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Induction of Bax Protein and Degradation of Lamin A during p53-Dependent Apoptosis Induced by Chemotherapeutic Agents in Human Cancer Cell Lines

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ABSTRACT. In this study, subcellular fractionation analysis was performed to investigate the intracellular localization of Bax protein. We demonstrated that Bax protein is localized primarily in the nuclear and heavy membrane fractions. The expression of Bax protein in the nuclear membrane was induced in wild-type p53 human cancer cells (COLO 205 and Hep G2) by a wide variety of cancer chemotherapeutic agents in order to scrutinize further the biologic function of the Bax protein in the nuclear membrane. We found that lamin A and poly-(ADP ribose) polymerase (PARP) protein degradation coincided when the Bax protein level was elevated in the nuclear membrane of cells affected by drug stimuli. By using anti-sense oligodeoxynucleotides specific to human Bax mRNA, we further demonstrated that inhibition of Bax expression could specifically block lamin A but not PARP cleavage in apoptotic cancer cells. BIOCHEM PHARMACOL **57**;2:143–154, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. apoptosis; etoposide; Bax; p53; PARP; lamin A

Over the past few years, there has been increasing recognition of (1) the important role of cell death in determining appropriate cell number and (2) how a lack of cell death under physiologic conditions can contribute to cellular transformation and malignant cell growth [1, 2]. Cell death permits the selective elimination of excess cells and permits the maintenance of tissue homeostasis in morphogenesis and in the immune system [reviewed in Refs. 3 and 4]. The majority of such cell deaths share common characteristics, such as reduction in cell volume, blebbing of the plasma membrane, chromatin aggregation, and finally fragmentation of DNA. This morphologically distinct form of cell death is called apoptosis [2].

In recent studies, we demonstrated induction of Bax, p53 and *p21/WAF1/CIP1* expression in human cancer cell lines using NO§ [5, 6]. Our results revealed that NO-induced apoptosis is inhibited by antioxidants (e.g. L-*N*-acetylcysteine) through modulation of intracellular glutathione, Bax, and Bcl-2 protein levels. We observed that NO-induced Bax protein elevation in human cancer cells is inhibited by L-*N*-acetylcysteine [6]. As in the previous study, the Bcl-2 protein was demonstrated and localized in the mitochondria, the endoplasmic reticulum and the nuclear membrane by immunohistochemical analysis [7–9]. However, the intracellular localization and the biologic function of Bax protein in cells in response to genotoxic stimuli need further study.

Numerous mammalian proteases that induce apoptosis have been identified [reviewed in Refs. 10–12]. Several studies have demonstrated that the death signal is transduced by a protease cascade [13]. For instance, activated TX (also called ICH-2 or ICE_{rel} II) cleaves proICE [14], and activated ICE cleave pro-CPP32/Yama [15]. Pro-CPP32/Yama has also been shown to be cleaved by granzyme B [16]. The known target of CPP32/Yama is PARP [15, 17]. As described above, one of the important questions in cell death research is whether the apoptotic cascade is driven by the action of a single IRP [14, 15, 18–20] or by multiple IRPs acting in concert [21, 22]. Clearly, IRPs are important components of the apoptotic machinery.

Identifying downstream substrates is the key to understanding the role of these IRPs *in vivo*. PARP was the first protein identified in a cell-free *in vitro* system as an apoptosis-specific IRP substrate [23]. The physiological significance of PARP cleavage in apoptosis remains uncertain [reviewed in Ref. 24]. Other substrates identified include various types of lamins (A, B, and C), the 70-kDa

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§ *Abbreviations:* IRP, ICE-related protease; MES, 2-[*N*-morpholino]ethane sulfonic acid; NO, nitric oxide; NRE, negative responsive element; ODNs, oligodeoxynucleotides; PARP, poly-(ADP ribose) polymerase; PMSF, phenylmethylsulfonyl fluoride; and prICE, protease resembling ICE.

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protein component of the U1 small ribonucleoprotein, α-fodrin, topoisomerase I, and β-actin [11, 13, 22]. However, the role of these substrates in apoptosis is not yet clear.

Nuclear lamins are intermediate filament proteins that constitute the main structural components of the nuclear lamina underlying the inner membrane and serve to organize the chromatin [25, 26]. The nuclear lamina is disassembled by different routes in mitosis and apoptosis. In mitosis, lamina disassembly is driven by phosphorylation of the lamin subunits, whereas in apoptosis, disassembly is accompanied by proteolytic cleavage of lamins [21, 27–29]. Nuclear lamins are cleaved to fragments in a cell-free extract system (S/M extracts) [21, 30] as seen in cells undergoing apoptosis [31]. A recent study demonstrated [32] that human lamin A was cleaved by Mch2α at a conserved sequence that corresponds to the site of lamin A cleavage in the S/M extract system [21]. These results imply that activity of lamin protease is a prerequisite for apoptotic nuclear disassembly.

In this study, we used immunofluorescence staining, subcellular fractionation, and western blotting to demonstrate that the Bax protein was induced and localized mainly in the nuclear envelope. However, factors and events downstream of Bax induction are yet to be elucidated. We suggest that induction of Bax protein in the periphery of the nucleus (nuclear lamina) may cause apoptosis. To determine whether the disassembly of the nuclear membrane occurred when Bax protein was induced, the cleavage of PARP and lamin A was investigated in various human cancer cells treated with chemotherapeutic drugs. In this study, Bax-specific antisense ODNs were used to block Bax protein expression and the biologic functions of Bax induction in apoptosis were scrutinized further.

MATERIALS AND METHODS

Chemicals

Actinomycin D, azaguanine, bromodeoxyuridine, budotitan, busulphan, cisplatin, cyclophosphamide, etoposide, methotrexate, and vincristine were purchased from the Sigma Chemical Co. 5-Fluorouracil and mitomycin C were purchased from the Merck Co. A protein assay kit was purchased from the Bio-Rad Co.

Cell Lines and Cell Culture

The cell line HT 29 (HTB-38; American Type Culture Collection was isolated from a moderately well-differentiated grade II human colon adenocarcinoma [33]. The cell line COLO 205 (CCL-222; American Type Culture Collection) was developed from a poorly differentiated human colon adenocarcinoma. Hep 3B cells (HB 8064; American Type Culture Collection) were derived from a human hepatocellular carcinoma line [34–36]. The cell line Hep G2 (HB 8065; American Type Culture Collection) was derived from a human hepatocellular carcinoma [34–36], and contained wild-type p53 [37]. The cell line CCD-922SK (CRL 1828; American Type Culture Collection) was derived from normal human fibroblasts. As in our previous reports [5, 6], the *p53* gene in the COLO 205 and CCD-922SK cells was cloned into the TA cloning vector (Invitrogen) and sequenced. The *p53* gene in COLO 205 and CCD-922SK cells was of the wild-type. In HT-29 cells, p53 is mutated in codon 273 [38]. The *p53* gene has been found to be partially deleted (7 kb) in Hep 3B cells [37]. Cell lines were grown at 37° in a 5% (v/v) carbon dioxide atmosphere in Eagle's minimal essential medium (for Hep 3B, Hep G2, and CCD-922SK cells), or RPMI 1640 (for COLO 205 and HT-29 cells), supplemented with 10% (v/v) fetal bovine serum; 50 μg/mL of gentamicin, and 0.3 mg/mL of glutamine.

