Induction of Apoptosis in Human Retinoblastoma Cells by Topoisomerase Inhibitors

Michela Giuliano, Marianna Lauricella, Erasmo Vassallo, Maria Carabillò, Renza Vento, and Giovanni Tesoriere

Purpose. To examine the apoptotic effect induced in human retinoblastoma Y79 cells by camptothecin, etoposide, and amsacrine, to examine the effect of these drugs on the expression of many apoptosis-related modulators, and to test the antiapoptotic effect exerted by insulin-like growth factor-I (IGF-I).

Methods. Morphologic features of apoptosis were demonstrated using acridine orange-ethidium bromide staining and electron microscopy. DNA fragmentation was determined by means of an in situ cell detection procedure (TdT-dUTP terminal nick-end labeling [TUNEL]) or by electrophoresis on agarose gels and was quantified by enzyme-linked immunosorbent assay. The expression of apoptosis-related modulators was studied by western blot analysis. The processing of latent p53 was examined by means of pulse-chase analysis.

Results. Camptothecin, etoposide, and amsacrine induced apoptosis in Y79 cells in a dose-dependent manner; camptothecin was the most efficacious compound. The effect, which was dependent on macromolecular synthesis, appeared after a lag of 8 hours and increased for as long as 24 hours. It was lower in cells treated with IGF-I, a potent mitogenic factor. Camptothecin and etoposide increased the p53 level after 4 hours of treatment, before the onset of apoptosis. This effect seemed to be a consequence of the conversion of latent p53 to one that is transcriptionally active. The drugs also induced an increase in p53-related proteins, such as p21, Bax, and IGF binding protein-3 (IGF-BP3), and caused a significant reduction of the Bcl-2 level. The latter effect was less evident in cells pretreated with IGF-I.

Conclusions. Topoisomerase inhibitors induce apoptosis in Y79 cells. This event is accompanied by a decrease in the expression of Bcl-2, a death antagonist, and an increase in that of Bax, a death agonist. A probable consequence of these modifications is the activation of ICE-like activity with degradation of poly-(adenosine diphosphate [ADP] ribose)-polymerase. Insulin-like growth factor-I exerts an antiapoptotic action in Y79 cells, and this function is most likely reduced by the overexpression of IGF-BP3 that is induced by drug treatment. (*Invest Ophthalmol Vis Sci.* 1998;39: 1300-1311)

Retinoblastoma is a highly malignant cancer of the retina that occurs in young children. The tumor, which originates from a primitive neuroectodermal cell,[1] can be transmitted as an autosomal dominant trait and occurs in a nonhereditary, sporadic form (unilateral) and in a hereditary (bilateral) form. The latter is often associated with additional neoplasms, such as osteosarcoma or soft-tissue sarcoma.[2,3] Retinoblastoma has been used as a prototypic model for the study of the genetic determination in cancer. Tumorigenesis is initiated by the inactivation of both alleles of a tumor-suppressor gene *RB-1*, which is located on chromosome 13q14 and which encodes a product with growth-suppression activity. The retinoblastoma gene product, a 105-kDa nuclear phosphoprotein, is expressed ubiquitously in normal mammal cells but has been absent in all retinoblastomas examined to date.[4] The present study was performed using the well-characterized Y79 human retinoblastoma cell line, in which the RB protein is completely absent.[5] Its deregulated proliferation is supported by a mitogenic loop that is dependent on insulin-like growth factor-I (IGF-I) and the type I IGF receptor (IGF-IR).[6]

Apoptosis, or programmed cell death, is a genetically determined process that has a significant role in the development of normal tissues. The process is characterized by morphologic and biochemical modifications consisting of cell shrinkage, membrane blebbing, chromatin condensation, and the internucleosomal degradation of DNA.[7] It is well known that ionizing radiation and many chemotherapeutic agents that are capable of inducing DNA damage exert a specific cytotoxicity on actively proliferating cells. This cytotoxic effect has now been attributed to the induction of apoptosis.

Several new chemotherapeutic agents that show promise in the treatment of many forms of advanced cancer have recently been adopted for use in the clinic. These drugs include inhibitors of topoisomerase I, in particular camptothecin

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and its analogs.[8] Camptothecin is a plant alkaloid that stabilizes topoisomerase I-DNA cleavable complexes.[9] It inhibits synthesis of DNA and RNA[10,11] and is capable of inducing apoptosis and fragmentation of DNA in many cancer cells.[12,13] An effective apoptotic action has also been demonstrated in many systems with the use of etoposide, an agent that inhibits topoisomerase II activity by stabilizing a covalent enzyme-DNA intermediate.[14-16]

The tumor-suppressor protein p53 exerts an arresting effect on the G1 stage of the cell cycle, and there is now increasing evidence that this protein may also induce apoptosis.[17] More specifically, a substantial increase in the total p53 level is required for the efficient activation of apoptosis after treatment with chemotherapeutic compounds or irradiation.[18] It has also been reported that another tumor-suppressor protein pRB may, on the contrary, inhibit programmed cell death and that inactivation of pRB results in apoptosis, which is mediated by the presence of functional p53.[19,20] The observation that pRB is absent in Y79 cells suggested to us that these cells, when treated with compounds capable of damaging DNA, may undergo programmed cell death through involvement of the p53 protein.

Despite the interest in this subject, only a few studies have been produced regarding apoptosis in retinoblastoma cells. In particular, Inomata et al.[21] examined the induction of apoptosis in cultured retinoblastoma cells by diverse drugs and they showed that only okadaic acid, an inhibitor of protein phosphatases, is capable of inducing DNA fragmentation in all of the cell lines tested. Also, sodium butyrate, a compound already known as an inducer of differentiation in Y79 cells,[22] has been reported to induce apoptosis in these cells.[23]

The present studies were carried out to assess the potential apoptotic effect of topoisomerase inhibitors in Y79 cells. In our findings, camptothecin and etoposide induced apoptotic fragmentation of DNA and cell death. Moreover, we analyzed the effects exerted by these compounds on the level of many apoptosis-related modulators, and we demonstrated that they favor the conversion of the latent form of p53 to one that is transcriptionally active. This event was accompanied by an increased expression of the transcriptional targets of p53. Finally, our report provides evidence of the antiapoptotic role exerted by IGF-I in Y79 cells.

METHODS

Cell Culture

The human retinoblastoma Y79 cell line used in this study was a kind gift from Adriana Albini (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). Cells were grown in suspension in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal calf serum and 1% pen-strep, in 75-cm^2 tissue culture flasks (Falcon, Cockeysville, MD) at 37°C in a moist atmosphere of 95% air-5% CO_2. The cultures were fed every other day. For monolayer cultures, 5×10^4 Y79 cells were seeded on 96-well culture plates (1.5×10^5 cells/cm^2) precoated with 5 μg/cm^2 poly-D-lysine (MWt 150,000-300,000) and cultured for 24 hours in 200 μl of serum-free medium consisting of a 2:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 5 μg/ml transferrin, 5 ng/ml sodium selenite, 10 mM HEPES, and 28 mM $NaHCO_3$ (pH 7.2-7.4). The drugs were added, and the incubation was protracted for the time indicated in the Results section. Topoisomerase inhibitors were dissolved in dimethyl sulfoxide and diluted to final concentrations in culture medium. Final concentrations of dimethyl sulfoxide never exceeded 0.04% and in routine control, exposure had no discernible effect in Y79 cells.

For the trypan blue exclusion assay, cells were removed from the wells after drug treatment, pelleted at 300g for 10 minutes and resuspended in phosphate-buffered saline (PBS). A 0.4% solution of trypan blue was added to the cells and the trypan blue-stained cells were counted with the aid of a hemocytometer.

