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E2F-1 Cooperates with Topoisomerase II Inhibition and DNA Damage To Selectively Augment p53-Independent Apoptosis

JOHN NIP,[1] DAVID K. STROM,[1] BRIAN E. FEE,[2] GERARD ZAMBETTI,[2] JOHN L. CLEVELAND,[2,3] AND SCOTT W. HIEBERT[1]*

Departments of Tumor Cell Biology[1] and Biochemistry,[2] St. Jude Children's Research Hospital, Memphis, Tennessee 38105, and Department of Biochemistry, University of Tennessee, Memphis, Tennessee 38163[3]

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Mutations in the retinoblastoma (pRb) tumor suppressor pathway including its cyclin-cdk regulatory kinases, or cdk inhibitors, are a hallmark of most cancers and allow unrestrained E2F-1 transcription factor activity, which leads to unregulated G_1-to-S-phase cell cycle progression. Moderate levels of E2F-1 overexpression are tolerated in interleukin 3 (IL-3)-dependent 32D.3 myeloid progenitor cells, yet this induces apoptosis when these cells are deprived of IL-3. However, when E2F activity is augmented by coexpression of its heterodimeric partner, DP-1, the effects of survival factors are abrogated. To determine whether enforced E2F-1 expression selectively sensitizes cells to cytotoxic agents, we examined the effects of chemotherapeutic agents and radiation used in cancer therapy. E2F-1 overexpression in the myeloid cells preferentially sensitized cells to apoptosis when they were treated with the topoisomerase II inhibitor etoposide. Although E2F-1 alone induces moderate levels of p53 and treatment with drugs markedly increased p53, the deleterious effects of etoposide in E2F-1-overexpressing cells were independent of p53 accumulation. Coexpression of Bcl-2 and E2F-1 in 32D.3 cells protected them from etoposide-mediated apoptosis. However, Bcl-2 also prevented apoptosis of these cells upon exposure to 5-fluorouracil and doxorubicin, which were also cytotoxic for control cells. Pretreating E2F-1-expressing cells with ICRF-193, a second topoisomerase II inhibitor that does not damage DNA, protected the cells from etoposide-induced apoptosis. However, ICRF-193 cooperated with DNA-damaging agents to induce apoptosis. Therefore, topoisomerase II inhibition and DNA damage can cooperate to selectively induce p53-independent apoptosis in cells that have unregulated E2F-1 activity resulting from mutations in the pRb pathway.

Imbalance between cellular proliferation and apoptosis is a hallmark of cancer. The transcription factor E2F-1 is a critical regulator of cell cycle progression, and it plays a pivotal role in the transition from G_1 to S phase of the cell cycle (1, 9, 11, 22, 44). The transcriptional activity of E2F-1 is negatively regulated by the product of the retinoblastoma tumor suppressor gene (pRb) (4, 14, 17, 51) or the related family members p107 and p130 (3, 6, 45) and is indirectly regulated by specific cyclins, such as the D-type cyclins, their associated kinases (cdks) (23, 34, 37, 45), and cdk inhibitors (p16 and p15) (15, 43). Hypophosphorylated pRb binds E2F, repressing its ability to activate genes involved in DNA synthesis and cell proliferation (e.g., dihydrofolate reductase, DNA polymerase α, thymidine kinase, and thymidylate synthase) (7, 35). However, when Rb is hyperphosphorylated, through the action of specific combinations of G_1 cyclins and their associated kinases (cyclin D-cdk4, cyclin D-cdk6, or cyclin E-cdk2), it releases E2F-1, which can then stimulate transcription and promote S-phase entry.

Naturally occurring mutations that involve pRb have been identified in nearly every type of human neoplasia (50). These changes include alterations of the E2F binding domain of pRb and the constitutive phosphorylation of pRb due to loss of cyclin-dependent kinase inhibitors (e.g., p16). Moreover, chromosomal translocations and amplifications that result in enhanced cyclin D1-cdk4 function and indirectly disrupt pRb are prevalent in carcinomas of the breast (12) and head and neck (2). Disruption in this pRb pathway also occurs in both lymphoid and myeloid leukemias (24). All of these alterations lead to unregulated E2F. For example, in two human leukemia cell lines, absence of p16 causes aberrant E2F-mediated regulation of thymidine kinase (16).

E2F-1 is the most well-characterized member of the E2F family (E2F-1 to E2F-5) and forms heterodimers with DP family members (DP-1 and DP-2) (29, 56). Enforced expression of E2F-1 in cooperation with activated *ras* can transform primary cells (21), and enforced expression of E2F-1 on its own can transform immortalized cells (46, 54). In the absence of growth factors, E2F-1 alone is sufficient to stimulate cells to complete G_1 and begin DNA synthesis (9, 22), yet this unscheduled entry into S phase is a potent trigger for apoptosis (25, 39, 44, 53). We have previously shown that E2F-1 overexpression is tolerated in interleukin 3 (IL-3)-dependent 32D.3 myeloid progenitor cells but that upon removal of IL-3, E2F-1 induces apoptosis (18). Moreover, E2F-1 and DP-1 can be coexpressed at low levels, but at high levels they can overcome the strong survival functions of IL-3 and serum and induce apoptosis. Overexpression of E2F-1 in 32D.3 cells also led to the accumulation of p53; however, p53 induction did not correlate with cell death (18). On the other hand, introduction of an E2F-1 expression plasmid into (10.1)Val5 mouse embryo fibroblast cells, which overexpress a temperature-sensitive (ts) mutant allele of p53, caused extensive apoptosis in these cells when p53 was in the wild-type conformation. Although $p53^{-/-}$ cells failed to undergo apoptosis in response to E2F-1 overexpression, again implicating p53 (25), human papillomavirus E6 failed to protect keratinocytes from E2F-1-induced death (33) and E2F-1 did not cooperate with ioninizing radiation to induce apoptosis (8). These results suggest that p53 and E2F-1 can cooperate to mediate apoptosis (53) but that other factors

* Corresponding author. Present address: Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146.

contribute. Furthermore, the cell death response differs greatly between fibroblasts and myeloid progenitor cells, as programmed cell death is a normal physiological component in the control of hematopoiesis.

