Adenovirus-mediated E2F-1 Gene Transfer Sensitizes Melanoma Cells to Apoptosis Induced by Topoisomerase II Inhibitors

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ABSTRACT

Melanoma has proven to be resistant to conventional chemotherapy; however, the mechanism of chemoresistance is still unclear. Recent reports show that the transcription factor, E2F-1, may play a role in mediating cytotoxicity of certain chemotherapeutic agents. We have shown in a previous study that adenovirus-mediated overexpression of E2F-1 can efficiently induce apoptosis in melanoma cells. In the present study, the effect of E2F-1 expression on drug sensitivity of melanoma cells was evaluated. Two human melanoma cell lines, SK-MEL-28 and SK-MEL-2, were treated with drugs (etoposide, Adriamycin, roscovitine, cisplatin, 5-fluorouracil, or cycloheximide), alone or in combination with adenoviral vectors expressing β-galactosidase (Ad-LacZ) or E2F-1 (Ad-E2F-1) at a multiplicity of infection of 1 in vitro. E2F-1 expression was confirmed by Western blot analysis. Sublethal concentrations of each drug alone or infection with Ad-E2F-1 alone produced <5% apoptosis by 3 days posttreatment. Conversely, cotreatment with Ad-E2F-1 and low concentrations of etoposide or Adriamycin markedly sensitized melanoma cells to apoptotic cell death. A slight enhancement of the cytotoxicity of roscovitine was demonstrated in combination with E2F-1 overexpression, but not to cisplatin, 5-fluorouracil, or cycloheximide. Ad-LacZ infection showed no obvious effects on drug sensitivity. Overexpression of p21 can block apoptosis induced by the combination chemogene therapy of Ad-E2F-1 and topoisomerase II poisons and does not require its proliferating cell nuclear antigen-binding ability. The protein synthesis inhibitor cycloheximide also has a cytotoxicity-protective effect against topoisomerase II inhibitor/E2F-1-induced apoptosis and suggests that new protein synthesis is required for this process. Topoisomerase II inhibitors also cooperated with Ad-E2F-1 to enhance antitumor activity in an in vivo model using xenografts in nude mice. When combined with Adriamycin or etoposide, E2F-1 adenovirus therapy resulted in an 87% or 91% decrease in tumor size, respectively, compared with controls (P < 0.002). Our results show that adenovirus-mediated E2F-1 gene transfer can sensitize melanoma cells to some chemotherapeutic agents, particularly topoisomerase II poisons, in vitro and in vivo. These results suggest a new chemosensitization strategy for melanoma gene therapy.

INTRODUCTION

Human malignant melanoma is a major medical problem, characterized by both rapidly rising incidence and growing lifetime risk. The present lifetime risk for Americans of developing malignant melanoma is ~1 in 87. The rapid increase in the incidence of melanoma has been paralleled by expanded research efforts to improve the outcome for patients with this disease (1, 2). Because of the limited success of surgical therapy in treating metastatic melanoma, the search for effective systemic therapy for melanoma has generated intense interest over the years. Single and combination chemotherapy regimens have yielded disappointing results (2, 3). The resistance of melanoma to multi-chemotherapeutic agent therapies in vivo and in vitro suggests the presence of intrinsic cellular resistant mechanisms. To date, cellular resistant mechanisms involved in melanoma chemoresistance remain ambiguous. The search for the mechanism of melanoma chemoresistance and treatments to overcome this resistance is the key to creating an effective chemotherapy strategy.

Recently, induction of the tumor suppressor E2F-1 in tumor cells was shown to correlate with chemosensitivity to topoisomerase II inhibitors, whereas loss of E2F-1 expression contributed to resistance to these chemotherapeutic agents (4). These data implicate E2F-1 in the mediation of cytotoxicity of some drugs in tumor cells. Additional studies report that E2F-1 may play a role in mediating apoptosis after exposure to DNA damage induced by chemotherapeutic agents and ionizing radiation (4–7). E2F-1 was first identified as a transcription factor that regulates a series of gene expression that promotes cell cycle transition from G1 to S-phase (8, 9). Early studies with E2F-1 suggested that this protein can function as an oncogene based on its ability to stimulate cell proliferation when overexpressed (10–12), transform rat embryonic fibroblasts in cooperation with the Ras oncogene, and promote tumorigenesis in mice (13, 14). Somewhat paradoxically, later studies showed that E2F-1 also has properties of a tumor suppressor. Homozygous E2F-1-null (knock-out) mice demonstrated increased cell proliferation and tumor formation in several tissues (15, 16), and overexpression of E2F-1 has been shown to induce apoptosis in many tumor cells (17–21). Therefore, E2F-1 appears to be involved in both cell proliferation and mediation of programmed cell death.