Cell viability was also determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) colorimetric assay,[24] as previously described.[6] Mitochondrial dehydrogenases reduce MTT dye to a purple formazan. The direct proportion between dye reduction and cell number extends from 1×10^3 to 5×10^5 cells/well. Four experiments were performed using the MTT assay to determine cell viability.

The determination of cloning efficiency was performed under the conditions described by Jarvis et al.[25] Pelleted cells were seeded in 6-well culture plates (400 cells/well in 2 ml medium) and formation of colonies, defined as clusters of 50 cells or more, was scored after 10 days of incubation. The experiment was performed three times. All of the media and the chemical reagents for cell culture were from Sigma (St. Louis, MO).

DNA Synthesis

To quantify thymidine incorporation into acid-insoluble fraction, 0.5 μCi/well ^{3}H-thymidine (6.7 Ci per millimole; DuPont NEN, Boston, MA) was added to monolayer cultures, prepared as reported earlier.[6] Cells were labeled for 1 hour at 37 °C, then harvested and rinsed with PBS. Preparation of trichloroacetic acid-insoluble fractions and measurement of radioactivity were performed as previously described.[26]

Quantification of DNA Fragmentation

Apoptosis was quantified using an enzyme-linked immunosorbent assay kit (ELISA; Boehringer Mannhein, Darmstadt, Germany) that measures the amounts of mono- and oligonucleosomes produced as a consequence of DNA fragmentation in the cytoplasmic fraction of lysed cells.[27,28] Cells, cultured in monolayers in serum-free medium, were collected and lysed according to the manufacturer's instructions. After centrifugation at 20,000g for 15 minutes, equal amounts of cytosolic fraction (0.3 μg protein) were applied to ELISA plates, which were coated with the capture antihistone monoclonal antibody (mAb; clone H11-4). After incubation for 90 minutes at room temperature and washing, anti-DNA conjugated with horseradish peroxidase was added, and the plates were incubated for another 90 minutes at room temperature. Visualization was achieved by addition of the substrate 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS). Optical density of the plates was read at 405 nm (test wave length) and at 490 nm (reference wave length). Quantitative values, reported as nucleosomal enrichment, were calculated as the ratio between the absorbance measured for drug-treated cells and that measured for control cells. Results are the mean values recorded in four experiments.

Acridine Orange–Ethidium Bromide Staining

Monolayer cultures in 96-well plates were used in the morphologic studies. After removal of the medium, the cells were rinsed and treated with a solution composed of 100 μg/ml acridine orange plus 100 μg/ml ethidium bromide. This procedure allows dead cells to be distinguished from viable cells. Acridine orange is taken up by living and dead cells and is intercalated into DNA, turning it green. Ethidium bromide is taken up only by nonviable cells and turns DNA orange, because ethidium staining predominates over acridine. Thus, viable cells appear green and dead cells have bright orange chromatin. After staining, cells were examined by fluorescence microscopy and photographed (Kodak Gold Royal 1000 ASA; Kodak, Rochester, NY) .

Electron Microscopy

Ultrastructural analysis of morphologic changes for evidence of apoptosis were performed on specimens fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.3) for 1 to 2 hours, rinsed twice in PBS, and postfixed in 1% osmium tetroxide. The cells were rinsed in PBS, dehydrated through a series of graded alcohols, embedded in epoxy resin (Epon 812), and polymerized at 60°C overnight. Semithin sections (0.5 μm) were mounted on slides and stained with warm methylene blue. Screening and photography were performed using a Zeiss microscope. Ultrathin sections (100 nm) were mounted on nickel grids and counterstained with uranyl acetate for 10 minutes and lead acetate for 1 to 2 minutes. The specimens were processed conventionally and analyzed by electron microscope (model 1220; JEOL, London, UK).

In Situ Cell Death Detection

To detect apoptotic DNA fragments, a kit (Boehringer Mannheim) was used for in situ cell death detection by TdT-dUTP terminal nick-end labeling (TUNEL). After the indication of Gavrieli et al.,[29] 3′ ends of DNA fragments were labeled with terminal deoxynucleotidyl transferase (TdT) and fluorescein-labeled deoxyuridine triphosphate (dUTP). Incorporated fluorescein was detected by an antifluorescein antibody conjugated with alkaline phosphatase. After substrate reaction, stained cells were analyzed under light microscope. Morphologic experiments were repeated four to six times, and typical data are presented.

DNA Fragmentation Analysis

Y79 cells (10^7) were lysed in 1 ml buffer consisting of 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 0.5% (wt/vol) *N*-lauroylsarcosine and incubated at 50°C for 1 hour with 125 μg/ml proteinase K and for 1 hour with 125 μg/ml RNase. DNA was extracted according to Martin et al.[30] Electrophoresis of 15 μg fragmented DNA was carried out on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. The DNA marker was a 123-bp DNA ladder (Sigma).

Western Blot Analysis

Cells were lysed for 30 minutes at 4°C with a solution (15 μl/10^6 cells) composed of 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in PBS (pH 7.4) and were sonicated three times for 10 seconds (Soniprep 150; MSE). Proteinase inhibitors were added as follows: 25 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium ortovanadate, 10 mM NaF, 25 μg/ml leupeptin, and 0.2 mM sodium pyrophosphate. Cell lysates were centrifuged at 15,000*g* for 20 minutes at 4°C. Equivalent amounts of protein (40 μg) were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoretic transfer to nitrocellulose, proteins were immunoreacted for 2 hours with primary antibodies, then incubated for 30 minutes with secondary antibodies conjugated with alkaline phosphatase, final concentration 330 ng/ml (Promega, Madison, WI). The primary antibodies were: 1 μg/ml (final concentration) mouse monoclonal anti-human p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA); 10 μg/ml PAb421 and 5 μg/ml PAb240, p53 monoclonal antibodies (Calbiochem, La Jolla, CA); 2 μg/ml mouse monoclonal anti-human p21, 1 μg/ml mouse monoclonal anti-human Bcl-2, 1 μg/ml goat polyclonal anti-human Bax, and 2 μg/ml goat polyclonal anti-human IGF-BP3 (all from Santa Cruz Biotechnology); and 5 μg/ml rabbit polyclonal anti-human poly-(adenosine diphosphate ribose)-polymerase (PARP; Boehringer Mannheim, Germany). Visualization was performed using nitro blue tetrazolium and bromochloroindoyl-phosphate. p53 and Bcl-2 bands obtained from western blots were quantified by densitometric analysis. Experiments using western blot analysis were repeated four to six times, and typical data are presented.

Pulse–Chase Analysis

For these experiments, a monolayer of Y79 cells (6×10^6cells/well) was seeded in serum-free medium on 6-well culture plates. After preincubation for 1 hour in methionine- and cysteine-free medium, cells were labeled for another hour with 100 μCi/ml ^{35}S-methionine and ^{35}S-cysteine (^{35}S *trans*-label; ICN, Aurora, OH) in 0.75 ml methionine- and cysteine-free medium and then chased for different times in complete medium. Finally, cells were washed with cold PBS, disrupted in 0.5 ml lysis buffer (1% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) and after 30 minutes on ice, centrifuged at 30,000*g*. Cell lysates, normalized to 1 mg/ml protein, were preabsorbed with protein A Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA). Then, 0.5 μg/ml DO-1 or 1 μg/ml PAb421 and protein A Sepharose were added. After 1 hour on ice, the immunoprecipitates obtained were washed with lysis buffer and submitted to 10% SDS-PAGE. Finally, the gels were fixed for 30 minutes in 7% acetic acid-0.5% glycerol, dried in an Easy-Breeze Cell Dryer (Genenco M. Medical, Firenze, Italy) and then exposed to film (Hyperfilm-MP; Amersham, Buckinghamshire, UK) for autoradiography in the presence of an intensifying screen at −70°C for 1 week.