As a model system to determine whether mutations of the pRb pathway might sensitize myeloid cells to various chemotherapeutic and radiotherapeutic strategies, we used 32D.3 cells overexpressing E2F-1. Enforced expression of E2F-1 caused a highly specific sensitivity to etoposide but not to other agents that induce DNA damage. Etoposide arrested the cells in S phase and triggered p53-independent apoptosis. Using a second inhibitor of topoisomerase II, we also demonstrated that both topoisomerase II inhibition and DNA damage are necessary to augment E2F-1-induced apoptosis. Thus, topoisomerase II may be a critical therapeutic target for tumors containing mutations that result in the loss of pRb function.

MATERIALS AND METHODS

Cell lines. Parental 32D.3 myeloid progenitor cells were maintained as suspension cultures in RPMI-1640 containing 10% fetal bovine serum (BioWhittaker, Walkersville, Md.), 2 mM L-glutamine (BioWhittaker), 1% penicillin G-streptomycin (Gibco-BRL, Life Technologies, Inc., Grand Island, N.Y.), and 20 U of IL-3 per ml at 37°C. The 32D.3 clones expressing E2F-1, hemagglutinin epitope-tagged human DP-1 (DP-1.1 and DP-1.6), and the E2F.138 mutant have been described previously (18). For clarity, the names of the E2F-1-overexpressing cell lines have been changed from E2F.1 and E2F.9 to E2F-1.1 and E2F-1.9, respectively. E2F-1.9 clones that express the ts p53 mutant (E2F-1.9/Val 135.11) were generated by co-transfection of the murine sarcoma virus long terminal repeat (LTR) ts p53 Val 135 expression construct (19) with the hygromycin expression plasmid pRMM and selection in G418 plus hygromycin (18). 32D.3 cells, stably transfected either with the spleen focus-forming virus (SFFV) LTR expression vector containing a human Bcl-2 cDNA (Bcl-2.4) alone or with the above vector and a dexamethasone-inducible pMAM-Neo (Clontech, Palo Alto, Calif.) E2F-1 expression vector, were made as described earlier (18).

Western blot (immunoblot) analysis and immunoprecipitation. For protein analysis of p53 and Bcl-2, cells were lysed for 15 min in TENN buffer (50 mM Tris-Cl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) containing the following protease inhibitors: 22 μg of aprotinin, 15 μg of pepstatin A, 30 μg of leupeptin, 100 μg of phenylmethylsulfonyl fluoride, and 10 μg of E-64 per ml. The lysates were then sonicated for 10 s with a Branson Sonifier 250 (Branson Ultrasonics Corp., Danbury, Conn.) set at 30% duty cycle. Cellular debris was removed by refrigerated centrifugation at 12,000 × *g* for 5 min, and lysates were snap frozen in dry ice and stored at −80°C.

Total protein concentration of the lysates was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.), and equal amounts of protein were immunoblotted with the indicated antibodies. Immune complexes were detected by the Amersham ECL detection system (Amersham Life Sciences Inc., Arlington Heights, Ill.). We used the following antibodies: an affinity-purified rabbit polyclonal against an N-terminal peptide (amino acids 4 to 21) of human Bcl-2 (reactive with mouse, rat, and human Bcl-2) (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) and a rabbit polyclonal affinity-purified antibody directed against the pRb-binding domain of E2F-1 (18).

To detect p53 protein, we immunoprecipitated from 1 mg of total protein with a monoclonal antibody directed to mouse p53 (PAb 421) and immunoblotted with a sheep anti-p53 polyclonal antiserum (Ab-7; Oncogene Science, Cambridge, Mass.).

Drug treatment and cell viability. The effects of antineoplastic drugs on cell viability were assessed by using a wide range of concentrations that includes the levels used previously in vitro (10, 32). The following drugs were used: cisplatin [*cis*-platinum (II)-diammine dichloride] (Sigma, St. Louis, Mo.), etoposide (VePesid) at 100 mg/5 ml (Bristol Laboratories, Princeton, N.J.), 5-fluorouracil (5-FU) at 500 mg/10 ml (SoloPak Laboratories, Elk Grove Village, Ill.), ICRF-193 (Zenyaku Kogyo, Tokyo, Japan), paclitaxel (Taxol) (Sigma), and topotecan (Smith-Kline Laboratories). Protein overexpression from the glucocorticoid-inducible vector transfectants was achieved by adding dexamethasone (25 μM) to the cultures. Cells were seeded at 5×10^5 cells/ml to maintain the highest levels of viability in the absence of drug over the 24-h assay time. Cells were incubated at 37°C, for 24 h, unless otherwise noted. Cell viability was determined by trypan blue dye exclusion, and on the basis of morphology, cell death was always due to the induction of apoptosis. For the morphological studies, cytospins of untreated and etoposide-treated (1 μM, 16 h) cells (5×10^4) were prepared as previously described (18), fixed with absolute methanol for 5 min, stained for 45 min with Giemsa stain (Accustain; Sigma), and mounted by using Permount (Fisher).

The ts p53 mutant-containing cell lines were equilibrated at the permissive temperature of 32°C (p53 in the wild-type conformation) or the nonpermissive temperature of 39°C (p53 in the mutant conformation) for 18 h before the antineoplastic drugs were added.

