibits apoptotic DNA fragmentation (Fig. 8B), the \textit{gadd153} mRNA level was increased when compared to cells treated with VP-16 alone. These results indicate

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\textbf{Fig. 3. Time course of \textit{gadd153} mRNA expression and DNA fragmentation in HL-60 and KCL22 cells treated with VP-16.} HL-60 (○) and KCL22 (●) cells were incubated for the indicated times with 10 and 100 \textmu M VP-16, respectively. \textit{A,} Northern blot analysis of \textit{gadd153} and \textit{α-actin} gene expression. \textit{B,} \textit{gadd153} mRNA:\textit{α-actin} mRNA ratio was calculated after densitometric scanning of Northern blots shown in \textit{A.} The value 1 was arbitrarily assigned to the ratio measured in untreated cells. \textit{C,} DNA fragmentation was quantified using a filter elution assay. Each point of the filter elution assay is the mean of three independent experiments performed in triplicate. Other results shown are representative of one of at least three independent experiments. Bars, SD.

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\textbf{Fig. 4. Time course of \textit{gadd153} mRNA expression in U937 (A) and K562 (B) cells treated with 1 \mu M daunorubicin.} Results shown are representative of one of two independent experiments. Lower panels, ethidium bromide-stained rRNA from each sample, indicating equivalent RNA loading.

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\textbf{Fig. 5. Time course of \textit{gadd153} mRNA expression and DNA fragmentation in HL-60 and KCL22 cells treated with VP-16.} HL-60 (○) and KCL22 (●) cells were incubated for the indicated times with 10 and 100 \textmu M VP-16, respectively. \textit{A,} Northern blot analysis of \textit{gadd153} and \textit{α-actin} gene expression. \textit{B,} \textit{gadd153} mRNA:\textit{α-actin} mRNA ratio was calculated after densitometric scanning of Northern blots shown in \textit{A.} The value 1 was arbitrarily assigned to the ratio measured in untreated cells. \textit{C,} DNA fragmentation was quantified using a filter elution assay. Each point of the filter elution assay is the mean of three independent experiments performed in triplicate. Other results shown are representative of one of at least three independent experiments. Bars, SD.
that the induction of gadd153 mRNA expression occurs downstream of CPI- and TPCK-sensitive events and upstream of ICE-like proteases-mediated events in the pathway leading to apoptosis of VP-16-treated U937 cells.

Effects of NAC on gadd153 mRNA Expression and Apoptotic DNA Fragmentation in U937 Cells Treated with VP-16. Many DNA-damaging agents result in cellular oxidative stress, either through the production of free radicals or by reaction with free sulfhydryl groups. We used NAC to test the role of reactive oxygen intermediates in mediating the gadd153 mRNA level increase following VP-16 treatment. At 25 μM for 2.5 h, NAC alone did not modify the gadd153 mRNA level (Fig. 9, upper and middle panels) but partially inhibited the gadd153 mRNA level increase in U937 cells treated with 100 μM VP-16 for 2.5 h (Fig. 9, upper and middle panels) or 30 μM VP-16 for 4 h (data not shown). Twenty-five mm NAC also partially inhibited VP-16-induced apoptotic DNA fragmentation (Fig. 9, lower panel). At the highest concentration (100 μM), NAC alone induced a 2-fold increase in gadd153 mRNA (data not shown) without inducing DNA fragmentation and efficiently prevented VP-16-induced DNA fragmentation (data not shown). Thus, the gadd153 mRNA level increase occurs downstream of the events inhibited by NAC in the pathway leading to apoptosis in VP-16-treated U937 cells.

Effects of Bcl-2 Overexpression on gadd153 mRNA Expression and Apoptotic DNA Fragmentation in HL-60 Cells Treated with VP-16. Overexpression of Bcl-2 oncoprotein was shown to delay drug-induced apoptosis in several cell systems by a presently unknown mechanism (30). The HL-60/IC2 subclone was genetically engineered to overexpress Bcl-2 protein. When treated with VP-16 (10 μM), gadd153 mRNA increase (Fig. 10) and apoptotic DNA fragmentation (data not shown) were both delayed in HL-60/IC2 cells compared to HL-60/neo cells, suggesting that gadd153 mRNA up-regulation occurs downstream of Bcl-2-controlled events in the pathway that leads to apoptosis.