Results

Induction of Apoptosis in Y79 Cells by Topoisomerase Inhibitors

In our experiments, we evaluated the effect of topoisomerase inhibitors on the viability of human retinoblastoma Y79 cells. In Figure 1, results are shown of experiments in which Y79 cells were treated with 2 μM camptothecin for different periods. The number of cells decreased progressively as time passed and was reduced by approximately 40% after 12 hours and by 84% after 24 hours of incubation. No viable cells were

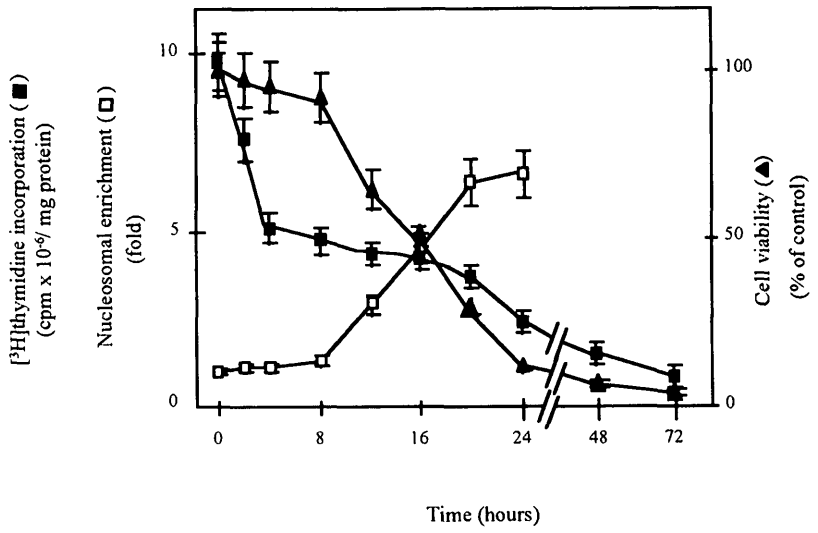

FIGURE 1. Effect of camptothecin on cell number (▲), DNA synthesis (■), and apoptosis (□) in Y79 cells. Cells (5×10^4) were cultured with 2 μM camptothecin for different periods. After incubation, cell number was evaluated by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide colorimetric assay (MTT), DNA synthesis was determined by measuring ^{3}H-thymidine incorporation into an acid-insoluble fraction, and apoptosis was evaluated by measuring DNA fragmentation with an enzyme-linked immunosorbent assay, as reported in the Methods section. Data are means ± SEM of four experiments.

detected by light microscopy (trypan blue exclusion assay) after treatment for three days with 2 μM camptothecin. Results of a viability test, performed by MTT assay, agreed with this observation. Treatment of Y79 cells with camptothecin resulted in a loss of clonogenicity, which correlated with a reduction in number of cells. Cloning efficiency, tested by an assay of colony formation, was equal to 20% of control cloning efficiency after 12 hours of incubation and to 10% after 24 hours (not shown).

Reduction of the number of cells was a consequence of the inhibition of cell proliferation and the induction of apoptosis. The two effects, however, were not simultaneous, as is suggested in Figure 1. DNA synthesis, measured by ^{3}H-thymidine incorporated into acid-insoluble fraction, declined rapidly during the first 4 hours of treatment, whereas the rate of apoptosis, assessed by an ELISA procedure that quantified DNA fragmentation, did not increase significantly in the first 8 hours of incubation. Thereafter, apoptosis increased progressively with passing time, reaching a value at 24 hours that was seven times greater than that measured at the beginning of incubation.

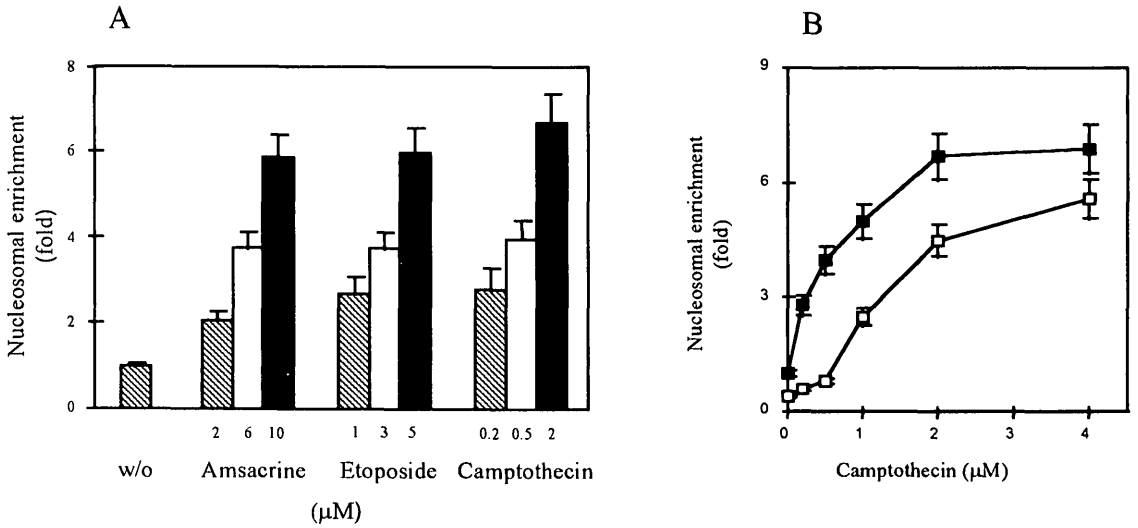

FIGURE 2. Apoptosis induced in Y79 cells that have been treated for 24 hours with topoisomerase inhibitors. (**A**) Comparison between the relative efficacy of different topoisomerase inhibitors. (**B**) Dose-dependent effect induced by camptothecin in cells that were cultured in serum-free medium without (w/o; ■) or with (□) 10 ng/ml insulin-like growth factor-I. Data are means ± SEM of four experiments.

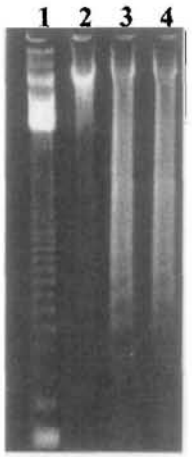

FIGURE 3. Induction of internucleosomal DNA fragmentation by topoisomerase inhibitors. DNA was extracted from the cells and analyzed by 1.5% agarose gel electrophoresis in the presence of ethidium bromide staining, as reported in the Methods section. DNA is shown from control cells (*lane 2*) and from cells treated for 24 hours with 2 μM camptothecin (*lane 3*) or 5 μM etoposide (*lane 4*). A 123-bp DNA ladder was used as a marker (*lane 1*).

The apoptotic effect induced in Y79 cells by topoisomerase inhibitors was dose dependent. A comparison between the relative efficacy of topoisomerase inhibitors (Fig. 2A) shows a fourfold increase in DNA fragmentation after 24 hours of incubation with 0.5 μM camptothecin; the same effect was observed with 3 μM etoposide or 6 μM amsacrine. Maximum induction (sevenfold increase) was elicited by 2 μM camptothecin (Fig. 2B).

Recently,[6] we demonstrated that Y79 cells produce IGF-I and IGF-II and contain the IGF-IR. The growth of these cells depends on an autocrine mechanism supported by IGFs and IGF-IR. In this study, we sought to establish whether treatment of cells with IGF-I could reduce the apoptotic effect exerted by camptothecin and etoposide. The apoptotic effect of camptothecin was less evident in cells pretreated for 24 hours with 10 ng/ml IGF-I (Fig. 2B), particularly at the lowest concentration of the drug. The antiapoptotic influence of IGF-I was also evident when etoposide was used to induce apoptosis (not shown).