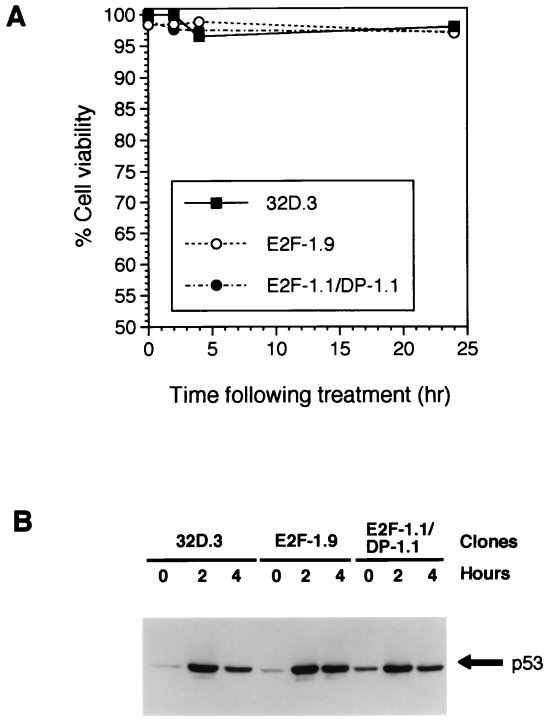


FIG. 1. Elevation of p53 levels by irradiation does not induce apoptosis in E2F-1-overexpressing myeloid progenitor cells. Myeloid progenitor cells (32D.3) and E2F-1-overexpressing clones maintained in IL-3-supplemented RPMI medium were irradiated with γ rays (5 Gy) and examined for cell viability (A) and p53 induction (B). Cell viability was measured by trypan blue dye exclusion. The p53 tumor suppressor was detected by immunoprecipitation-Western analysis of 0.5% Nonidet P-40 extracted cellular lysates. The antibodies PAb 421 and Ab-7, which both recognize mouse p53, were used in the immunoprecipitation and immunoblotting procedures, respectively. Immunocomplexes were isolated and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE under reducing conditions. Detection of p53 on the immunoblots was achieved by a chemiluminescence system (ECL; Amersham).

Cell cycle analyses. Drug-treated cells (10^6) were centrifuged and resuspended in 1 ml of propidium iodide staining solution (0.05 mg of propidium iodide per ml, 0.1% sodium citrate, 0.1% Triton X-100) (28). Immediately prior to flow cytometric analysis, each sample was treated at room temperature with DNase-free RNase (Calbiochem, San Diego, Calif.) at a final concentration of 5 μg/ml for 30 min. Samples were then filtered through a 40-μM-pore-size nylon mesh. Fluorescence (λ = 563 to 607 nm) emitted from propidium iodide-DNA complexes was measured from approximately 2×10^5 cells with a Becton Dickinson FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, Calif.). The ModFit computer program (Verity Software House, Topsham, Maine) was used to determine the percentage of cells within the G_1, S, and G_2-M phases of the cell cycle. Events with fluorescence levels below a relative DNA content of 1 (2N) were excluded from the display histograms by electronic gating.

RESULTS

Induction of p53 by ionizing radiation does not cooperate with E2F-1 to induce apoptosis. Previously, we have shown that overexpression of E2F-1 was tolerated in IL-3-dependent 32D.3 myeloid progenitor cells, but increased activity of E2F-1 by coexpression of its heterodimeric partner DP-1 overrode the strong survival signals of IL-3 and serum and induced apoptosis. Induction of E2F-1 activity also led to induction of p53 (18). To determine whether p53 accumulation cooperates with E2F-1 to trigger apoptosis in myeloid cells, E2F-1-overexpressing cell lines and appropriate control lines were irradiated with 5 Gy of γ rays. Cell viability and p53 protein expression were then determined (Fig. 1). We found that the level of basal p53 expression was higher in the E2F-1.1–DP-1.1 clone