Our previous studies show that adenovirus-mediated E2F-1 gene transfer efficiently induces apoptosis in human melanoma cells in a dose-dependent manner (19). In this study, the effect of the induction of exogenous E2F-1 expression by adenovirus-mediated gene transfer on the chemosensitivity of melanoma was evaluated. Our results indicate that adenovirus-mediated E2F-1 gene transfer can markedly sensitize melanoma cells to some chemotherapeutic agents, particularly the topoisomerase II inhibitors. Topoisomerase II inhibitors also cooperated with Ad-E2F-1 to enhance antitumor activity in an in vivo nude mouse model. These results suggest a new chemosensitization strategy for melanoma gene therapy.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human melanoma cell lines SK-MEL-28 (wild-type p53), SK-MEL-2 (mutant p53), and A2058 were purchased from American Type Culture Collection (Rockville, MD). The SK-MEL-2 and SK-MEL-28 cell lines were cultured in α-MEM, and the A2058 cell line was cultured in DMEM. The human colon cancer cell line RKO was purchased from American Type Culture Collection and cultured in DMEM. All media were supplemented with 10% heat-inactivated FBS, and penicillin/streptomycin (100 units/ml). All cell culture reagents were obtained from Life Technologies, Inc. (Bethesda, MD). Cells were cultured in a 5% CO2 incubator at 37°C, and the medium was changed every 3 days.

The abbreviations used are: FBS, fetal bovine serum; CMV, cytomegalovirus; PCNA, proliferating cell nuclear antigen; 5-FU, 5-fluorouracil; MOI, multiplicity of infection; mAb, monoclonal antibody; CPP32, caspase-3; PARP, poly(ADP-ribose) polymerase; FACS, fluorescence-activated cell sorting; pflu, plaque-forming units; Rb, retinoblastoma protein.
Adenoviral Vectors. Four replication-defective recombinant adenoviral vectors were used. Ad5CMV-E2F-1 vector has the E1 subunit deleted and contains the transgene E2F-1 under the control of the CMV promoter (17). Ad5CMV-WAF1 (generously provided by Dr. Wafik S. El-Deiry, University of Pennsylvania, Philadelphia, PA) contains the transgene full-length p21WAF1/CIP1 under the control of the CMV promoter. Ad5CMV-WAF1/H11002 (generously provided by Dr. Wafik S. El-Deiry, University of Pennsylvania, Philadelphia, PA) contains the COOH-terminal deletion mutant of p21WAF1/CIP1 under the control of the CMV promoter, which is defective in its ability to bind PCNA (22). Ad5CMV-LacZ (generously provided by Dr. Brent French, University of Virginia, Charlottesville, VA) was used as a control vector that expresses nuclear-localized β-galactosidase under control of the same promoter (23). All vectors were propagated in the 293 cell line, and titers were determined by standard plaque assays (24).

Chemotherapeutic Agents. The following chemotherapeutic agents were used. Cisplatin (cis-platinum(DI)-diammine dichloride), etoposide (Ve-Pesid), Adriamycin (doxorubicin), 5-FU, and cycloheximide [3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide] were purchased from Sigma Chemical Co. (St. Louis, MO), Roscovitine [2-[1-(D)-hydroxymethyl-1-propylamino]-6-benzamino-9-isopropylpurine] was purchased from Calbiochem (La Jolla, CA), Cisplatin, 5-FU, and cycloheximide were prepared fresh in 0.9% saline for each experiment. Adriamycin was dissolved at 5 mM in H2O and stored at 20°C. Etoposide was dissolved at 100 mM in DMSO and stored at 20°C. Roscovitine was dissolved at 10 mM in DMSO and stored at 20°C. All drugs were diluted in culture medium before addition to the cell cultures.