Oligonucleosomal fragmentation was also demonstrated using agarose gel electrophoresis, which showed laddering of DNA from cells treated for 24 hours with camptothecin or etoposide, whereas laddering was absent in the untreated control cells (Fig. 3).

The morphologic features of apoptosis are described in Figure 4. The presence of condensed, crescent-shaped chromatin associated with nuclear membrane was detected by light microscopy and transmission electron microscopy.

A morphologic analysis was also performed by staining the cells with acridine orange-ethidium bromide (Fig. 5A). After this procedure, apoptotic cells were identified by the presence of nuclei with condensed or fragmented chromatin, a typical feature of apoptotic cells. This aspect was found in viable (green stained) and dead cells (orange stained). Exposure of the cells for 24 hours to 0.5 μM camptothecin, the concentration that induced a fourfold increase in DNA fragmentation (Fig. 2A), caused the death of approximately 40% of cells (not shown). Moreover, it was observed that when the cells were exposed to 2 μM camptothecin, the concentration that caused a sevenfold increase in DNA fragmentation, approximately 70% of cells appeared apoptotic when stained with acridine orange (Fig. 5A). These observations suggest that DNA fragmentation was associated with an increased number of cells exhibiting morphologic features of apoptosis.

The fragmentation of DNA in cultured Y79 cells was also detected by TUNEL assay. This assay labels the 3′-OH ends of DNA that result from the endonucleolytic cleavage that occurs during apoptosis. Y79 cells cultured without drugs were TUNEL negative (Fig. 5B). In contrast, large populations of cells treated for 24 hours with 2 μM camptothecin or 5 μM etoposide were TUNEL positive.

Effect of Actinomycin D and Cycloheximide on Apoptosis Induced by Topoisomerase Inhibitors

The apoptotic effect exerted by topoisomerase inhibitors was dependent on mRNA and protein synthesis. Increasing the concentration of actinomycin D, a transcription inhibitor, or that of cycloheximide, a translation inhibitor, reduced the apoptotic effect exerted by topoisomerase inhibitors. At a concentration of 5 μM, actinomycin D and cycloheximide both provided Y79 cells with complete protection against cell death caused by camptothecin (Fig. 6) or etoposide (not shown).

Effect of Topoisomerase Inhibitors on the Levels of p53, p53-Related Proteins, and Bcl-2

To examine the impact of topoisomerase inhibitors on p53, we evaluated the level of the protein in Y79 cell extracts by means of western blot analysis, using three antibodies: DO-1, specific for the amino-terminal domain; PAb421, specific for the carboxyl-terminal regulatory domain; and PAb240, which reacts with an epitope (residues 212–217) present at the DNA-binding domain. When DO-1 was used (Fig. 7A) the level of a protein component with the relative mobility of p53 increased in cells treated for 24 hours with amsacrine (lane 2) or etoposide (lane 3). With 2 μM camptothecin, the most efficacious compound, the level of p53 increased by 70% after 4 hours of incubation (lane 4), reached an increase of 150% after 8 hours of incubation (lane 5), and attained its maximum level (+200%) after 24 hours (lane 6). A comparison between the time dependence of the effects exerted by camptothecin on apoptosis and on the p53 level (Fig. 7C) caused us to conclude that the increase in p53 preceded the increase in apoptosis. The effect of camptothecin on the level of p53 was dose dependent and at 2 μM, reached levels three times greater than levels in untreated control cells (Fig. 7B). With etoposide, the effect on p53 level was also dose and time dependent (not shown). When PAb421 (Fig. 7D) or PAb240 (Fig. 7E) antibodies were used for analysis, the level of p53 was not modified after treatment of the cells with camptothecin or etoposide.

It is well known that p53 activates the transcription of various genes, including *p21waf-1*, *Bax*, and the gene for IGF-BP3.[31] Through the production of these proteins, p53 exerts its functions: p21 is responsible for the antiproliferative effect of p53 and transcriptional activation of Bax, and IGF-BP3 is associated with the induction of apoptosis.

Western blot analysis showed that after 24 hours of treatment with camptothecin, the level of p21, Bax, and IGF-BP3 markedly increased (Fig. 8). When the cells were treated with

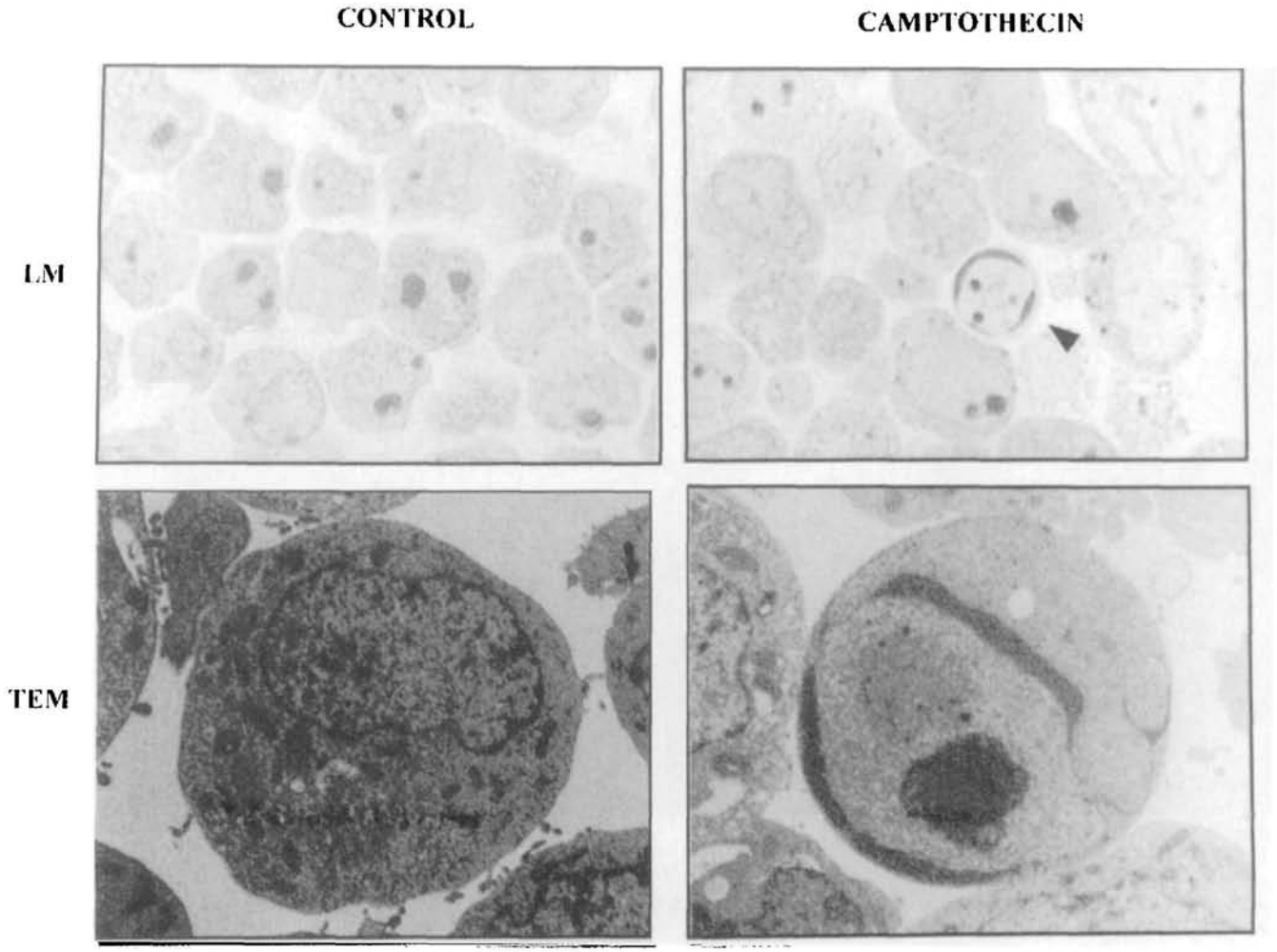

FIGURE 4. Cytologic profiles of Y79 cells treated with camptothecin. Y79 cells were cultured for 24 hours with 2 μM camptothecin. Homogeneous hemilunar marginations, typical of apoptosis, were observed in treated cells. In light microscopy, the crescent is indicated by an *arrow*. *LM*, light microscopy (original magnification, ×100); *TEM*, transmission electron microscopy (original magnification, × 10,000).

etoposide, a similar effect was observed, but it was less evident than the effect of camptothecin (not shown).