Drug Treatment

Logarithmically growing human cancer cells were grown at 37° in a 5% (v/v) carbon dioxide atmosphere. Etoposide or other chemotherapeutic drugs were added from a 1000-fold concentrated stock in DMSO. After various periods of incubation, cells were sedimented for protein preparation or for DNA fragmentation analysis.

Indirect Immunofluorescence Staining

Cells were plated on glass cover slips in 12-well culture dishes. After treatment with various agents, the cells were fixed for 10 min in acetone-ethanol (1:1, v/v) at −20° and air-dried. For immunofluorescence, the slides were incubated for 45 min at 37° in a humidified chamber with a primary antibody, diluted 1:30 in PBS with 0.05% (v/v) Tween 20. The cover slips were washed three times at room temperature with PBS, incubated for another 30 min with biotinylated goat anti-rabbit antibody [diluted 1:100 in PBS and 0.05% (v/v) Tween 20], and washed again. Finally, the cover slips were incubated for 10 min with fluorescein isothiocyanate-conjugated streptavidin, washed thoroughly, and mounted with Glycergel (Dako Corp.).

Fractionation of Cellular Extracts

Cells in 100-mm dishes were detached by adding 2 mL of 0.25% (w/v) trypsin. Then a PBS solution (8 mL) containing 0.01% (w/v) calcium chloride and 0.01% (w/v) magnesium sulfate was added to the trypsinized cells. After centrifuging the cell suspension at 500 *g* for 5 min, the pellet was washed with PBS and centrifuged again. The pellet was resuspended in 0.5 to 1.5 mL of MES buffer [39] containing protease inhibitors. Cells were then homogenized in a Dounce homogenizer with pestle A (30 strokes). Lysis of more than 99% of the cells was verified based on trypan blue dye uptake. The samples were transferred to Eppendorf centrifuge tubes (1 mL maximum/1.5-mL tube) and centrifuged to pellet the nuclei at 500 *g* for 5 min in a swinging-bucket rotor at 42°. The nuclear pellet was then resuspended in 0.5 to 2 mL of 1.6 M sucrose containing

either 50 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$ according to the method of Blobel and Potter [40] or 17 mM morpholinopropanesulfonic acid (pH 7.4) and 2.5 mM EDTA. After underlayering with 1–2 mL of 2.0 to 2.3 M sucrose containing the same buffer, the nuclear suspension was centrifuged at 150,000 *g* for 60 min in a Beckman SW50.1 rotor with or without adapters for 1.5-mL tubes, depending on the volume. The resulting nuclear pellets were again suspended in 1.6 M sucrose solution and the procedure was repeated prior to lysing nuclei in 0.1 to 0.3 mL of a 1% (v/v) Triton X-100-containing buffer [39].

The supernatant resulting from the initial low-speed centrifugation was subjected to centrifugation at 10,000 *g* (average) for 15 min at 4° to obtain the HM fraction (pellet), and this supernatant was then centrifuged for 60 min at 150,000 *g* (SW50.1) to obtain the LM (pellet) and C (supernatant) fractions. The HM and LM fractions were resuspended in 1% Triton-containing buffer [39] and either analyzed immediately for enzyme activities or frozen at −80° for subsequent immunoblot assays.

Determination of LDH Levels

LDH activity in N, HM, LM, and C fractions was measured using a kit from the Sigma Chemical Co. (No. 500). Data from the assays were normalized for total protein content where indicated.

SDS-PAGE and Immunoblotting

Equal amounts of proteins (50 μg) (estimated using a Bio-Rad protein assay kit) were separated by 12% (w/v) SDS-PAGE on 0.75 mm mini-gels (Midget System; Pharmacia Inc.) and were transferred to an immobilon P membrane (Millipore Corp.) with a semidry electroblotting apparatus (TE70; Hoefer Scientific Instruments) at 2 mA/cm^2 for 40 min in 25 mM Tris-HCl, pH 8.3; 192 mM glycine; and 20% (v/v) methanol. The membrane was blocked overnight at room temperature with blocking reagent [20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% (v/v) Tween 20; 4% (w/v) non-fat dry milk; and 0.1% (w/v) sodium azide].

Antibodies and Immunoblot Analysis

Antibodies used for immunoblot assays including polyclonal rabbit antisera (used at 0.1%, v/v) specific for human Bax protein (Ab-1, CAN Bioscience Co.) were raised against the synthetic peptide corresponding to amino acids 150–165 of human p21-Bax and rabbit polyclonal anti-F1-β-ATPase (0.05%, v/v) [41]. Mouse monoclonal antibodies included 7H8.2C12 specific for cytochrome *c* (0.1%, v/v) [42]; and antibodies specific for lamin A (JOL4, Serotec Co.) and PARP (ABR Co.) [43]. SDS-PAGE and electroblotting to nitrocellulose filters were performed as described above. Filters were incubated for 1 hr with primary antibodies, washed three times, and then incubated with alkaline phosphatase-conjugated secondary antibody (immunoglobulin G) in PBS and 0.5% (v/v) Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins were visualized by incubating with the colorigenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Chemical Co.).

Measurement of Caspase-3 Protease Activity

Caspase-3 was assayed by the cleavage of DEVD-AFC, a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of PARP [44]. Cells (5×10^6 to 10^7 per flask) were cultured overnight in the presence of etoposide, Bax-specific antisense ODNs, and zDEVD-fmk, washed once with 5 mL of Hanks' balanced salt solution, and resuspended in 1 mL of NP40 lysis buffer. After 2 hr in lysis buffer at 4°, the insoluble material was pelleted at 15,000 *g*, and a sample of the lysate was tested for protease activity. For the assay, 20–40 μL of cell lysate was added to each tube containing an 8 μM concentration of substrate in 1 mL of protease buffer (50 mM HEPES, 10% sucrose, 10 mM dithiothreitol, and 0.1% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate, pH 7.4). After 45 min at room temperature, yellow-green fluorescence at 505 nm (excitation 400 nm), due to the release of AFC, was quantitated in a Perkin–Elmer LS50 spectrophotometer. Optimal amounts of added lysate and the duration of the assay were determined in preliminary experiments.

Determination of Apoptosis by Terminal Deoxynucleotide Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Staining

Cells were fixed with 4% paraformaldehyde and permeabilized with ethanol/acetone. After washing with PBS, cells were incubated with terminal deoxynucleotide transferase and fluorescein-conjugated dUTP at 37° for 1 hr. Cells were washed, and free DNA ends were visualized by fluorescence microscopy.