Adenovirus Infections and Combined Treatments. For infections, 1 × 105 cells were plated in 10-cm tissue culture plates. The following day, the medium was reduced, and the adenoviral vectors were added in 1 ml of α-MEM at a MOI of 1 for Ad-E2F-1 and Ad-LacZ and a MOI of 10 for Ad-WAF1 and Ad-WAF1/H11002. Mock infection was performed by treatment of cells with vehicle (medium) only. After 1 h of incubation at 37°C, the medium was removed, and 10 ml of fresh α-MEM-5% FBS was added. For the combined treatments, cells were infected with viruses as above, 1 h later, the medium was replaced with 10 ml of fresh α-MEM-5% FBS containing chemotherapeutic agents at the appropriate concentrations. Cells were then cultured at 37°C and harvested after 3 days for analysis.

Western Blot Analysis. Cells were harvested 3 days after treatments, and cell lysates were prepared as described previously (19). Equal amounts of cellular protein (50 μg) were electrophoresed in SDS-polyacrylamide gels and transferred to a hydrophilic polyvinylidene difluoride (PVDF) membrane (Amersham, Arlington Heights, IL). The membrane was first incubated with three primary antibodies, mouse anti-E2F-1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA), mouse antihuman CIP2 p32 mAb (Transduction Lab, Lexington, KY), mouse antihuman p21 and antihuman PARP mAb (Calbiochem, Oncogene Research Products, Cambridge, MA), and then with antirabbit and antimouse immunoglobulin, peroxidase-linked, species-specific whole antibody (Amersham Research Products, Cambridge, MA). The membrane was first incubated with nonimmune rabbit IgG and then with antimouse immunoglobulin, peroxidase-linked, species-specific whole antibody (Amersham). The membrane was then incubated with horseradish peroxidase-conjugated antirabbit or antimouse IgG (Amersham). Chemiluminescence reagents were used to detect the signals according to the manufacturer’s instructions (Amersham).

Single and Combination Therapy Dose-Response Assays. The cytotoxicity of drugs was determined by measuring the conversion of the tetrazolium salt WST-1 to formazan according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). Briefly, cells were plated into 24-well plates in triplicate wells (2 × 104 cells/well) and infected with adenoviral vectors (Ad-E2F-1 or Ad-LacZ) at a MOI of 1 or treated with mock infection. One h later, cells were exposed to Adriamycin, etoposide, 5-FU, cycloheximide, cisplatin, or roscovitine at various concentrations. 5-FU-treated cells were cultured in α-MEM supplemented with 10% dialyzed FBS. After 2 days of incubation at 37°C, WST-1 was added to each well and incubated at 37°C for 3 h. The number of viable cells was assessed by measuring the conversion of the tetrazolium salt WST-1 to formazan through measurement of absorbance at 415 nm. Result are expressed as the percentage of the absorbance of control (untreated) cells.

Cell Viability Assays. Cells were plated in 10-cm plates and treated as above. Cell morphology was observed by standard light microscopy. Cell viability was determined at day 3 by trypan blue exclusion. Cells were stained with trypan blue (final concentration, 0.2%) for 5 min and counted using a hemocytometer.

Cell Cycle Analysis and Apoptosis Assays. Adherent and nonadherent cells were harvested, washed once with PBS, and fixed in 70% (v/v) ethanol at −20°C for up to 1 week. Cells were pelleted, washed once with PBS, and resuspended in propidium iodide solution [50 μg/ml propidium iodide and 0.5 mg/ml RNase in PBS (pH 7.4)] for 30 min in the dark. FACS analysis was performed at 3 days after treatments. Flow cytometric analysis was performed on a FACSscan Flow Cytometer (Becton Dickinson, San Jose, CA). The data from 10,000 cells were collected and analyzed using Cell Quest Cell Cycle Analysis Software. The subdiploid population was calculated as an estimate of the apoptotic cell population. Several methods were used to confirm apoptotic cell death, including cellular morphology, the in situ terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay, and the PARP cleavage assay, and were performed as described previously (19).