The proteolytic cleavage of PARP, assured by ICE-like protease activity, is considered a biochemical marker of apoptosis.[32] Figure 8 illustrates that treatment of Y79 cells with 2 μM camptothecin induces proteolysis of PARP (116 kDa) with the concomitant generation of an 85-kDa fragment. A similar result was obtained with cells treated with etoposide.

It has been reported that members of the Bcl-2 family control cell survival by exerting a protective effect against apoptosis.[33,34] We demonstrated, by means of western blot analysis, that topoisomerase inhibitors decreased the level of Bcl-2 in Y79 cells. The effect was dose dependent (not shown). The level of Bcl-2, which appeared unmodified after 4 hours of incubation with 2 μM camptothecin, decreased by 55% after 8 hours and by 73% after 24 hours (Fig. 9A). Similar results were obtained when the cells were treated with 5 μM etoposide (not shown).

We investigated whether the antiapoptotic effect of IGF-I modifies the action of camptothecin on the expression of the apoptosis modulators analyzed in the study. When Y79 cells were pretreated for 24 hours with IGF-I, camptothecin induced the same marked increases in p53, p21, and IGF-BP3 (not shown) as occurred in the absence of IGF-I. Instead, effect of camptothecin or etoposide in decreasing the level of Bcl-2 was much reduced when the cells were pretreated with IGF-I (Fig. 9B).

Stimulation by Topoisomerase Inhibitors of the Processing of p53 Protein

The influence of cycloheximide on the effect exerted by camptothecin and etoposide on p53 level was analyzed by means of western blot analysis. The analysis was performed by using the previously described DO-1, which is specific for the amino-terminal domain, as the primary antibody (Fig. 10). When Y79 cells were cultured in the presence of 5 μM cycloheximide for 24 hours, the level of p53 declined to 25% (lane 2) of the value measured in control cells (lane 1). Twenty-four hours of incubation with camptothecin or etoposide to Y79 cells increased the level of p53 by 200% (lane 3) and 180% (lane 5), respectively, compared with the control value. When cycloheximide was added with camptothecin or etoposide, the effect exerted by these compounds on p53 diminished, but the level of the protein remained much higher than that in control cells—

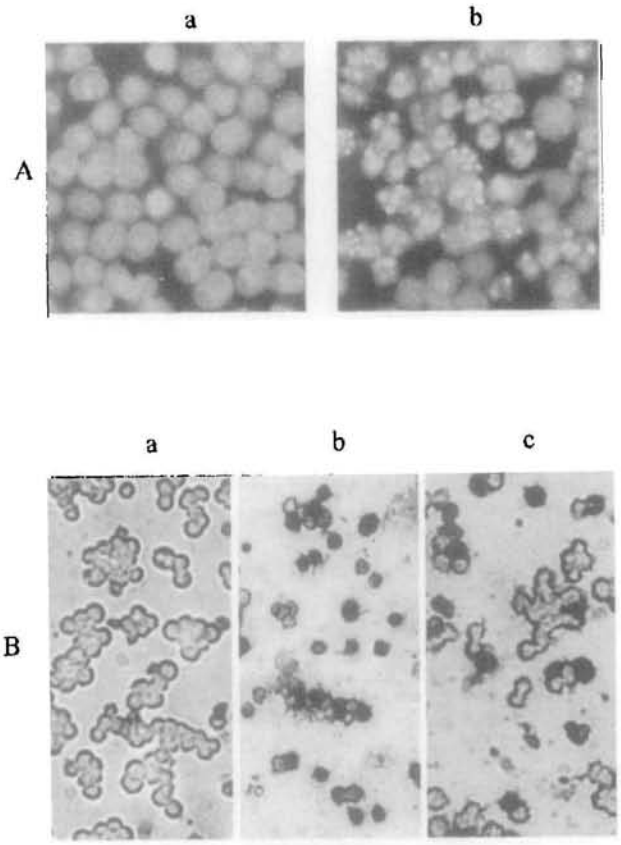

FIGURE 5. Morphologic analysis of Y79 cells cultured in the presence of topoisomerase inhibitors. (**A**) Cells stained with an acridine orange–ethidium bromide fluorescent dye and photographed under fluorescence microscope. (**B**) Cells processed for TdT-dUTP terminal nick-end labeling (TUNEL) and photographed under light microscope. Control cells (**a** in **A** and **B**) and cells cultured for 24 hours with 2 μM camptothecin (**b** in **A** and **B**) or 5 μM etoposide (**c** in **B**).

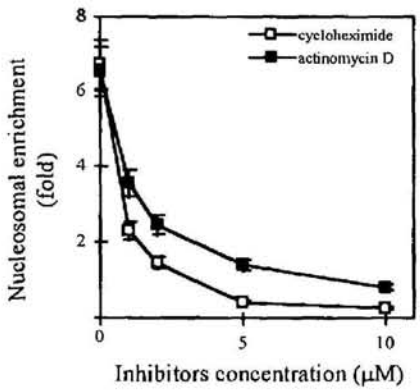

FIGURE 6. Inhibitory effects of actinomycin D and cycloheximide on apoptosis induced by camptothecin in Y79 cells. Cells were incubated for 24 hours with 2 μM camptothecin together with increasing concentrations of actinomycin D (■) or cycloheximide (□). DNA fragmentation was measured by means of an enzyme-linked immunosorbent assay, as reported in the Methods section. Data are means ± SEM of four experiments.