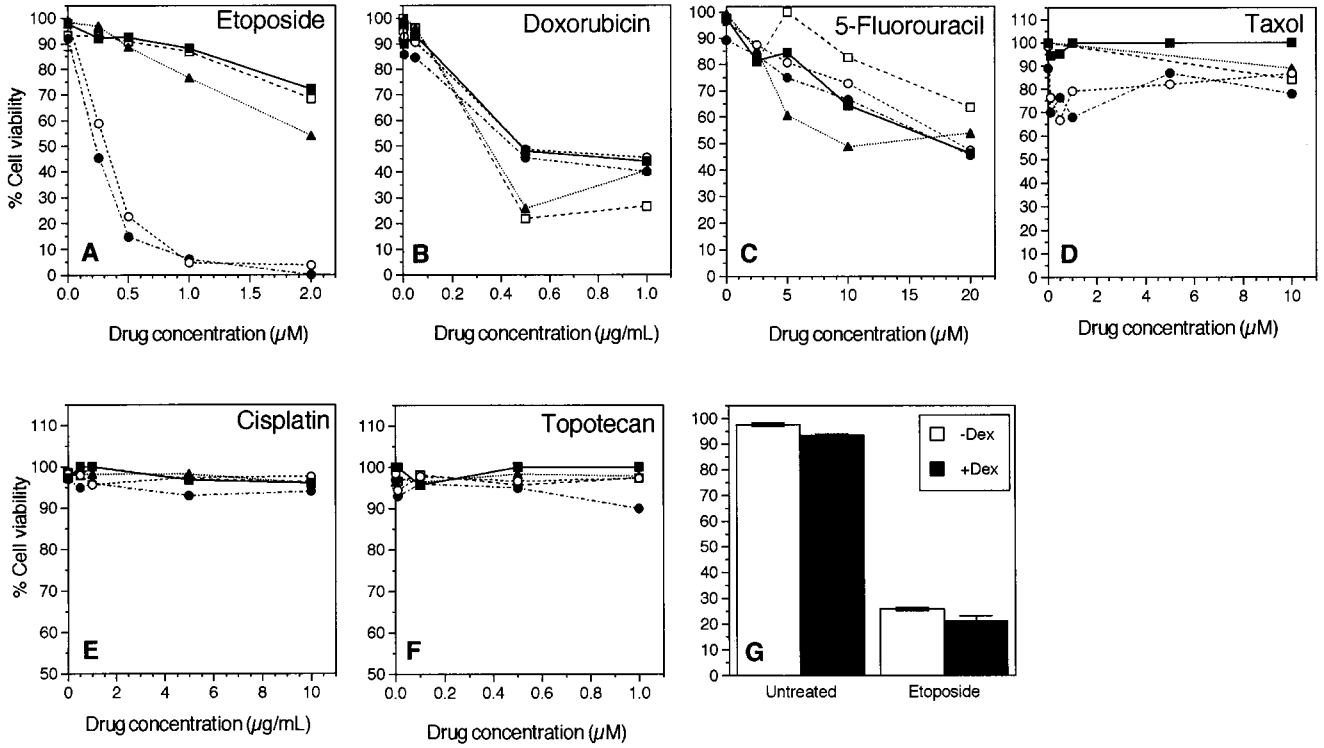


FIG. 2. Enforced expression of E2F-1 in 32D.3 cells preferentially sensitizes cells to etoposide-mediated apoptosis. Antineoplastic drugs at the concentrations indicated were added to 5×10^5 cells/ml in 24-well tissue culture plates. Protein expression from the glucocorticoid-inducible pMAM-Neo transfectants was achieved by the addition of 25 μM dexamethasone. After a 24-h incubation at 37°C, cell viability was assessed by trypan blue dye exclusion. A representative experiment of three is shown (A through F). The cell lines used were 32D.3 (■), E2F-1.9 (○), E2F-1.1–DP-1.1 (●), DP-1.6 (▲), and 138.2 (□). Cell viability of E2F-1.1–DP-1.1 cells following etoposide treatment (1 μM) for 24 h was measured as described above in the absence or presence of dexamethasone (25 μM) (G). A representative experiment is shown (means ± standard deviations [SD] of duplicate samples).

than in the parental 32D.3 cells, which confirms our previous finding that E2F-1 overexpression induces p53 protein (18). In all cell lines analyzed, p53 expression was maximally induced by 2 h postirradiation and began to decrease by 4 h (Fig. 1B). Despite the induction of high levels of p53 protein, indicative of DNA damage, the viability of all the cell lines remained greater than 95%.

Etoposide selectively sensitizes cells that overexpress E2F-1 to undergo p53-independent apoptosis. The p53 induction in response to a pulse of ionizing radiation is transient (Fig. 1B). To determine whether prolonged accumulation of p53 cooperated with E2F-1 to induce apoptosis, the viability of E2F-1-overexpressing cells was determined 24 h after the cells were treated with various concentrations of different classes of antineoplastic drugs used for treating myeloid leukemia (Fig. 2). Treatment with the topoisomerase II inhibitor etoposide resulted in a specific increase in the level of apoptotic cell death (up to 100% at 2 μM) in cells expressing E2F-1 or E2F-1 plus DP-1, compared to the parental 32D.3 cells, cells overexpressing DP-1, or cells overexpressing an E2F-1 mutant that does not bind DNA (E2F-138, Fig. 2A). Cell death was also observed in cells treated with doxorubicin, an anthracycline antibiotic-topoisomerase I and II inhibitor (Fig. 2B) and in cells treated with 5-FU, a pyrimidine analog (Fig. 2C). However, this loss in cell viability was not specific to E2F-1-overexpressing cells. Treatment with paclitaxel, which stabilizes microtubules (Fig. 2D); cisplatin, a DNA cross-linking agent (Fig. 2E); topotecan, a topoisomerase I inhibitor (Fig. 2F); or cytosine arabinonucleoside (Ara-C), a cytosine analog (data not shown) did not augment the levels of apoptosis in E2F-1-expressing cells versus control cells. The cytotoxic effects of etoposide were confirmed in long-term growth assays, where treatment of cells for 24 h with etoposide led to a complete loss of viability within 36 h for E2F-1-overexpressing cells. In contrast, topotecan treatment only transiently blocked cell growth and did not cause a loss in viability, and the cells eventually began to proliferate (data not shown). The cooperation between E2F-1 and etoposide was evident at both moderate and high levels of E2F-1 plus DP-1, as similar results were obtained in the presence or absence of dexamethasone (Fig. 2G).

Enforced E2F-1 expression can promote continuous S-phase entry in the absence of mitogens (9, 18, 22). To determine whether these drugs had an effect on E2F-1-induced cell cycle progression, we analyzed the cell cycle distribution of the cell lines following drug treatment (Fig. 3A). Treatment with etoposide caused some control 32D.3 cells to accumulate in S phase (54.4%); however, a far greater percentage of E2F-1 and E2F-1–DP-1 overexpressing cells accumulated at this stage of the cell cycle (80.5 and 80.4%, respectively). Etoposide also caused an accumulation of cells in G_2-M. Given the large number of cells undergoing apoptosis (Fig. 2A), the S-phase peak may be due to DNA fragmentation of cells that have reached G_2-M. E2F-1 overexpression moderately reduced the number of cells in G_1 after etoposide treatment. Doxorubicin also caused an accumulation of cells in G_2-M, while enforced E2F activity increased the number of cells in S phase and decreased the proportion of cells in G_1. Exposure of cells to paclitaxel induced a striking G_2-M block that was unaffected by E2F-1 expression. Although enforced expression of E2F-1 caused significant S-phase accumulation of cells treated with