In Vivo Combined Treatment of Transplanted Human Melanoma Cells. To evaluate the ability of Ad5CMV-E2F-1 plus systemic etoposide or Adriamycin to suppress tumor growth in vivo, tumors were formed by injecting 5 × 106 A2058 cells into athymic BALB/c nu/nu male mice (6–8 weeks of age; Charles River Laboratories, Wilmington, MA). The A2058 human melanoma cell line was used for animal experiments because SK-MEL-28 and SK-MEL-2 cells do not form tumors well in nude mice. Anesthesia was induced by i.p. injection of ketamine (37.5 mg/kg) and xylazine (5 mg/kg). The cells were injected s.c. into the bilateral flanks of the mice. Six days later, palpable tumors were randomized to be directly injected with Ad5CMV-E2F-1 (1 × 105 pfu), Ad5CMV-LacZ (1 × 105 pfu), or control (0.9% saline); s.c. injections were performed every 3 days, with a total of four treatments. Each injection of purified virus was diluted in total volume of 100 μl of 0.9% saline and administered in a single pass of a 27-gauge hypodermic needle using gentle, constant infusion pressure. Beginning on day 7, mice were treated with etoposide (1.5 mg/kg), Adriamycin (1 mg/kg), or 0.9% normal saline via the i.p. route once every 3 days, for a total of four treatments. Tumors were measured every 3 days, and tumor size was calculated using the following formula: length (mm) × width (mm)/2. Animal survival was closely monitored, and no signs of systemic toxicity were observed in animals receiving the recombinant adenoviruses followed by the treatment with topoisomerase II inhibitors. Animal experiments were performed in accordance with institutional guidelines and approved by the University of Louisville Institutional Animal Care and Use Committee.

Statistical Analysis. Statistical analysis was performed by Student’s t test. P < 0.05 was considered statistically significant.

RESULTS

Resistance of Melanoma Cell Lines to Anticancer Agent-induced Apoptosis. Defects in the apoptotic machinery are often associated with chemoresistance (25). In this study, we wanted to first evaluate in vitro chemosensitivity of human melanoma cell lines to a panel of chemotherapeutic agents. Two human melanoma cell lines (SK-MEL-28 and SK-MEL-2) were treated with cisplatin, Adriamycin, etoposide, 5-FU, cycloheximide, or roscovitine. Cytotoxicity was evaluated by WST-1 assay. As shown in Fig. 1, both cell lines responded to the drugs in a dose-dependent manner. Although Adriamycin and etoposide can inhibit cell growth by causing G2 arrest, apoptosis was not effectively induced in SK-MEL-28 and SK-MEL-2 cell lines treated with these agents (Fig. 2). Both cell lines were extremely resistant to 5-FU and cycloheximide; no cytotoxicity was observed even at very high doses of either drug (>1 mM for 5-FU and 4 μg/ml for cycloheximide). Cell cycle profiles of both cell lines were unaffected by treatment with 5-FU, cisplatin, or cycloheximide (data not shown). Roscovitine slightly delayed SK-MEL-28 cells in G2 phase and induced apoptosis in SK-MEL-2 at doses of ≥30 μM (data not shown).

Adenovirus-mediated E2F-1 Gene Transfer Sensitizes Melanoma Cell Lines to Adriamycin, Etoposide, and Roscovitine, but not to Cisplatin, 5-FU, and Cycloheximide. Recent reports showed that endogenous E2F-1 induction correlates with tumor cell sensitivity to some DNA-damaging agents (4–7). Our Western blot results showed that endogenous E2F-1 was not elevated in response to drug treatments in either melanoma cell line (Fig. 3). We wanted to investigate whether increased levels of exogenous E2F-1 in melanoma cells could enhance their sensitivity to chemotherapeutic drugs. Our previous data showed that Ad-E2F-1 infection alone efficiently induced apoptosis in both cell lines in a dose-dependent manner. In this
study, we first determined the optimal concentration of Ad-E2F-1 to use that would allow significant levels of exogenous E2F-1 protein without inducing apoptosis. As shown in Fig. 4, at a MOI of 1, Ad-E2F-1 infection causes a marked increase in E2F-1 protein in both cell lines, but no significant toxicity was detected at this concentration. Therefore, the following combination treatments were performed at an Ad-E2F-1 virus concentration that gave a MOI of 1.