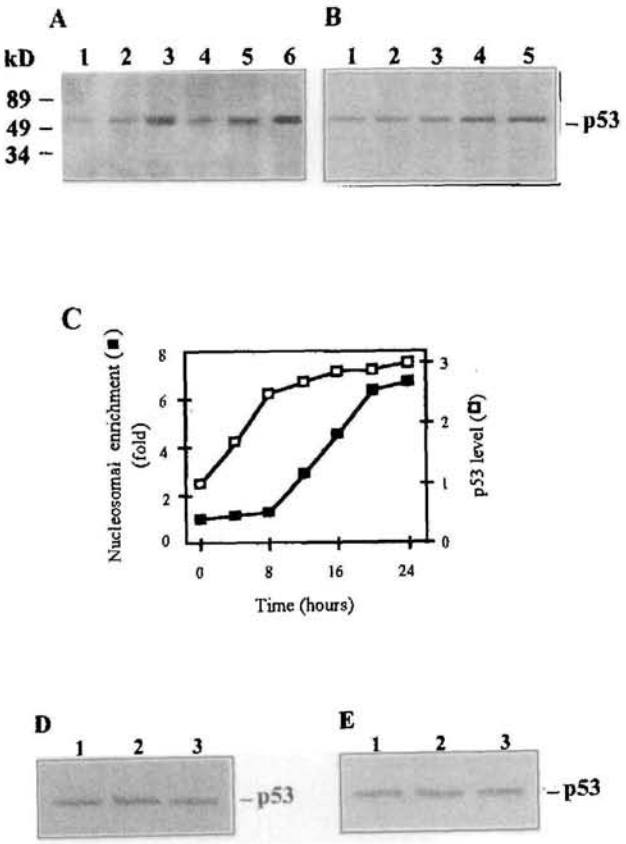

FIGURE 7. Induction of p53 by topoisomerase inhibitors in Y79 cells. Extracts were prepared from cells treated with topoisomerase inhibitors. As reported in the Methods section, identical amounts of protein (approximately 40 μg) were used for western blot analysis, using DO-1 (**A**, **B**), PAb421 (**D**), or PAb240 (**E**) antibodies. (**A**) Control cells (*lane 1*); cells treated for 24 hours with 10 μM amsacrine (*lane 2*) or 5 μM etoposide (*lane 3*); and cells treated for 4 hours (*lane 4*), 8 hours (*lane 5*), or 24 hours (*lane 6*) with 2 μM camptothecin. (**B**) Control cells (*lane 1*) and cells treated with various concentrations of camptothecin: 0.5 μM (*lane 2*), 1 μM (*lane 3*), 2 μM (*lane 4*), or 10 μM (*lane 5*). (**C**) Time-dependent induction of p53 by 2 μM camptothecin in comparison with the effect exerted on apoptosis. p53 bands obtained from western blots using DO-1 antibody were quantified by densitometric analysis; p53 level at time 0 was considered to be 1. (**D**, **E**) Control cells (*lane 1*) and cells treated for 24 hours with 2 μM camptothecin (*lane 2*) or 5 μM etoposide (*lane 3*).

110% (lane 4) and 105% (lane 6) of the control value, respectively. These results indicate that topoisomerase inhibitors stimulated the production of an immunodetectable form of p53 from a latent from, by means of different mechanisms, which are either dependent or independent of synthesis of new protein.

In an attempt to determine the mechanisms of the increase in p53 content mediated by camptothecin and etoposide, we performed a pulse-chase experiment (Fig. 11). Y79 cells, cultured in serum-free medium, were starved for 1 hour in methionine- and cysteine-free medium and then pulse-labeled for 1 hour with ^{35}S-methionine and ^{35}S-cysteine. After washing, the cells were fed with fresh medium and incubated in the presence or absence of camptothecin or etoposide for 0, 5, 10, 15, or 24 hours. Finally, labeled p53 was immunopre-

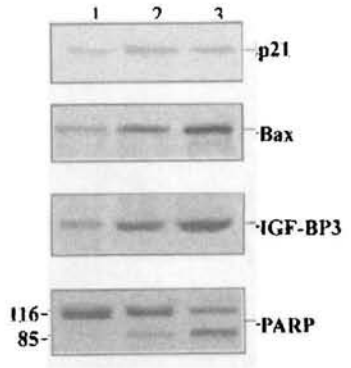

FIGURE 8. Effect of camptothecin treatment in Y79 cells on the expression of p21, Bax, and insulin-like growth factor-binding protein-3 (IGF-BP3) and on the degradation of poly-(adenosine diphosphate ribose)-polymerase (PARP). Cells were incubated for 24 hours in the presence of camptothecin. At the end, cell extracts were prepared, and identical amounts of protein (40 μg) were used for western blot analysis. Shown are control cells (*lane 1*) and cells treated with 0.5 μM (*lane 2*) or 2 μM (*lane 3*) camptothecin.

cipitated by DO-1, which reacts with an epitope (residues 37–45) present in human wild-type and mutant p53 at the amino-terminal transactivation domain. The immunoprecipitate was subjected to SDS–PAGE and detected by autoradiography. The relative intensities of the p53 bands determined by densitometric analysis are reported in Figure 11D. The half-life of p53 in cells cultured without drugs was calculated to be approximately 8 hours (Fig. 11A). When the cells were incubated with 2 μM camptothecin (Fig. 11B) or 5 μM etoposide (data not shown), the level of radioactive p53 increased during the first 10 hours of the chase period (120%). This effect could be the result of the processing of p53, which exposed the epitope to the antibody. After 10 hours of incubation, the level of p53 progressively decreased until 24 hours had passed, although it remained significantly higher (65%) than the level measured at the beginning of the chase period. We think that this reduction was a consequence of the degradation of p53, which most likely prevailed over the production of immunoprecipitable p53 in the second part of the experiment. These considerations were supported by experiments in which cycloheximide, an inhibitor of protein synthesis, was added with

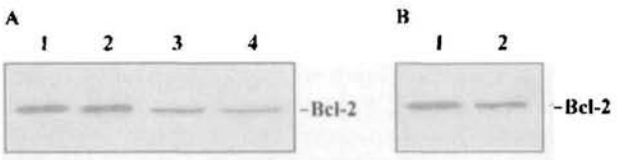

FIGURE 9. Effects of apoptotic drugs on Bcl-2 content in Y79 cells, treated or untreated with insulin-like growth factor-I (IGF-I). Y79 cells were grown for 24 hours in a serum-free medium in the absence (**A**) or in the presence (**B**) of 10 ng/ml IGF-I. Two micromolar camptothecin was added, and the incubation continued for different periods. Cell extracts were prepared, and identical amounts of protein were used for western blot analysis. (**A**) Control cells (*lane 1*); cell is treated with camptothecin for 4 hours (*lane 2*), 8 hours (*lane 3*), or 24 hours (*lane 4*). (**B**) Control cells (*lane 1*); cells are treated with camptothecin for 24 hours (*lane 2*).

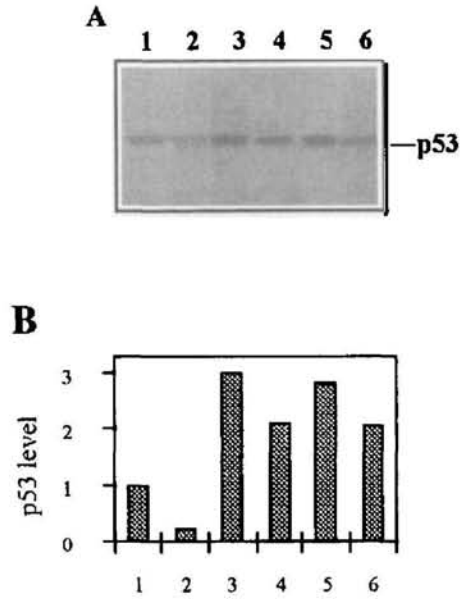

FIGURE 10. Effect of cycloheximide on the p53 level in Y79 cells cultured in the presence of topoisomerase inhibitors. Extracts were prepared from Y79 cells treated for 24 hours with 2 μM camptothecin or 5 μM etoposide in the presence or absence of 5 μM cycloheximide. (**A**) Western blot analysis. (**B**) Densitometric analysis of p53 bands. Y79 cells cultured without drugs (*lanes 1, 2*), with camptothecin (*lanes 3, 4*), or with etoposide (*lanes 5, 6*). Five micromolar cycloheximide was added to samples 2, 4, and 6.

camptothecin. In this case (Fig. 11C), the level of radioactive p53 continued to increase beyond 10 hours of incubation and at 24 hours, reached an increase of 140% compared with the value measured at time 0. The results seem to indicate that the addition of cycloheximide prevented p53 from degradation by newly synthesized enzymes and showed that camptothecin and etoposide stimulated processing of p53 during the entire incubation period. When cycloheximide alone was added to Y79 cells, no increase in the level of radioactive p53 was seen (not shown).