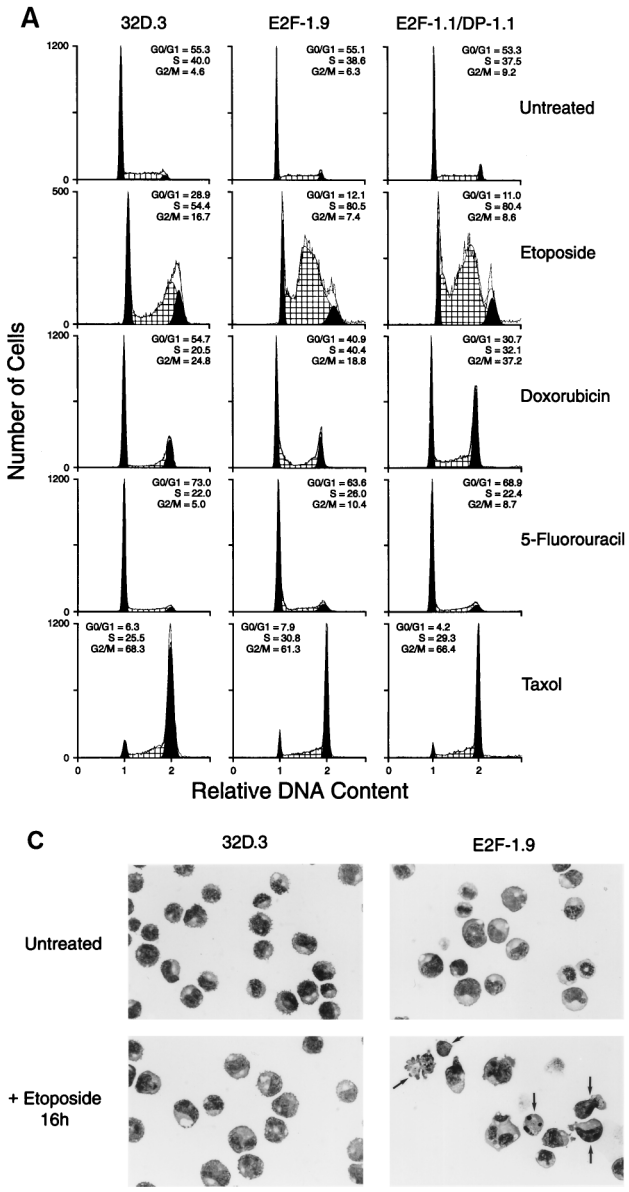


FIG. 3. Antineoplastic drug-induced cell cycle arrest and p53 induction do not enhance E2F-1-induced apoptosis. Cells treated with chemotherapeutic drugs were analyzed for cell cycle kinetics (A) and p53 induction (B). For the cell cycle analyses, cells (at 0.5×10^6 cells/ml) were treated for 24 h with either 1 μM etoposide, 0.035 μg of doxorubicin per ml, 10 μM 5-FU, or 5 μM paclitaxel. Cells (10^6) were then centrifuged and stained with propidium iodide. The stained DNA from approximately 2×10^5 cells was quantitated with a FACScan flow cytometer (Becton Dickinson). The percentages of cells in G_1, S, and G_2-M phases of the cell cycle were determined by the ModFit program (Verity Software House). From the same experiment, samples of drug-treated cells were analyzed for p53 induction by immunoprecipitation-Western blotting, as described in the legend to Fig. 1. Cytospin preparations of parental 32D.3 (transfected with pMAM-Neo vector alone [Neo]) and E2F-1.9 cells were cultured in the absence (C, top row) or presence (C, bottom row) of etoposide (1 μM) for 16 h and stained with Giemsa stain. Apoptotic cells (arrows) were assessed by light microscopy. Drug-treated cells, but not untreated control cells, showed characteristic signs of apoptosis. Magnification, ×40.

etoposide or doxorubicin, cells treated with the antimetabolite 5-FU, which inhibits the function of the transcriptional targets of E2F-1, arrested in G_1. Thus, E2F-1 overexpression affected the cell cycle profiles of only those cells treated with etoposide or doxorubicin (Fig. 2 and 3A). This phenotype correlated with inhibition of topoisomerase II but not with the E2F-1-specific induction of cell death.

To determine whether p53 status correlated with etoposide-induced apoptosis or cell cycle arrest, p53 levels were determined by immunoblot analysis. In these myeloid cells, all of the antineoplastic drugs induced p53 to high levels, although the kinetics of induction varied (Fig. 3B). The decrease in p53 levels observed at later time points in the etoposide-treated E2F-1-expressing cell lines is likely due to the significant cell death. Importantly, drugs such as cisplatin, which induces DNA damage, were not toxic to the E2F-1-expressing cells, suggesting that p53 induction alone does not promote E2F-1-induced apoptosis. Etoposide-treated E2F-1.9 cells showed morphologies characteristic of apoptotic cells, including formation of cells with micronuclei (Fig. 3C).

The relevance of p53 as a mediator of apoptosis in E2F-1-overexpressing cells that had been exposed to etoposide (1 μM) was directly tested by using cell lines that overexpress both E2F-1 and a ts p53 mutant (Val 135). The ts p53 protein is in the wild-type conformation at 32°C and in the mutant conformation at 39°C, where it functions in a transdominant-negative manner. The mutant p53 was expressed from the murine sarcoma virus LTR promoter at over 50 times the endogenous levels of wild-type p53 (data not shown). At 32°C, there was no significant enhancement of apoptosis in response to etoposide, paclitaxel (5 μM), or topotecan (1 μM) (Fig. 4, top panel). At 39°C, etoposide accelerated the rate of apoptosis of 32D.3 cells as well as that of cells overexpressing E2F-1 or E2F-1 plus transdominant-negative mutant Val 135 p53. Thus, etoposide-induced apoptosis in cells overexpressing E2F-1 is p53 independent.

Bcl-2 overexpression rescues etoposide-induced apoptosis in E2F-1-overexpressing cells. While E2F-1 overexpression induces p53 (18), it reduces levels of the apoptosis inhibitor Bcl-2 by fivefold. Cells overexpressing E2F-1 plus DP-1 express even lower levels of Bcl-2 (5) (compare lanes marked "0 hr" in Fig. 5C).