Analysis of DNA Fragmentation

Treated or mock-treated cells were grown in 9-cm Petri dishes. Both attached and unattached cells were harvested, washed twice with ice-cold PBS, resuspended in TNE (10 mM Tris-HCl, pH 7.6; 140 mM sodium chloride; and 1 mM EDTA), and lysed at 37° in 4 mL of extraction buffer [10 mM Tris-HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 20 μg/mL of pancreatic RNase (Boehringer Mannheim Corp.); and 0.5% (w/v) SDS]. After 2 hr, proteinase K was added at a final concentration of 100 μg/mL, and the mixture was incubated for another 3 hr at 50°. The DNA was extracted twice with equal volumes of phenol and once with chloroform-isoamyl alcohol (24:1, v/v). The DNA was then precipitated with 0.1 vol. of sodium acetate (pH 4.8) and 2.5 vol. of ethanol at −20° overnight, and pelleted at 13,000 *g* for 1 hr. Samples were electrophoresed in a 1.5%

(w/v) agarose gel, and DNA was visualized by ethidium bromide staining.

Synthesis of ODNs and Antisense ODN Inhibition Experiment

The ODNs were as follows: a Bax-specific antisense ODN (5′-TGCTCCCCGGACCCGTCCAT-3′) complementary to the translation initiation site of human Bax mRNA versus a sense ODN (5′-ATGGACGGGTCCGGGGAG CA-3′). As an additional control for sequence specificity, a random sequence with the same base composition as the antisense ODN (CGCCGCCATCATCGTCTCCG) was used. Human Hep G2 cells were cultured at 37°, in a 5% (v/v) CO_2 humidified atmosphere at a concentration of 400,000 cells/mL in 24-well culture plates (Costar) (1 mL/well) in Eagle's minimal essential medium containing 10% (v/v) fetal bovine serum and antibiotics. Some wells were pretreated with various concentrations (1, 5, and 10 μM) of ODNs to human Bax mRNA (anti-Bax) and some wells with the same concentration of control or sense ODNs. After a 12-hr incubation with ODNs, etoposide (1 μg/mL) was added to all wells except the untreated wells. The cells were harvested and assessed by western blotting and DNA fragmentation analysis.

RESULTS

Induction of Bax Protein by Chemotherapeutic Drugs in Human Cancer Cells

As shown in Fig. 1, Bax protein was induced by etoposide in the perinuclear portion of CCD-922SK, Hep G2, and COLO 205 cells (with the wild-type *p53* gene) (Fig. 1, B, D, and H, arrowhead) but not in the Hep 3B and HT 29 cells (with deleted *p53* and mutant-type *p53* respectively) (Fig. 1, F and J). The kinetics of the Bax protein induction in Hep G2 cells were investigated by indirect immunofluorescence staining with the polyclonal anti-Bax antibody. The results showed that a significant Bax induction occurred in less than 12 hr and reached its maximum between 24 and 48 hr after exposure to etoposide (Fig. 2A). Treatment of Hep G2 cells with etoposide resulted in morphological changes consistent with the process of apoptosis. These morphological changes were accompanied by progressive internucleosomal degradation of DNA to yield a ladder of DNA fragments (Fig. 2B). The protein lysates of the cells treated with etoposide were separated by SDS-PAGE and stained with Coomassie blue, and the bulk of the cellular polypeptide remained intact (Fig. 2C).

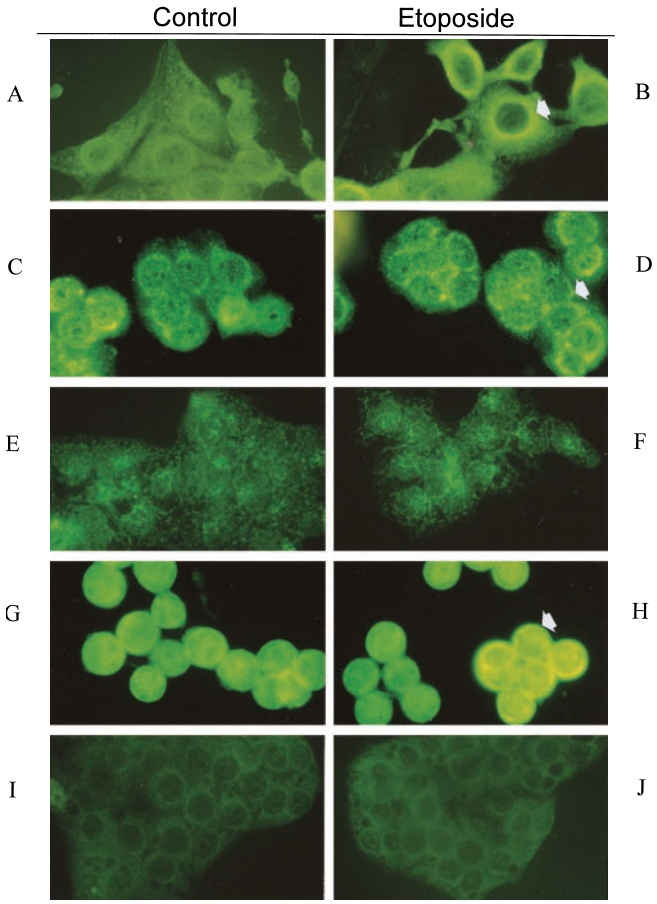

FIG. 1. Immunofluorescence staining of Bax protein in CCD-922SK (A and B), Hep G2 (C and D), Hep 3B (E and F), COLO 205 (G and H), and HT 29 (I and J). The cells in the left-hand panels (A, C, E, G and I) were mock-treated as controls. The cells in the right-hand panels (B, D, F, H and J) were treated with etoposide (1 μg/mL). After 24 hr, the cells were fixed and stained with Bax-specific antibody by using the biotin-streptavidin system. Induction of Bax protein in CCD-922SK (B), Hep G2 (D), and COLO 205 (H) cells were indicated by the arrowhead.

Localization of the Bax Protein Primarily in the N and HM Fractions

To further confirm the observations described above, subcellular fractionation analysis was performed to study the intracellular localization of the Bax protein. Crude fractionation of these cells to yield N, HM, LM, and C fractions revealed that Bax protein was located primarily in the N fractions (Fig. 3A). A small amount of 21-kDa material reacting with anti-Bax antibody also was detected in the HM fraction during these immunoblot assays where subcellular fractions were normalized for total protein content. Similar results were obtained using three different anti-Bax antibodies (Ab 1, 2, and 3, CAN Bioscience Co.) directed against nonoverlapping epitopes (data not shown). The presence of Bax protein in the nuclear compartment could not be attributable to residual unlysed cells, based on LDH measurements (Fig. 3D). It persisted despite centrifuging the nuclei twice through 2.0 to 2.3 M sucrose. Despite attempts at rigorous purification, however, nuclear preparations from these cancer cells frequently appeared to be contaminated with mitochondria, since p50-F1-β-ATPase and p12-cytochrome *c* also were detected by immunoblotting using specific antibodies (Fig. 3, B and C).