Different results were obtained when labeled p53 was immunoprecipitated by PAb421 antibody, which reacts with an epitope (residues 371–380) that is present at the carboxyl-terminal regulatory domain in human wild-type and mutant p53. The intensity of the radioactive p53 band decreased during the pulse-chase period in untreated (Fig. 11E) and treated cells (Fig. 11F). In the latter case, however, the half-life of p53 was 11 hours, compared with 8 hours in control cells. The relative intensities of p53 bands in cells treated with 2 μM camptothecin in comparison with p53 bands in control cells are shown in Figure 11G.

DISCUSSION

The Effect of Camptothecin and Etoposide on Apoptosis and on the Level of p53

In the present study, we showed for the first time that topoisomerase inhibitors can induce efficacious programmed cell death in human retinoblastoma cells. This conclusion is suggested by the appearance of morphologic features of apoptosis

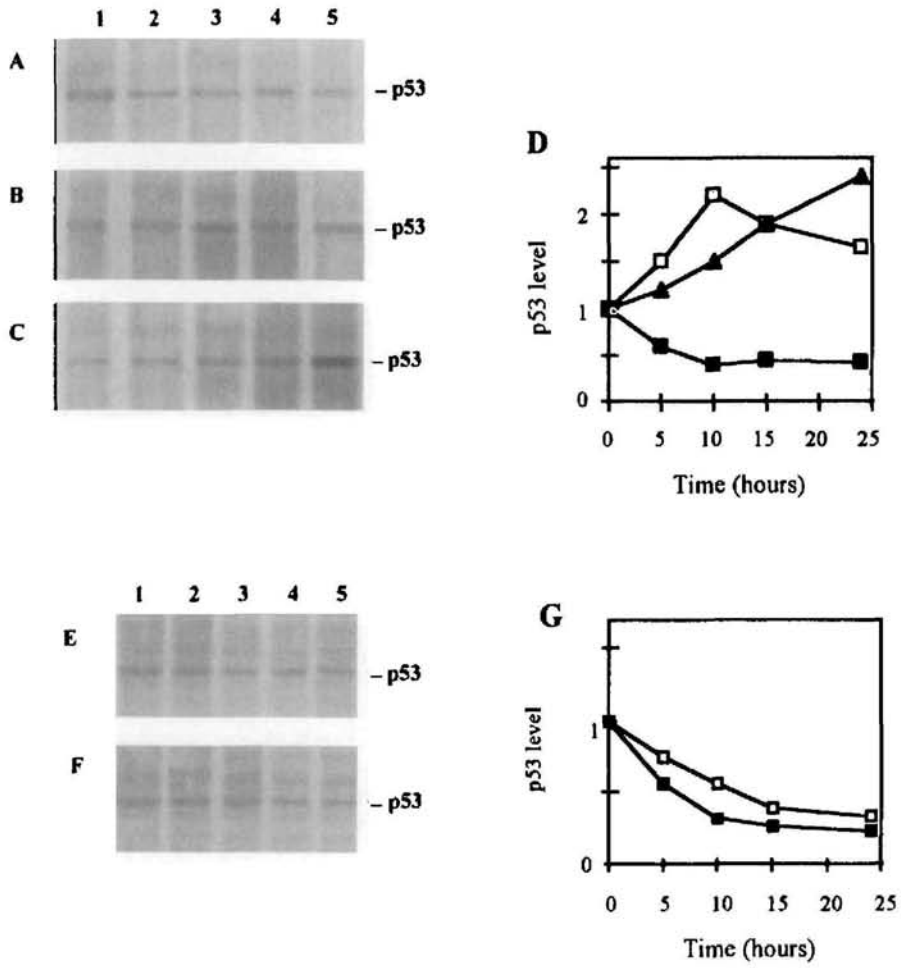

FIGURE 11. Pulse-chase analysis of p53 in Y79 cells treated with camptothecin. Cells were pulse-labeled with ^{35}S-methionine-^{35}S-cysteine, as reported in the Methods section. Thereafter, the cells were incubated in complete Dulbecco's modified Eagle's medium, without addition of drugs (**A**, **E**), with 2 μM camptothecin (**B**, **F**), or with 2 μM camptothecin plus 5 μM cycloheximide (**C**). Cells were harvested at time 0 (*lane 1*), 5 hours (*lane 2*), 10 hours (*lane 3*), 15 hours (*lane 4*), and 24 hours (*lane 5*) of the chase period. Immunoprecipitation of p53 was performed, as reported in the Methods section, using DO-1 (**A**, **B**, **C**, **D**) or PAb421 antibody (**E**, **F**, **G**). The immunoprecipitate proteins were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel and autoradiographed. The relative intensity of single p53 bands, determined by densitometric analysis, is shown in **D** and **G**. The p53 level at time 0 was considered to be 1. (**D**) (■) refers to **A**, (□) to **B**, and (▲) to **C**. (**G**) (■) refers to **E**, and (□) refers to **F**.

that correspond to biochemical hallmarks of this process, such as DNA fragmentation and nucleosomal enrichment. In our studies, the most active compound was camptothecin; etoposide and amsacrine were effective at much higher concentrations.

Treatment of Y79 cells with camptothecin or etoposide induced a marked increase in p53 level. This effect, which was evident after 4 hours of treatment, clearly anticipated the induction of cell death.

It is well known that p53 protein, the product of a tumor-suppressor gene, exerts an antiproliferative effect by inducing an arrest in the cell cycle or apoptosis,[17] two separate functions of the protein. These events can be caused by means of transactivation-dependent or -independent mechanisms. Moreover, it has been demonstrated that the level of p53 increases in the cells as a consequence of DNA damage. Whether this increase provokes cell cycle arrest or apoptosis is determined by several factors, such as the amount of DNA damage, the level of p53, and the availability of survival factors.[35,36] Cells with DNA damage should be eliminated in p53-dependent apoptosis, when they are forced into a replicative cycle by activated oncogenes.[37] In particular, p53 is required for the efficient activation of apoptosis after irradiation or treatment with chemotherapeutic compounds such as 5-fluorouracil, etoposide, and adriamycin.[18]

p53 plays a deciding role in inducing apoptosis in cells with no pRB, as has been suggested by Howes et al.[38] They demonstrated that p53 is required in apoptosis that has been induced in the photoreceptor cells of transgenic mice by the functional inactivation of pRB determined by the expression of E7 protein. In line with this suggestion, Nork et al.[39] have provided evidence that, in human retinoblastoma, p53-immunoreactive cells are observed in close association with cells undergoing apoptosis. Moreover, the involvement of p53 in

apoptosis induced in retinoblastoma cell lines has been suggested recently by Kondo et al.,[40] who demonstrated the overexpression of p53 and its associated protein WAF-1 after γ-radiation of the cells.

Our results demonstrate that the increase in p53 level, which was induced by topoisomerase inhibitors, was correlated to changes occurring at the amino-terminal domain of p53. This conclusion is suggested by results of western blot analysis or pulse-chase experiments performed using DO-1, which specifically binds to an epitope located at this domain. The increasing effect was not observed when we used PAb421 and PAb240, which bind to other epitopes. Our findings also suggest that an amount of p53 was present in the Y79 cells in a latent state and that treatment with topoisomerase inhibitors induced the conversion of this form into a new form that was immunoprecipitable by DO-1. Our results further indicate that this effect was at least partially dependent on protein synthesis.