DISCUSSION

One effect of DNA damage is the transient cell growth arrest. This arrest occurs at any phase of the cell cycle, depending on the stimulus and its intensity. It reflects the time used by the cell to try to repair the damaged DNA template before replication and mitotic chromosome segregation (31). Then the cell either progresses again in the cell cycle or undergoes apoptotic cell death. We previously observed that the resistance of leukemic cell lines to VP-16-induced apoptosis increased with their ability to arrest cell cycle progression in response to similar levels of DNA damage induced by the drug (2). At high doses, VP-16-induced apoptosis occurred within a few hours in the most sensitive cell lines, before any measurable effect on their cell cycle progression. Cells that were more resistant to apoptosis induction by this drug were staggered in their cell cycle progression when treated with equitoxic doses and died later by apoptosis. The gadd153 gene, whose expression is induced by a wide variety of DNA-damaging agents including VP-16 (20), has been previously involved in cell cycle arrest (14, 16, 18). We hypothesized that this gene could have a role in the efficient cell cycle arrest observed in the cell lines that were the most resistant to VP-16-induced apoptosis. Actually,
could mediate apoptosis (3–9, 34–36). Whether gad153 could be one of the cell cycle-related proteins that are also involved in cell death pathways independently of their cell cycle effects remains to be demonstrated. Overexpression of gad153 gene is not a consequence of apoptotic DNA fragmentation since ICE-like proteases peptide inhibitors prevent this fragmentation without inhibiting the gad153 mRNA increase in VP-16-treated U937 cells.

The gad153 gene was previously reported to be expressed in virtually every mammalian cell lines (14) but its constitutive expression is low in most untreated cells. The mRNA level of gadd genes has been shown to be regulated both at the transcriptional and the...
Posttranscriptional levels. The gadd153 promoter was shown to be activated rapidly and specifically by DNA damage induced by UV-mimetic agents, DNA cross-linking and alkylating agents, DNA intercalators, and topoisomerase inhibitors (20), suggesting that transcriptional mechanisms accounted for the induction of gadd genes in cells treated with DNA-damaging agents. Conversely, signals responsible for withdrawal from an active proliferative state stabilized gadd transcripts (14, 20, 21). However, treatment with MMS or UV radiation was shown to stabilize gadd153 mRNA in actively proliferating cells, an effect that was not observed in growth-arrested cells (37). In VP-16-treated U937 cells, gadd153 mRNA appears to be regulated mainly at the transcriptional level. Stabilization of gadd153 mRNA was observed only in the first hour of exposition to actinomycin D following VP-16 treatment and decreased rapidly, although gadd153 mRNA remained elevated in cells treated by VP-16 without actinomycin D. Cycloheximide did not modify gadd153 induction in VP-16-treated cells, suggesting that de novo protein synthesis was not required. However, we observed that cycloheximide itself increased the gadd153 level, an effect that has been described also in rat pancreatic islets (38), without potentiating VP-16-induced gadd153 mRNA level increase.

Identification of ICE, a protease that cleaves pro-interleukin 1β to produce the mature cytokine, as the mammalian homologue of the Caenorhabditis elegans cell death protein ced-3, suggested a central role of proteolytic events in apoptotic cell death (39). Overexpression of recombinant ICE induces apoptosis in rat-1 fibroblasts, implicating this protease in signaling cell death (40). Actually, ICE and ced-3 are members of a large family of cysteine proteases that differ from other protease in their sequence, structure, and substrate specificity. These proteases could be the key members of a larger proteolytic pathway that is modulated by various protease inhibitors, both in whole cells and in a cell-free system (29). TPCK, a serine threonine protease inhibitor, inhibits the early steps of the apoptotic pathway triggered by cytotoxic drugs in human leukemic cells (41). Calpain, another protease that regulates G1 (42), was also involved in apoptosis triggering in some cell systems (43). Both TPCK and the CPI N-acetyl-leucyl-leucyl-norleucinal prevented the gadd153 mRNA level increase and apoptotic DNA fragmentation in VP-16-treated U937 cells. In contrast, inhibition of apoptotic DNA fragmentation by ICE-like protease inhibitors does not prevent the gadd153 mRNA increase. These results indicate that the gadd153 mRNA increase occurs downstream of CPI- and TPCK-sensitive events and upstream of ICE-like proteases-mediated events in the pathway leading to apoptosis of VP-16-treated U937 cells (29).