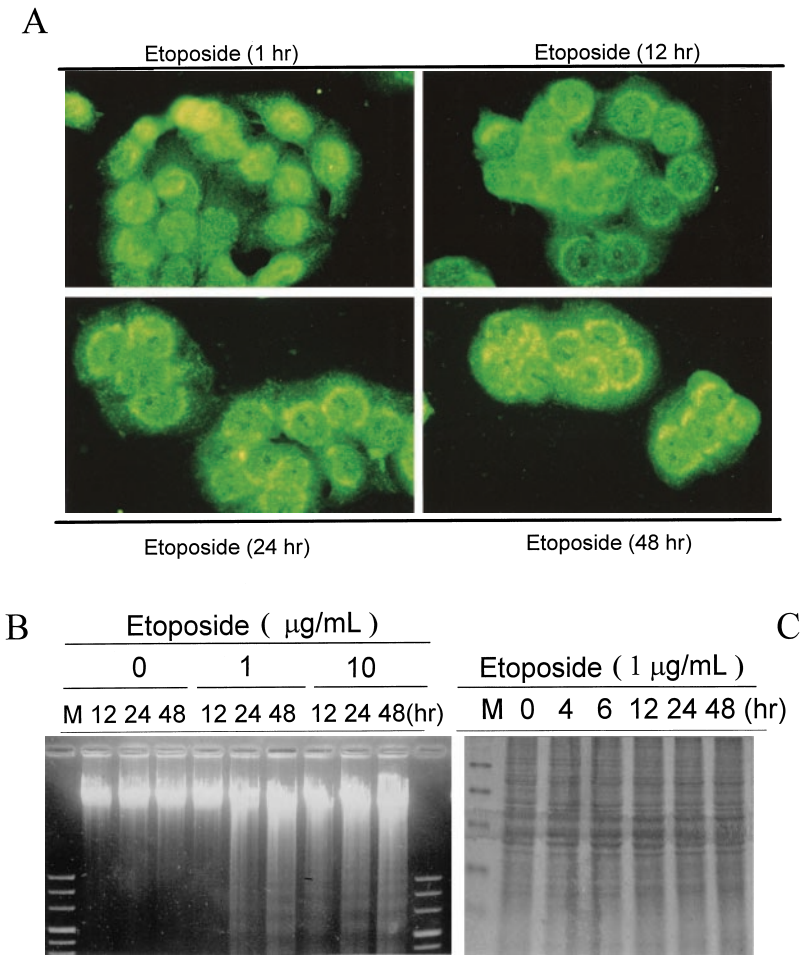

FIG. 2. Studies of the kinetics of the Bax protein induction in Hep G2 cells (A). Immunofluorescence staining of Bax protein in Hep G2 cells. The cells were treated with etoposide (1 μg/mL) for 1, 12, 24, and 48 hr, respectively. After treatment, the cells were fixed and stained with Bax-specific antibody by using the biotin-streptavidin system (B). DNA fragmentation analysis in Hep G2 cells treated by etoposide in a concentration- and time-dependent experiment (C). Total cell lysates were prepared from Hep G2 cells that were treated by etoposide in a time-dependent manner. Protein extracts were separated by SDS-PAGE and were stained with Coomassie blue to see the bulk of the cellular polypeptide.

Simultaneous Occurrence of Bax Protein Induction, and Degradation of PARP and Lamin A

As seen in Fig. 4A, the Bax protein in the nuclear fraction was induced and translocated from the cytosol (containing HM, LM, and C fractions) to the N fraction 24–48 hr after etoposide exposure. This was consistent with the immunofluorescence observations shown in Fig. 2. We further explored the possibility that induction of Bax protein in the nuclei (nuclear lamina) may also be correlated with nuclear lamina protein degradation. Western blotting analysis revealed that the M_r 116,000 PARP molecule was degraded to a relatively stable M_r ~85,000 fragment (Fig. 4B) 24 hr after etoposide treatment. Similar cleavage of PARP was observed at etoposide concentrations of 0.1 μg/mL (data not shown). Cleavage of lamin A was also observed 24 hr later (Fig. 4C) with a time course that paralleled DNA fragmentation (Fig. 4D) when cells were treated with etoposide.

To determine whether the rise of caspase-3 protease activity was essential for the induction of apoptosis by etoposide, two membrane-permeable inhibitors of caspases were used. zVAD-fmk is based on the peptide sequence at the cleavage site of interleukin 1β (mediated by caspase-1), whereas zDEVD-fmk corresponds to the sequence at the cleavage site of PARP (mediated by caspase-3) [44]. In Hep G2 cells incubated with either zVAD-fmk (data not shown) or zDEVD-fmk (Fig. 5, inset), there was suppression of caspase-3. As seen in Fig. 5, there was a marked increase in the activity of caspase-3 12 hr after addition of etoposide. However, caspase-3 activity was not inhibited with combined treatment with Bax-specific antisense ODNs and etoposide (Fig. 5). These results were consistent with Fig. 6 C, which shows that PARP protein degradation was not prevented by Bax-specific antisense ODNs in Hep G2 cells treated by etoposide. In contrast, caspase-3 activity was inhibited completely in Hep G2 cells, when the cells were treated simultaneously with etoposide and caspase-3 inhibitor, zDEVD-fmk (Fig. 5).

Inhibition of Lamin A But Not PARP Degradation by Blockade of Bax Protein Expression

By using the antisense ODN inhibition strategy, we investigated the correlation between Bax induction and lamin A and PARP degradation in Hep G2 cells. Hep G2 cells were pretreated either with various concentrations (1, 5, and 10 μM) of antisense ODNs to human Bax mRNA (anti-Bax) or with the same concentration of sense and random control ODNs (Fig. 6A). After a 12-hr incubation period with or without ODNs, etoposide was added to all cells

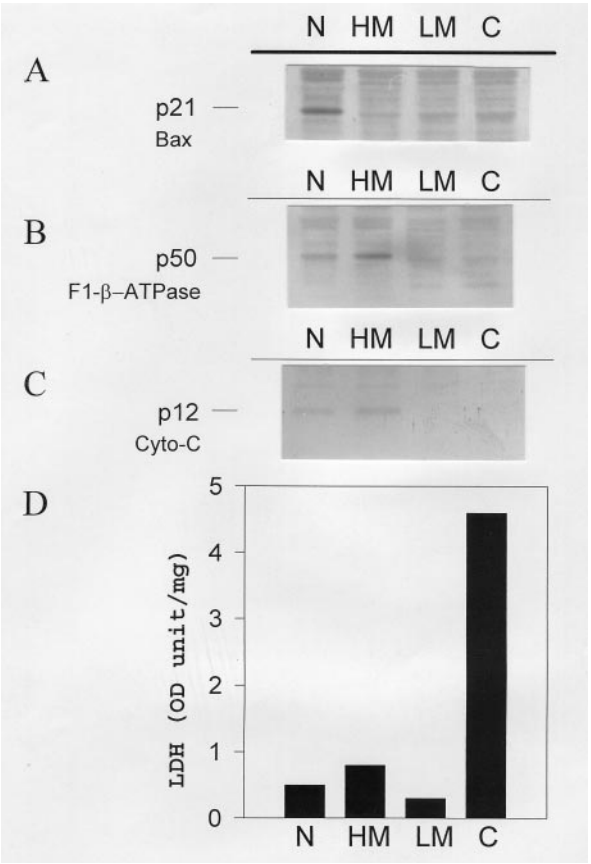

FIG. 3. Subcellular fractionation of p21-Bax in human Hep G2 cells. Nuclear (N), heavy membrane (HM), light membrane (LM), and cytosolic (C) fractions were prepared from Hep G2 cells and resuspended in lysis buffer containing 1% (v/v) Triton X-100 [45]. Subcellular lysates were normalized for protein contents (50 μg/lane) and subjected to SDS-PAGE using 12% (w/v) gel. An aliquot of each fraction was also assayed for LDH activity (D). Proteins in gels were transferred to nitrocellulose filters, which were cut into sections for immunoblot assays using antibodies specific for (A) P21-Bax, (B) p50-F1-β-ATPase, and (C) p12-cytochrome c. The relative positions on blots are shown in kilodaltons (kDa).