Many investigators have demonstrated that the activity of p53 is regulated by several mechanisms at different levels. Normal cells contain p53 protein in a latent state that can be activated by UV radiation or by the intervention of highly specific peptides.[41,42] A transcriptionally inactive p53 form has been seen in undifferentiated teratocarcinoma cells that undergo p53-mediated apoptosis in response to DNA damage.[43] Moreover, p53 is present in the cells as inactive complexes in which it is associated with heat shock proteins,[44] protein kinases, and phosphatases.[42] In a significant proportion of human cancers, p53 is associated with MDM-2, a protein that binds p53 and inhibits its transactivation activity.[45]

Currently, it is not possible to specify the mechanism by which topoisomerase inhibitors induce the conversion in Y79 cells of the latent form of p53 to a form that is recognized by DO-1. It has recently been suggested that genotoxic stress can stimulate the processing of the latent form of p53. [46] Evidence has been provided that p53 is sensitive to stress or DNA damage through phosphorylation at its N terminus.[47] In particular, it seems that topoisomerase-directed drugs are capable of enhancing the level of the delta isoform of casein kinase-1. This enzyme is involved in the phosphorylation of a group of the sites at the N terminus of p53.[48]

The Effect of Camptothecin and Etoposide on the Expression of p21, Bax, Insulin-like Growth Factor–Binding Protein 3, and Bcl-2

Our results provide evidence that camptothecin and etoposide induced the production of a new form of p53 that is transcriptionally active. Treatment with both compounds increased the level of p53 and the levels of three transcriptional targets of this protein: p21, an antiproliferative factor, and Bax and IGF-BP3, two apoptosis modulators. Furthermore, treatment with topoisomerase inhibitors induced a concomitant reduction in Bcl-2, a protein that promotes cell survival and inhibits apoptosis.[33,34] This effect took place after the increase in the p53 level, but it anticipated the increase in apoptosis. It therefore seems possible that this effect may be a consequence of the direct action of p53,[49] although it is known that the expression of Bcl-2 protein can also be regulated by other mechanisms that are independent of p53 function.[50]

To obtain a better understanding of the mechanism by which camptothecin and etoposide induce apoptosis in Y79 cells, it is necessary to analyze the role of the modulators that seem to be involved in this process. It has been demonstrated that p21 is responsible, at least partially, for the antiproliferative effect of p53.[35] In many systems, p53 provokes a G1 arrest by transactivating the expression of p21, which inhibits G1 cyclin-dependent kinases, the enzymes involved in the phosphorylation of pRB. As a consequence, the pRB-E2F complex cannot be broken down, and the cells are prevented from entering the S phase of the cycle. In Y79 cells, which have no pRB, the increase in p21 after treatment with apoptotic drugs, indicates that this protein could be involved in another function that is still unknown.

Bcl-2 is a well known antiapoptotic factor[51,52] that belongs to one of the most important classes of apoptosis-regulating gene products, including proapoptotic and antiapoptotic factors.[33,34] Bax, a Bcl-2 family member that functions as a proapoptotic factor, is capable of producing homodimers and heterodimers with the cell death antagonists Bcl-2 and Bcl-X_L. The relative abundance of the Bcl-2 family of cell death agonists compared with the number of antagonists determines the susceptibility of cells to an apoptotic stimulus. In Y79 cells, topoisomerase inhibitors induce an increase in the expression of Bax and a reduction in Bcl-2. Thus, the ratio of cell death agonists to antagonists increases, and the cells become susceptible to apoptosis.[34]

The Bcl-2 family acts at the level of the outer mitochondrial membrane as a regulator of intracellular ion fluxes and as an inhibitor of the ICE-like proteases that are required for some mechanisms of cell death.[52] The reduction in Bcl-2 and the concomitant increase in the level of agonist modulators induces the activation of proteases that are involved in the degradation of PARP.[34,53] Our study demonstrates that treatment of Y79 cells with topoisomerase inhibitors stimulated the degradation of PARP with the appearance of an 85-kDa fragment. It seems likely that this event in Y79 cells can also be considered a consequence of the increase in the ratio of cell death agonists to antagonists.

Antiapoptotic Role of Insulin-like Growth Factor-I

Results reported in this study suggest that IGF-I, aside from its function in cell growth,[6] is capable of opposing the apoptotic effect of topoisomerase inhibitors. Moreover, our recent unpublished results show that αIR3, a monoclonal antibody capable of inhibiting the mitogenic activity of IGF-I,[6] does not modify the antiapoptotic effect exerted by the same compound. Our results agree with the conclusions of Rubin and Baserga[54] and Sell et al.,[55] who demonstrated that IGF-IR, activated by its ligands, protects cells from apoptosis in vivo and in vitro and that the domains of the IGF-I receptor required for its antiapoptotic function are distinct from those required for its proliferation or transformation.[56]

To explain the mechanism of the antiapoptotic action of IGF-I in Y79 cells, we ascertained that IGF-I modified neither the inhibitory effect exerted by camptothecin and etoposide on the synthesis of DNA, nor their effect on the content of p53. Instead, our results are consistent with a role for IGF-I in regulating the level of Bcl-2, because treatment with IGF-I protected Y79 cells from the reduction in Bcl-2 level induced by camptothecin or etoposide. This conclusion agrees with the recent observation of Singleton et al.[57] that IGF-IR plays a role in keeping Bcl-2 at a normal level.

It is interesting that p53 also mediates apoptosis by inducing the production of IGF-BP3,[31] a protein that has a high affinity with IGF-I and interferes with IGF signaling. In the

current study, treatment of Y79 cells with apoptotic drugs markedly increased the level of IGF-BP3. This suggests that through the production of this protein, the antiapoptotic action of IGF-I can be controlled and its protective effect on the expression of Bcl-2 can be removed.

Although the results described in this study are inconclusive and many aspects deserve greater attention and further experiments, the apoptotic mechanism of topoisomerase inhibitors in Y79 cells is partially evidenced by our results. Our conclusion is that the drugs induce a reduction in Bcl-2 expression and an increase in that of p53. p53 stimulates, through a transactivation mechanism, the expression of Bax protein. Thus, among the members of the Bcl-2 family, the death-agonist factors become prevalent, leading to the activation of ICE-like activity with degradation of PARP. The growth of Y79 cells is stimulated by an autocrine mechanism dependent on IGF-I and IGF-IR. IGF-I also exerts a clear antiapoptotic effect that is most likely reduced by the increased expression of IGF-BP3, induced by a transactivation mechanism dependent on p53.

The Role of Apoptotic Drugs in Chemotherapy for Retinoblastoma

Retinoblastoma is the most common intraocular neoplasm of childhood. Treatment includes chemotherapy that is intended to reduce tumor size and allow local treatment with photocoagulation, cryotherapy, or both. In this regard, the combination of etoposide with carboplatinum is particularly efficacious.[58] Intensive treatment with these compounds has been effective, even in the advanced stages of tumor growth and in extraocular forms.[59] Data in the present report suggest that the antitumor action of etoposide could be a consequence of its apoptotic effect. Our results also demonstrate that camptothecin is much more efficacious than etoposide in inducing apoptotic death in Y79 cells. However, camptothecin and its analogs are not yet used in the treatment of retinoblastoma.

We are currently investigating other compounds capable of inducing death in retinoblastoma cells. Our recent results demonstrate that cisplatin and carboplatin, whose apoptotic action is well known,[60,61] are effective in inducing cell death in Y79 cells, although resistance occurs after prolonged treatment (unpublished data, 1998). Other studies in progress in our laboratory concerning sodium butyrate confirm that this compound may induce differentiation[22] or apoptosis[23] in Y79 cells and that its apoptotic effect is independent of p53. Finally, we have demonstrated the apoptotic effect induced by ceramide[62] and by compounds that stimulate breakdown of sphingomyelin (i.e., tumor necrosis factor and interleukin 1-β). The purpose of all these studies is to ascertain whether the administration of some of these compounds together with topoisomerase inhibitors can produce a clear apoptotic effect at minimal doses of camptothecin or etoposide.

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