A high expression of Bcl-2 was related to poor clinical outcome in several human malignant diseases (44). Bcl-2 protein overexpression was shown to delay apoptotic cell death in tumor cells treated with cytotoxic drugs (30). Bcl-2 was shown recently to delay activation of ICE-like proteases in various apoptotic pathways (45). Here, we show that Bcl-2 overexpression also delays the gadd153 mRNA increase in VP-16-treated cells. ICE-like proteases' inhibition does not prevent gadd153 mRNA increase. Altogether, these data indicate that Bcl-2 either modulates gadd153 expression and activity of ICE-like proteases by two different pathways or acts upstream of both events in the apoptotic pathway triggered by VP-16.

Bcl-2 is not involved in the slow induction of apoptosis in K562 and KCL-22 cells, since the protein is not expressed in K562 cells while it is highly expressed in both U937 and HL-60 cells. Bax, a Bcl-2-related protein that induces apoptosis, is equally expressed in the four studied cell lines (data not shown). In both K562 and KCL-22 cell lines, the chimeric bcr-abl gene is responsible for the synthesis of gadd153 AND VP-16-INDUCED APOPTOSIS

Fig. 8. Effects of Z-VAD-CH2F and Z-DEVD-CH2F on gadd153 mRNA induction and apoptotic DNA fragmentation in VP-16-treated U937 cells. U937 cells were treated with 30 µM VP-16 for 4 h in the absence (●) or in the presence of 20 µM (○) and 50 µM (△) of Z-VAD-CH2F or Z-DEVD-CH2F, respectively. A, Northern blot analysis of the gadd153 mRNA level. Ethidium bromide staining of rRNA from each sample indicates equivalent RNA loading. B, apoptotic DNA fragmentation measured using a filter elution assay (means of three independent experiments performed in triplicate). Bars, SD.
a 210-kDa fusion protein that displays uncontrolled Abelson tyrosine kinase activity. BCR-ABL was shown to inhibit apoptosis induced by a variety of agents including VP-16 (46). Whether this protein is responsible for the decreased gadd153 mRNA expression in VP-16-treated K562 and KCL-22 cells remains speculative.

Many DNA-damaging agents result in cellular oxidative stress either through the production of free radical or by reaction with sulfhydryl groups. NAC, a free radical scavenger which is rapidly converted within the cells to the reduced form of glutathione, prevents induction of the gadd153 gene by cadmium chloride, an agent presumed to generate oxidative stress by decreasing intracellular glutathione levels (32). More recently, protection by NAC was suggested to be due to a direct effect of the compound on mitochondrial integrity and function (47). Whatever the mechanism, NAC and other antioxidants such as diethyldithiocarbamate impair the apoptotic process in various cell systems, including cell lines deprived from their trophic factor (47), U937 cells treated with tumor necrosis factor α (48, 49), and HL-60 cells treated with topoisomerase inhibitors (50). At 25 µM, NAC partially prevented the gadd153 mRNA level increase and apoptotic DNA fragmentation in VP-16-treated U937 cells, suggesting that gadd153 mRNA increase occurred downstream of the cellular mechanisms inhibited by NAC in the apoptotic pathway. At a higher concentration (100 µM), NAC alone increased the gadd153 mRNA level. The sulphydryl agent DTT was also reported to induce an increase in the gadd153 mRNA level without related toxicity, suggesting that cellular thiol-disulfide content could play a role in gadd153 induction, independently of the apoptotic pathway (51).

gadd153 mRNA level regulation could involve several different pathways, depending on the stimulus, its intensity, and the cell line. Here, we show that the gadd153 mRNA level increase is associated with apoptotic DNA fragmentation rather than cell cycle regulation in human leukemic cell lines treated with VP-16. This increase is a late event that occurs downstream of NAC-, CPI-, TPCK-, and Bcl-2-sensitive steps and upstream of ICE-like protease-involving events in the apoptotic pathway. Transfection of gadd153 was recently shown to increase gastric cancer cells sensitivity to both VP-16- and taxotere-induced apoptosis (52). The gadd153 mRNA level increase in human tumor samples treated with cytotoxic drugs was also related to a better clinical response to these drugs (53). The role of gadd153 in the cascade of events that trigger apoptosis in cytotoxic drug-sensitive cells remains speculative. CHOP l0/Gadd153 protein could act as both an inhibitor of C/EBP-mediated activation of some target genes and a direct activator of others (17, 54). Modulation of presently unknown target genes by the protein could be one of the events that contribute to drug-induced cell death. This effect could be cell specific, depending on the expression level of the C/EBPs (19). Altogether, these
data suggest that gadd153 could play a role in determining the sensitivity of malignant cells to chemotherapeutic agents.

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Increased *gadd153* Messenger RNA Level Is Associated with Apoptosis in Human Leukemic Cells Treated with Etoposide

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