To determine the significance of this reduced Bcl-2 expression on etoposide-induced apoptosis, we generated clones which overexpressed both E2F-1 and Bcl-2. Bcl-2 and E2F-1 protein levels were determined by Western blot analysis (Fig. 5A). All clones (Fig. 5A, upper panel, lanes 3 to 6) overexpressed Bcl-2 to the same level as that expressed by a clone of SFFV–Bcl-2-transfected 32D.3 cells (Bcl-2.4, Fig. 5A, upper

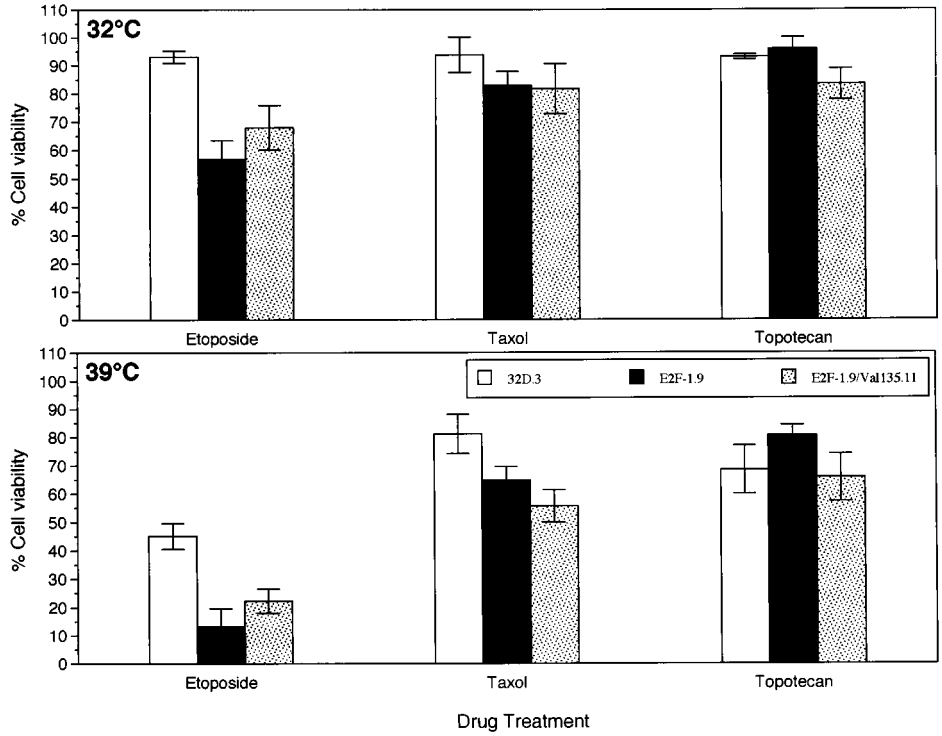


FIG. 4. Enforced expression of wild-type or transdominant negative $p53^{tsval135}$ does not modulate E2F-1–etoposide-induced apoptosis. E2F-1-overexpressing cells transfected with a ts p53 mutant (Val 135) were used to determine the importance of p53 in mediating drug-induced apoptotic cell death. At the permissive temperature of 32°C, p53 is in its wild-type conformation, whereas at 39°C it is in a mutant conformation. Drug treatment was carried out at 32°C (top panel) and 39°C (bottom panel), and cell viability was determined 24 h posttreatment. Cells were equilibrated at either 32 or 39°C overnight before the drugs were added (etoposide [1 μM], paclitaxel [5 μM], or topotecan [1 μM]). The results shown are means ± SD of triplicate experiments.

panel, lane 2). This level of Bcl-2 expression was significantly higher than that of the parental 32D.3 cells (Fig. 5A, upper panel, lane 1). Figure 5A also shows that E2F-1 was overexpressed in the presence or absence of dexamethasone in these cells (Fig. 5A, lower panel, lanes 1 to 8). These levels of E2F-1 were comparable to those of the E2F-1-overexpressing cell lines (data not shown).

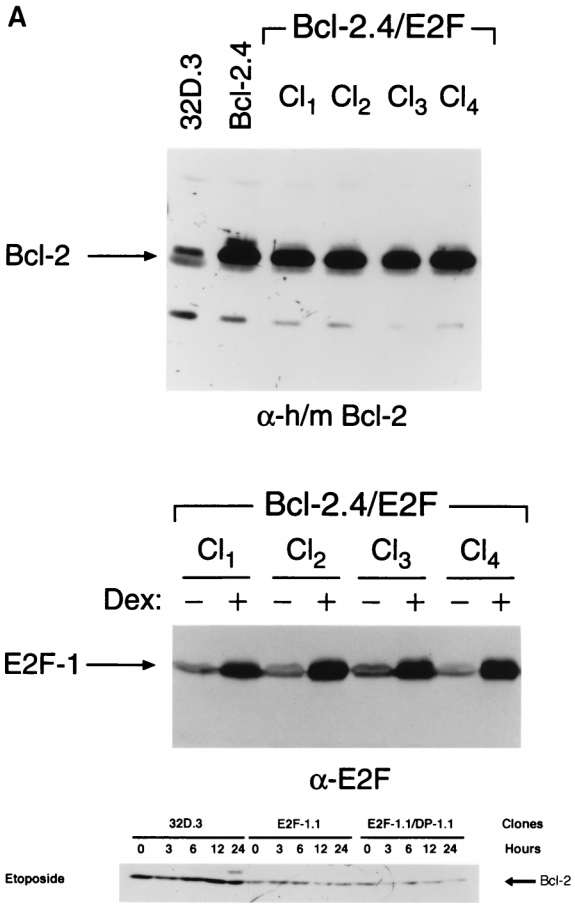
Following treatment with etoposide, the E2F-1-overexpressing cells showed a marked decrease in viability, as expected (see also Fig. 2). However, overexpression of Bcl-2 in these cells (Bcl-2.4–E2F-1.1) protected them from etoposide-induced apoptosis (Fig. 5B). Similar results were obtained whether E2F-1 was introduced into a Bcl-2-overexpressing clone or Bcl-2 was introduced into an E2F-1-overexpressing cell line (data not shown). Bcl-2 coexpression also rescued the E2F-1 cells from nonspecific apoptosis mediated by other chemotherapeutic agents, such as 5-FU and doxorubicin (data not shown). We also examined the Bcl-2 protein level in the E2F-1-expressing cells at several time points following exposure to etoposide (Fig. 5C) and found that Bcl-2 was not reduced following drug treatment in any of the cell lines tested. Thus, reduced Bcl-2 levels appear to lead to a general (not etoposide-specific) sensitivity to apoptosis in E2F-1-overexpressing cells.