except the mock-treated control group, which was treated with DMSO. The cells were harvested 24 hr after exposure to etoposide. We demonstrated that Bax protein expression was detectable only in the cells treated with sense ODNs (Fig. 6A, lane 2) and in the mock-treated control group (Fig. 6A, lanes 3 and 7). Bax-specific antisense ODNs strongly inhibited Bax protein expression (Fig. 6A, lanes 1, 4, 5, and 6). Interestingly, the Bax specific antisense ODNs showed the specific effects of inhibition in lamin A cleavage induced by etoposide (Fig. 6B, lanes 4–6). In contrast, this specific effect did not occur in the cleavage of PARP (Fig. 6C, lanes 4–6). These results described above imply that etoposide-induced Bax protein induction is correlated with lamin A degradation.

As described above, DNA fragmentation characteristics were observed in Hep G2 cells treated under various conditions. Figure 6 D shows that neither antisense-, sense-, nor mock-treated cells underwent apoptotic DNA fragmentation (Fig. 6D, lanes 1, 2, and 7). However, when the Hep G2 cells were treated with etoposide alone, Bax protein induction, lamin A degradation, PARP cleavage and DNA ladder formation were observed simultaneously (Fig. 6, A, B, C, and D, lane 3).

As seen in Fig. 6D, DNA fragmentation was only partially inhibited in Hep G2 cells pretreated with Bax-specific antisense ODNs (Fig. 6D, lanes 4–6). To further confirm such observations, the percentage of apoptotic cells treated by Bax-specific antisense ODNs was calculated. As seen in Fig. 7, the percentage of apoptotic cells increased (from 4.1 ± 0.7 to 45 ± 4.2) in cells treated with etoposide for 24 hr. However, the percentage of apoptotic cells decreased when cells were treated with Bax-specific antisense ODNs (from 2.5 ± 0.2 to 16 ± 4.1) or a specific inhibitor of caspase-3, zDEVD-fmk (from 2.2 ± 0.3 to 21 ± 3.5) at 24 hr after combination with etoposide treatment. This was a consistent finding as seen in Fig. 6D, and might reflect that other apoptosis-inducing factors are involved. We further evaluated the percentage of apoptotic cells by simultaneously treating with Bax-specific antisense ODNs,

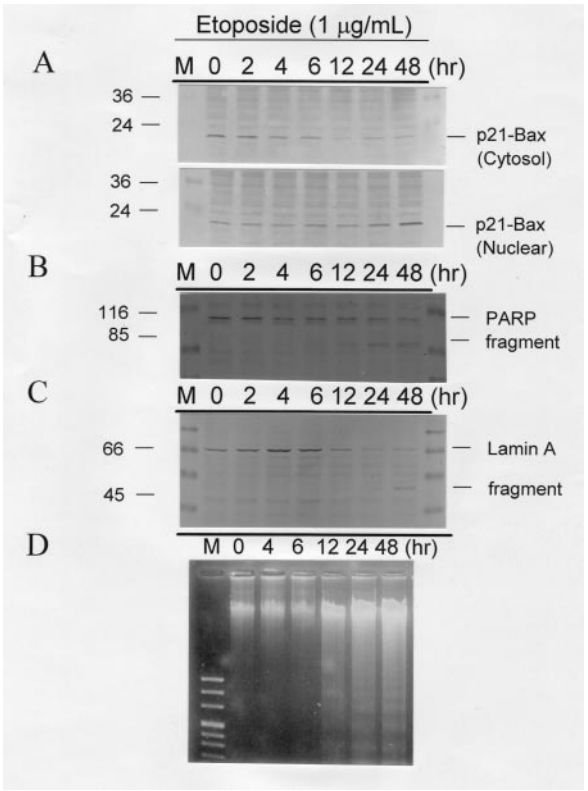

FIG. 4. Simultaneous occurrence of Bax protein induction, PARP degradation, lamin A cleavage, and DNA fragmentation in apoptotic Hep G2 cells. (A) Western blot analysis of Bax protein in the cytosolic (containing HM, LM, and C fractions) and nuclear fractions of Hep G2 cells with etoposide (1 μg/mL) exposure at the indicated times. (B) PARP degradation and (C) nuclear lamin A cleavage were observed initially 12 hr after treatment with etoposide (1 μg/mL). (D) DNA fragmentation analysis in Hep G2 cells treated with etoposide at the same timepoints as in (A), (B), and (C), except for the 2-hr time point.

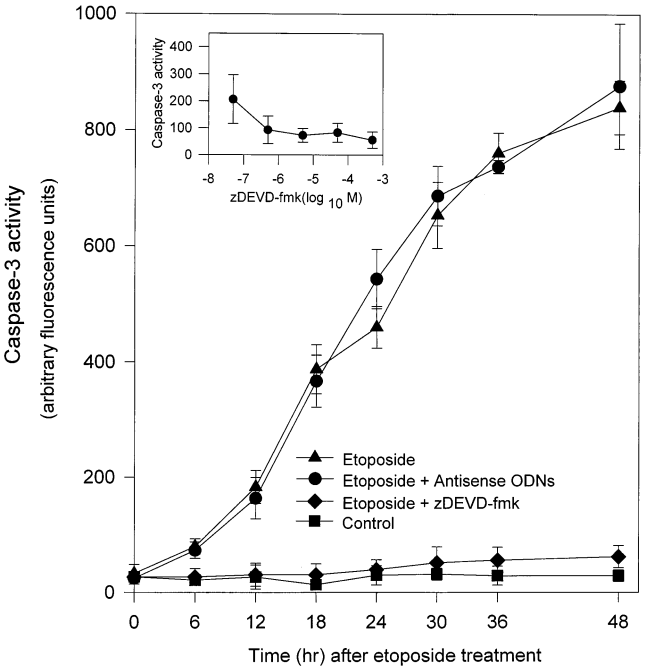

FIG. 5. Increase in caspase-3 activity during etoposide-induced apoptosis. Hep G2 cells were cultured with etoposide (1 μg/mL), caspase-3 inhibitor (zDEVD-fmk, 1 μM), or Bax-specific antisense ODNs (10 μM) at the indicated times and the caspase-3 activity was quantified. The inset shows concentration-dependent inhibition of caspase-3 activity by zDEVD-fmk in the Hep G2 cells. Each point represents a mean of triplicate culture flasks; bars, SD.

zDEVD-fmk and etoposide (Fig. 7). Our results demonstrated that the apoptotic effects induced by etoposide in Hep G2 cells were prevented almost completely (from 2.5 ± 0.2 to 6.6 ± 1.2) when Bax and caspase-3 activity were inhibited completely (Fig. 7).