Topoisomerase II is a critical target for augmenting E2F-1-induced apoptosis. Because antineoplastic drugs often have pleiotropic cellular effects, we used a second topoisomerase II inhibitor to determine the role of this enzyme in E2F-1-induced apoptosis. The bisdioxopiperazine, ICRF-193, traps topoisomerase II in a closed clamp conformation such that it is unable to bind DNA and is thus in a configuration inaccessible to etoposide (40, 41). To test whether etoposide acts solely through topoisomerase II to selectively induce cell death in E2F-1-expressing cells, we pretreated E2F-1-overexpressing cells with 6 μM ICRF-193 for 6 h before adding 1 μM etoposide (Fig. 6). ICRF-193 protected the E2F-1-expressing cells from etoposide-mediated programmed cell death.

Etoposide not only inhibits topoisomerase II but also stabilizes DNA strand breaks. Therefore, we asked whether the combination of topoisomerase II inhibition and DNA damage could selectively induce apoptosis in E2F-1-expressing cells. Cells were treated with DNA-damaging agents (Ara-C [10 μM] or cisplatin [5 μg/ml]) alone or together with ICRF-193. Treatment of E2F-1-overexpressing cells with 6 μM ICRF-193 (a dose that maximally inhibits mammalian cell topoisomerase II activity) (20) caused a reduction in the numbers of cells in S phase, an accumulation of cells in G_2-M (data not shown), and a slight decrease in viability. However, the combination of ICRF-193 and DNA damage significantly decreased the viability of E2F-1 (Fig. 7) and E2F-1-plus-DP-1 overexpressing cells (data not shown) compared to cells singly treated with only ICRF-193, Ara-C, or cisplatin. Thus, the combined effects of topoisomerase II inhibition and DNA damage selectively potentiate E2F-1-mediated apoptosis.

DISCUSSION

Enforced E2F-1 expression in 32D.3 cells leads to an accumulation of modest levels of p53 (18). In fibroblasts, induction of high levels of p53 by DNA-damaging agents induced apoptosis, and overexpressing dominant negative forms of p53 prevented E2F-1-mediated apoptosis (30, 39, 53). However, in



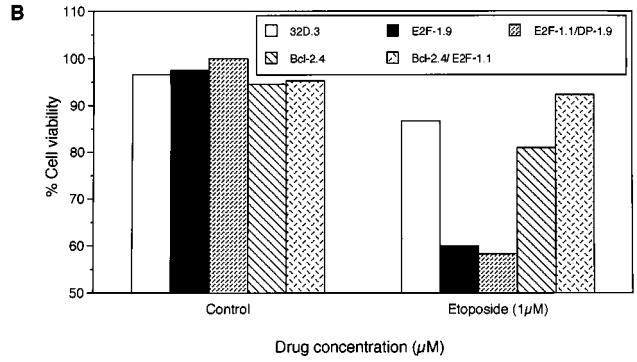


FIG. 5. Enforced expression of Bcl-2 protects against apoptosis in E2F-1-overexpressing cells. 32D.3 cells transfected with the SFFV LTR expression vector containing human Bcl-2 cDNA were transfected with the pMAM-Neo E2F-1 expression vector, and four clones were selected for further study. (A) Western blotting analysis reveals the Bcl-2 (upper panel) and E2F-1 (lower panel) protein levels in the transfectants. E2F-1 levels were measured in the presence (+) and absence (−) of dexamethasone. (B) Cell viability after 24-h exposure to etoposide (1 μM) was determined by trypan blue exclusion. A representative experiment of three independent experiments is shown. Cell lines used are indicated in the inset. (C) 32D.3 cells and E2F-1 overexpressors were treated with 1 μM etoposide and their Bcl-2 levels were analyzed at various times following treatment by Western blotting. Total protein at 50 μg per lane was loaded and run out under reducing conditions on SDS–10% PAGE gels.

diploid, IL-3-dependent 32D.3 myeloid progenitor cells, induction of high levels of p53 by ionizing radiation or by different chemotherapeutic drugs failed to potentiate E2F-1-induced apoptosis. Furthermore, overexpression of wild-type p53 with E2F-1 did not enhance apoptosis mediated by etoposide or by the other drugs tested, and expression of a dominant inhibitory mutant of p53 did not protect the cells from death, suggesting that p53 does not trigger apoptosis of these cells (Fig. 4). Interestingly, in most solid tumors, pRb mutations are accompanied by mutations in p53 (42, 52). However, while p16 and pRb mutations are found in acute myelogenous leukemia, p53 is rarely mutated (38).

The enhanced sensitivity of the E2F-1-expressing cells to etoposide may be the result of molecular events during late S phase. Topoisomerase IIα is cell cycle regulated, with levels peaking in late S phase, whereas topoisomerase IIβ predominates in quiescent cells (47). In fibroblasts, at late S phase, cyclin A-kinase phosphorylates DP-1, the heterodimeric partner of E2F-1, and suppresses the DNA binding activity of E2F-1, which allows the cells to complete S phase (26, 31, 55). Mutations in E2F-1 that block cyclin A binding trigger an S-phase cell cycle checkpoint that leads to cell cycle arrest, apoptosis, and sensitivity to DNA-damaging agents (27, 30). In an analogous manner, topoisomerase II inhibition coupled with DNA damage and unregulated E2F-1 may also trigger this S-phase checkpoint to induce p53-independent apoptosis.