Significant Cleavage of Lamin A in Cells with Wild-Type p53 Undergoing Apoptosis

According to a recent report [45], p53 was demonstrated to be a direct transcriptional activator of the *Bax* gene. However, to our knowledge, there has been no evidence to indicate whether or not the wild-type p53 protein in cells plays a definite role in the regulation of Bax protein expression in response to DNA-damaging agents. To exclude the possibility that Bax protein elevation and cleavage of lamin A occurred only in the Hep G2 cells, the correlations among p53 status, Bax protein induction, lamin A degradation, and DNA fragmentation induced by etoposide were investigated in human cancer cell lines with different p53 status [5, 6] (Fig. 8). We found that Bax was easily induced in cells containing wild-type p53 (COLO 205 and Hep G2) after etoposide exposure (Fig. 8A). Cleavage of nuclear lamin A and internucleosomal DNA degradation were also apparent in these two cells (Fig. 8, B, and C). In contrast, Bax protein elevation and lamin A protein degradation were induced only slightly in the *p53* deleted (Hep 3B) and mutated (HT 29) cells (Fig. 8A). These results could be due to a slower apoptotic response in the *p53*-mutated and -deleted lines.

Cleavage of Lamin A in Hep G2 Cells Treated with Different Cytotoxic Agents

As described above, cleavage of the lamin A protein occurred when Bax protein was induced. To ascertain whether the association of Bax induction and cleavage of lamin A was unique to etoposide-induced apoptosis, Hep G2 cells were treated with a variety of cytotoxic agents as shown in Table 1. These compounds are listed in Table 1 according to their putative modes of action. The protein lysates of the cells treated by drugs were normalized to equal amounts of protein (50 μg) according to the levels of α-tubulin shown in Fig. 9A. Group I drugs contained alkylating agents, which mainly attack DNA (mitomycin C and cyclophosphamide) and protein (busulphan). The Bax protein was induced by mitomycin C and cyclophosphamide (Fig. 9A, lanes 2, and 3) but apparently was not induced by busulphan (Fig. 9A, lane 4). Group II was composed of drugs, such as cisplatin and budotitan, which can form metal complexes and cause DNA strand breaks, followed by apoptosis. In Fig. 9A, lane 8, cells treated with budotitan showed an induced accumulation of Bax in the N fraction. Group III drugs contained DNA-intercalating agents that bind to topoisomerase II. Actinomycin D, which forms cleavable complexes with topoisomerase II and cellular DNA, apparently was able to induce Bax accumulation (Fig. 9A, lane 9). This result also was seen in the ethidium bromide treatment group (Fig. 9A, lane 7). Among group IV drugs, etoposide, which forms a ternary complex with DNA and topoisomerase II, eventually caused DNA strand breaks and apoptosis (Fig. 9A, lane 6). Most of the agents that induced Bax protein induction also caused lamin A protein degradation (Fig. 9B). Despite the diverse mechanisms of these agents, DNA fragmentation was observed simultaneously when nuclear lamin A degradation occurred (Fig. 9C).

DISCUSSION

Elevation and Translocation of Bax Protein from the Cytosol to the Nuclear Fraction during Apoptosis

In previous studies [46, 47], Bax protein levels did not change during apoptosis. It seems plausible that a posttranslational activation of Bax occurs during apoptosis. Another study indicated that Bax activity may be regulated at the level of intracellular localization, with Bax moving to the membranes of organelles after induction of apoptosis [48]. In our study, redistribution of intracellular Bax from the cytosol to the nuclear fractions during apoptosis was also demonstrated in Hep G2 cells. In the previous studies, Bax protein elevation also was induced in various types of tumor cells by radiation and chemotherapeutic drugs in a p53-dependent manner [49, 50], suggesting an important role

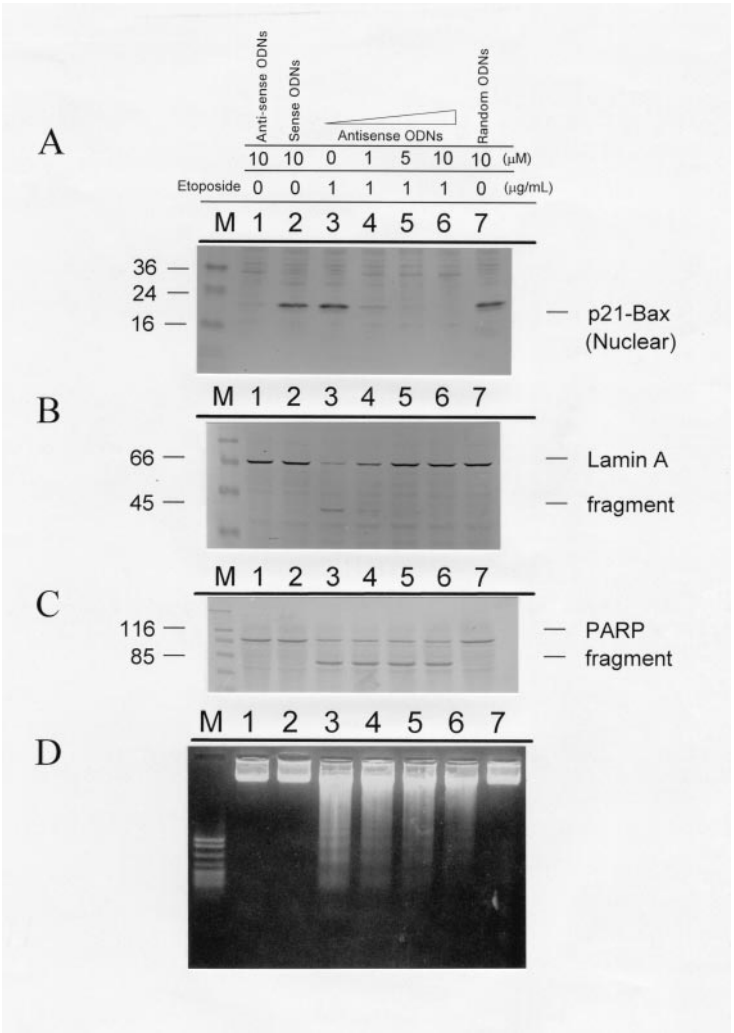

FIG. 6. Inhibition of lamin A but not of PARP degradation by Bax-specific antisense ODNs. The Hep G2 cells were treated with the Bax-specific antisense, sense, or random oligodeoxynucleotides. After a 12-hr period, etoposide (1 μg/mL) or DMSO was added to the cells. The protein lysate and total DNA of the cells were harvested 24 hr after etoposide treatment and analyzed as follows: (A) Bax protein expression; (B) cleavage of lamin A protein; (C) PARP degradation; and (D) DNA fragmentation analysis.

for the Bax protein in apoptosis induced by genotoxic stress. As the previous reports demonstrated, p53 is a direct transcriptional activator of the *Bax* gene [51, 45]. On the other hand, a dominant NRE regulated by p53 was also found in the *Bcl-2* gene [52]. To further explore the role of p53 in the regulation of Bax expression during apoptosis, in this study cells of different p53 status were investigated for their sensitivity to drug-mediated induction of Bax protein. Our results demonstrated that elevation of Bax in Hep G2 and COLO 205 cells (with wild-type p53) was significant after drug treatment. In contrast, Bax levels in Hep 3B (with deleted p53) and HT 29 cells (with mutant-type p53) did not increase significantly after drug treatment. This gave rise to the possibility that p53 elicits apoptosis by shifting the balance between Bcl-2 and Bax [53].

A recent study demonstrated that Bax had a punctate, organellar localization visualized by immunohistochemistry [54]. In that study, coexpression of high levels of Bcl-2 led to a punctate distribution of Bax, suggesting that Bax association with organelle membranes required Bcl-2. In another study [48], the genes encoding human Bcl-2, Bcl-XL and Bax were fused to that of the green fluorescent protein and demonstrated movement of Bax from localization into the living cells mainly in the mitochondria during apoptosis. Our findings with subcellular fractionation and immunocytochemical analysis seem to conflict with previous reports that suggest that Bax may be localized in mitochondria membranes during apoptosis. One possible explanation for this discrepancy may be that in our work, nuclear fractions were contaminated with mitochondria. However, we also observed disproportionate amounts of Bax in the nuclear fraction relative to mitochondria markers such as p50-F1-β-ATPase and p12-cytochrome *c* (Fig. 3A, B, and C), suggesting that mitochondrial contamination cannot entirely account for the pool of Bax found in the nuclear fraction. A previous study [48] also showed that there are areas where the green fluorescent protein-Bax complex is punctate in the cytosol but that do not label with mitochondrial dye in an overlay observation.

Role of Caspase in Execution of DNA Fragmentation during Apoptosis

Caspase or caspase-like cleavage of multiple intracellular proteins has been described during the past few years [11]. Activation of caspase results in the cleavage of substrates

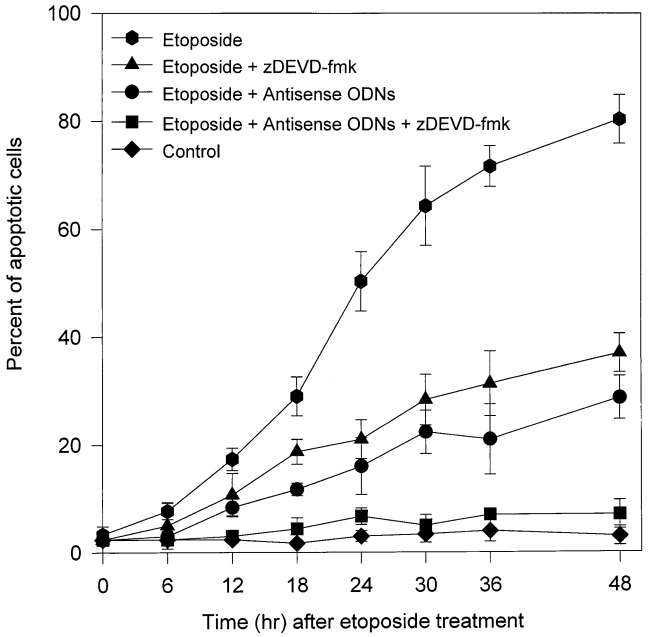

FIG. 7. Involvement of caspase-3 in apoptosis induced by etoposide in Hep G2 cells. Hep G2 cells were cultured in the presence of etoposide (1 μg/mL), Bax-specific antisense ODNs (10 μM), or caspase-3 inhibitor (zDEVD-fmk, 1 μM), and combined treatment at the indicated times. The graph shows nearly complete inhibition of etoposide-induced apoptosis by combined treatment with Bax-specific antisense ODNs and caspase-3 inhibitor. Each point represents a mean of triplicates culture flasks; bars, SD.

that are thought to play a role in maintaining cytoplasmic and nuclear integrity, such as lamin A and PARP, α-fodrin, gelsolin, and focal adhesion kinase [32, 55, 56]. However, the functional significance of these cleavage events in the morphological changes and nuclear degradation that are hallmarks of apoptosis is less clear. As described above, apoptosis also is accompanied by the internucleosomal degradation of chromosomal DNA [57–59]. Recent studies first identified a caspase-activated deoxyribonuclease (CAD) and its inhibitor (ICAD) [60, 61]. In these studies, caspase-3 cleaved ICAD and inactivated its CAD-inhibitory effect. These results indicate that caspase-3 can induce DNA fragmentation via degradation of ICAD.

In this study, we tried to determine whether the rise of caspase-3 protease activity was essential for the induction of DNA fragmentation during apoptosis. A marked increase

TABLE 1. Induction of Bax in the nuclear envelope of Hep G2 cells after treatment with various clinically applied cancer therapy drugs

Agent class*	Agent	Concentration (μg/mL)
	Untreated cells	0
I	Mitomycin C	10
	Cyclophosphamide	10
	Busulphan	10
II	Cisplatin	10
	Budotitan	10
III	Actinomycin D	2
	Ethidium bromide	10
IV	Vincristine	1
	Etoposide	1
V	5-Fluorouracil	10
	Methotrexate	10
	Bromodeoxyuridine	1
	Azaguanine	10

*Class of agents tested: I, alkylating agents; II, metal complexes; III, intercalating agents; IV, *Vinca* alkaloids; and V, nucleotide analogues.

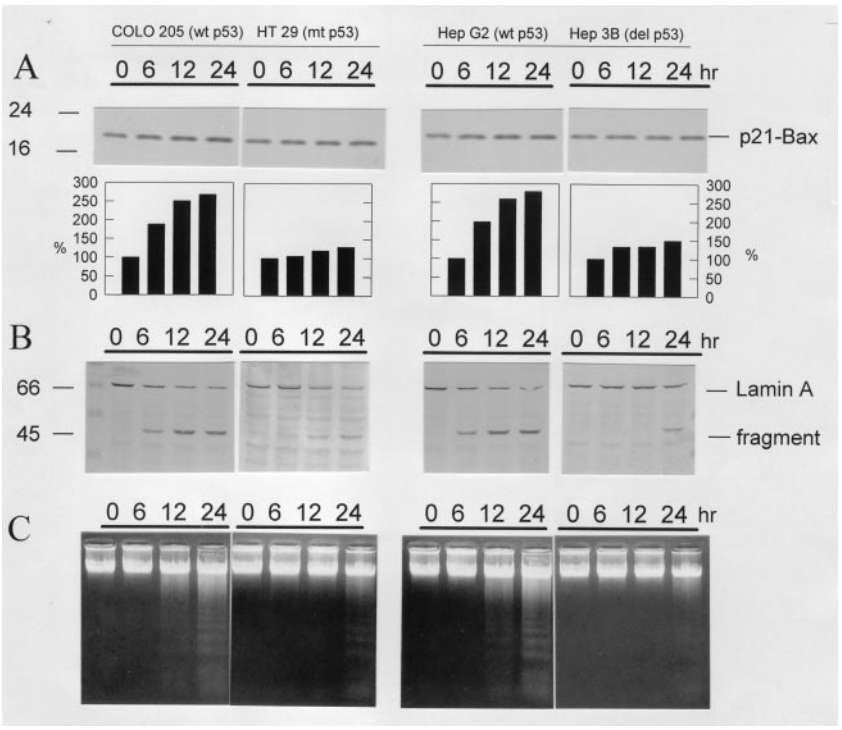

FIG. 8. Bax protein expression, lamin A degradation, and DNA fragmentation analysis in human cancer cells (with different p53 status) in response to etoposide (1 μg/mL) at various time points. (A) Bax protein was induced in the nuclear fraction of the COLO 205 and Hep G2 cells but not in the HT 29 and Hep 3B cells. Proteins were normalized to 100 μg/lane, and the amount of Bax was determined by western blot analysis and video densitometry (lower panel). Each blot is representative of three similar experiments. (B) Lamin A protein degradation, and (C) DNA ladder formation were observed consistently with the Bax protein induction as shown in (A).