$p53^{-/-}$ murine lymphoma cells undergo apoptosis following treatment with anticancer drugs or γ irradiation (48). However, these cells are protected from programmed cell death by the Bcl-2 oncoprotein, a known inhibitor of apoptosis (49). The ratio of Bcl-2 to heterodimeric partners, such as Bax, regulates apoptosis (36). Bcl-2 protein levels are significantly reduced in the 32D.3 myeloid cells overexpressing E2F-1 or E2F-1 plus DP-1 (5), and Bcl-2 overexpression rescues cells overexpressing E2F-1 from etoposide (Fig. 5C). However, etoposide did not further reduce Bcl-2 levels (Fig. 5), indicating that Bcl-2 is not a mediator of the specific killing by etoposide. However, the reduced levels of Bcl-2 likely contribute to E2F-1-induced apoptosis seen in the absence of IL-3 or apoptosis induced by E2F-1-plus-DP-1 expression in the presence of IL-3 (18).

ICRF-193 is a bisdioxopiperazine compound that binds to topoisomerase II in its closed protein clamp configuration,

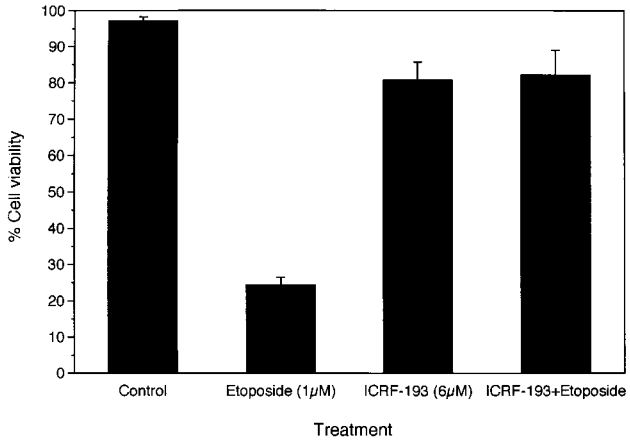


FIG. 6. Preincubation with ICRF-193 abrogates apoptotic effect of etoposide on E2F-1-overexpressing cells. E2F-1.1 cells (5×10^5 cells/ml) were incubated with medium alone (Control), with etoposide (1 μM), with ICRF-193 (6 μM), or with ICRF-193 and etoposide. Cells treated with both agents were given ICRF-193 first, followed 6 h later by etoposide (1 μM). ICRF-193 or etoposide as single-agent treatments were given at the same time as the etoposide was added to the double-agent wells. Cell viability was assessed by trypan blue exclusion 24 h later. Each bar represents the mean ± SD of duplicate experiments.

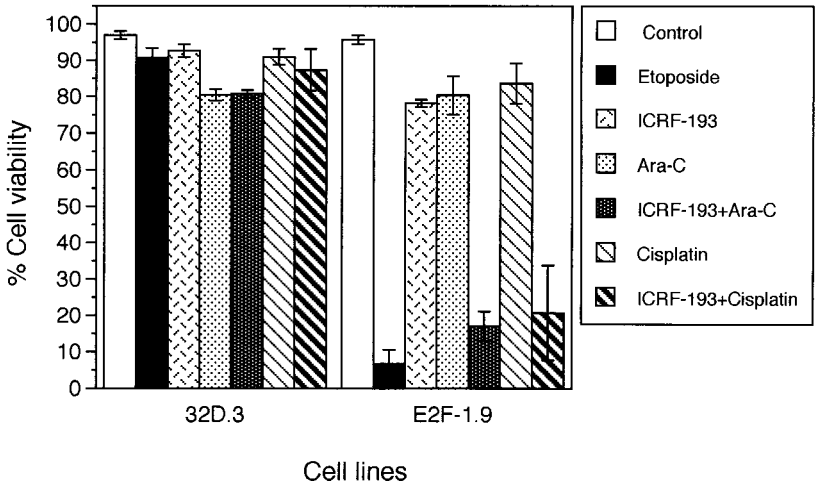


FIG. 7. Topoisomerase II inhibition and DNA damage synergize to enhance apoptosis in E2F-1-overexpressing cells. Cell lines were treated either with 6 μM ICRF-193 alone or with ICRF-193 followed 6 h later by DNA-damaging drugs (Ara-C [10 μM] or cisplatin [5 μg/ml]). After 24 h, cell viability was determined by trypan blue dye exclusion. The results of treatment with etoposide (1 μM), Ara-C, or cisplatin alone are also shown for comparison. Control levels represent the viability of cells not exposed to ICRF-193 or DNA-damaging drugs. The results shown are means ± SD of duplicate experiments.

which renders it incapable of cleaving DNA (40, 41). Pretreatment of E2F-1-overexpressing cells with ICRF-193 protected them from etoposide, thus pinpointing topoisomerase II as the only relevant target of etoposide. ICRF-193 alone induced cell cycle arrest but was not sufficient to enhance apoptosis of the E2F-1 clones to the levels seen with etoposide. On the other hand, addition of the DNA-damaging agent Ara-C or cisplatin, following ICRF-193 pretreatment, augmented apoptosis in the E2F-1- and E2F-1-plus-DP-1-expressing cells relative to the same cells treated with ICRF-193, Ara-C, or cisplatin alone (Fig. 7). Thus, as with etoposide, topoisomerase II inhibition together with DNA damage is needed to augment E2F-1-induced apoptosis.

Mutations in the pRb pathway are a hallmark of cancer. Enforced E2F-1 expression in myeloid progenitor cells confers preferential sensitivity to p53-independent apoptosis mediated by the chemotherapeutic agent etoposide. Etoposide inhibits topoisomerase II and is used to treat acute lymphoblastic leukemia and a variety of solid tumors, including ovarian, small-cell lung, and testicular cancers (13). Based on our data, we speculate that for a subset of tumors, ICRF-193 pretreatment could sensitize tumor cells to DNA-damaging agents.

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