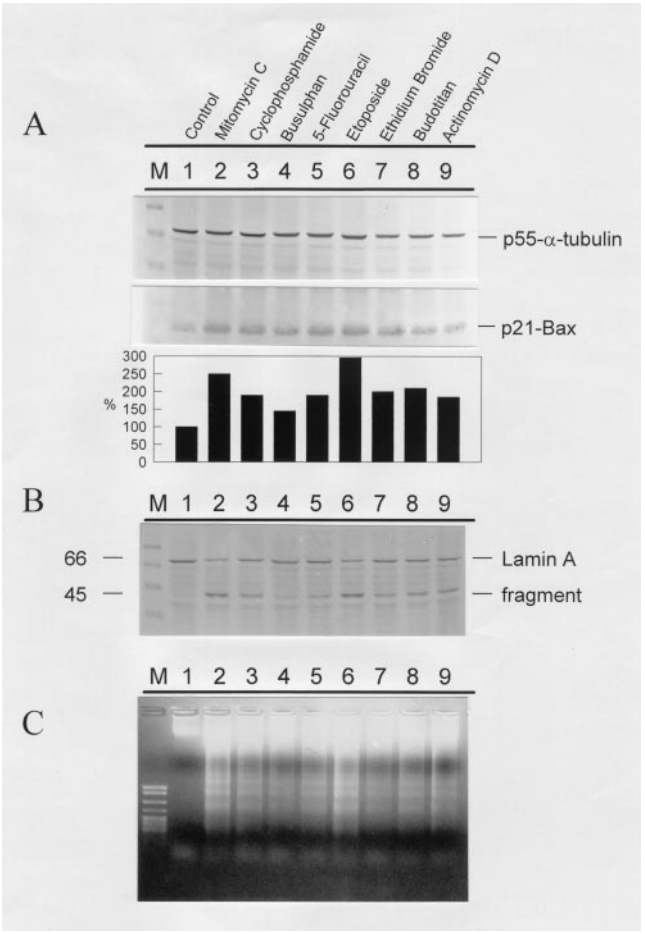

FIG. 9. Bax protein expression and lamin A degradation in Hep G2 cells in response to various types of cancer chemotherapeutic drugs. (A) Bax protein was induced in the nuclear fraction of Hep G2 cells treated with mitomycin C (10 μg/mL), cyclophosphamide (10 μg/mL), busulphan (10 μg/mL), 5-fluorouracil (10 μg/mL), etoposide (1 μg/mL), ethidium bromide (10 μg/mL), budotitan (10 μg/mL) or actinomycin D (2 μg/mL). The total proteins were normalized to 100 μg/lane, and the amount of Bax was determined by western blot analysis and video densitometry (lower panel). The α-tubulin level was shown as an internal control. Each blot is representative of three similar experiments. (B) Lamin A protein degradation and (C) DNA ladder formation in cells treated as described above.

in the capacity of caspase-3 was observed 12 hr after addition of etoposide. These results are consistent with Fig. 6, which shows that PARP protein degradation and DNA fragmentation occurred simultaneously. As seen in Fig. 6D, DNA fragmentation was only partially inhibited in Hep G2 cells pretreated with Bax-specific anti-sense ODN. These observations indicate that another factor (such as caspase-3) may be involved in the DNA fragmentation process. To demonstrate that, we further evaluated the percentage of apoptotic cells by simultaneous treatment with Bax-specific antisense ODNs, caspase-3 inhibitor (zDEVD-fmk), and etoposide. Apoptotic effects induced by etoposide can be prevented almost completely when Bax and caspase-3 activity are completely inhibited.

Role of Caspase in Bax-Induced Apoptosis

Recent reports indicate that Bcl-2 family members control the release of cytochrome *c* and apoptosis-inducing factor from mitochondria during apoptosis and contribute to cell death by caspase activation [62, 63]. Overexpression of Bcl-2 has been reported to block the release of cytochrome *c* from mitochondria and in doing so to prevent apoptosis [64, 65]. In a recent study [66], the loop domain of Bcl-2 was cleaved by caspase-3 *in vitro*, and the carboxy-terminal of the Bcl-2 cleavage product (BH3 domain) triggered apoptosis. The BH3 domain appears to contribute to the proapoptotic activity of BAK, Bax, and other BH3-containing proteins [67]. Similar observations in a recent report suggested that Bax protein is targeted to organelle membranes [68], and in particular to mitochondria [69], by a COOH-terminal hydrophobic region. In this context, it is possible that upon Bax insertion into membranes, Bax may form channels or pores allowing the release of factors such as cytochrome *c* from within the mitochondria to propagate the apoptotic pathway. In the future, it will be crucial to elucidate how Bax insertion into the membrane is controlled.

In this study, Hep G2 cells were treated with etoposide for various lengths of time. Two days after drug exposure, PARP had undergone extensive degradation to an M_r 85,000 fragment. At the same time, the level of lamin A, the major structural protein of the Hep G2 nuclear envelope, had also degraded. By using the antisense ODN inhibition strategy, we demonstrated that Bax-specific antisense ODNs specifically inhibited the lamin A cleavage induced by etoposide. In contrast, this specific effect did not occur in the cleavage of PARP. Such results in the observation of lamin A degradation raise the possibility that there is an additional cellular hydrolase that is normally present in the extranuclear compartment of the cell. Translocation of Bax protein from the cytosol to the membrane of organelles is responsible for drug-induced apoptosis. Insertion of Bax in the membrane might be expected to activate hydrolase, which would result in extensive hydrolysis of lamin A in the nuclear lamina. The reports described above and our data suggest that Bax localization to the membrane of organelles is a vital aspect of its death-promoting ability.

Elevation of Bax Protein Levels in a p53-Dependent Manner by Agents That Cause DNA Damage

In our recent study [47], apoptosis was induced by various stimuli (such as staurosporine, quinacrine, and cycloheximide) in Hep G2 cells. Most of these stimuli induced both p53 and Myc protein expression. However, none of the stimuli altered the expression levels of Bcl-2 and Bax protein. These results are consistent with our observations here that busulphan (a protein alkylating agent) and vincristine (which binds to tubulin and blocks the mitotic process) apparently did not induce Bax protein expression

in Hep G2 cells as compared with the mitomycin C treated group. Other studies and ours also demonstrated that agents that cause DNA damage (such as NO and γ-radiation) might elevate p53 and Bax levels and eventually cause apoptosis [5, 6, 70]. It seems that the induction of Bax is dependent on the competent function of a wild-type *p53* gene in DNA-damaging agent-treated cells.

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