Melanoma cells were infected with adenovirus vectors (Ad-E2F-1 or Ad-LacZ), and then treated with a panel of drug treatments as above. As shown in Fig. 5, adenovirus-mediated E2F-1 gene transfer resulted in marked sensitization of melanoma cells to low concentrations of etoposide and Adriamycin. The IC50 of etoposide was reduced 25- and 12-fold for SK-MEL-28 and SK-MEL-2 cells, respectively, infected with Ad-E2F-1. In addition, the IC50 of Adriamycin was reduced 3-fold in SK-MEL-28 and 10-fold in SK-MEL-2 after Ad-E2F-1 infection. The differences between Ad-E2F-1/topoisomerase II inhibitor treatment and topoisomerase II inhibitors alone or LacZ/topoisomerase II inhibitor treatments was statistically significant (P < 0.02). Adenovirus-mediated E2F-1 gene transfer slightly increased the sensitivity of both melanoma cell lines to roscovitine, but the increase was not statistically significant (P > 0.3). No such effect was observed with 5-FU, cycloheximide, or cisplatin (data not shown). Ad-LacZ infection did not significantly affect drug sensitivity (P > 0.4).

E2F-1 Enhances Chemosensitivity by Synergistic Effects on Apoptotic Cell Death. To investigate the mechanism of increased chemosensitivity by adenovirus-mediated E2F-1 gene transfer, cell viability and cell cycle analyses were performed. Accumulation of E2F-1 protein in melanoma cells was confirmed by Western blotting (Fig. 6A). Cell morphology showed that few melanoma cells treated with a combination of Ad-E2F-1 and etoposide were still adherent to the dish and that most exhibited cell shrinkage, whereas >90% of cells treated with Ad-E2F-1 or drugs alone were adherent to the dish (Fig. 6B). Moreover, a trypan blue exclusion assay showed that topoisomerase II inhibitors (etoposide or Adriamycin) alone and Ad-E2F-1 (MOI = 1) alone killed <20% of cells, whereas infection with Ad-E2F-1 followed by these drug treatments markedly reduced viability by >50% compared with control treatments (P < 0.01; Fig. 6C). The combination of drugs and Ad-LacZ did not significantly affect cell viability compared with drug treatments alone (P > 0.05). Combined treatment with Ad-E2F-1 (MOI = 1) and topoisomerase II poisons (etoposide and Adriamycin) had a synergistic effect on DNA fragmentation as well. Cell cycle analysis showed that cells cotreated with Ad-E2F-1 and topoisomerase II inhibitors developed a sub-G1 population, indicative of apoptosis. (Fig. 7A). Furthermore, the 89-kDa PARP cleavage product was detected (Fig. 7B), thus confirming that cell death was attributable to apoptosis.

![Fig. 1. Dose-response curve of melanoma cell lines to chemotherapeutic agents. SK-MEL-28 (●) and SK-MEL-2 (□) cells were exposed to drugs for 2 days. Cytotoxicity of drugs was determined by WST-1 assay. Results are expressed as a percentage of controls (untreated cells), and values represent the means ± SD (bars) obtained from triplicate determinations.](image1)

![Fig. 2. Adriamycin and etoposide can inhibit melanoma cell growth by G1 arrest, but cannot efficiently induce apoptosis. SK-MEL-2 and SK-MEL-28 cells were treated with Adriamycin (Adr) and etoposide (Etop) at indicated doses. Three days after treatment, cells were harvested and then subjected to FACS analysis. The subdiploid population was calculated as an estimate of the apoptotic cell population (%Apop).](image2)

![Fig. 3. Absence of induction of endogenous E2F-1 by topoisomerase II inhibitors in human melanoma cells. SK-MEL-28 and SK-MEL-2 melanoma cells were treated with Adriamycin (Adr) or etoposide (Etop) at indicated concentrations and harvested on day 2. SK-MEL-28 and SK-MEL-2 cells were exposed to indicated concentrations of drugs for 2 days. Cytotoxicity of drugs was determined by WST-1 assay. Results are expressed as a percentage of controls (untreated cells), and values represent the means ± SD (bars) obtained from triplicate determinations.](image3)
Cycloheximide Has a Protective Effect on Cell Death Induced by Combination of Ad-E2F-1 and Topoisomerase II Inhibitors. Some reports have indicated that cycloheximide (a protein synthesis inhibitor) and actinomycin D (a RNA synthesis inhibitor) block etoposide- and Adriamycin-induced apoptosis in certain cell lines, suggesting that new protein and RNA synthesis are required in topoisomerase II inhibitor-induced apoptosis (26–28). To detect whether the same physical process is involved in cell death induced by combined treatment with Ad5-E2F-1 and topoisomerase II inhibitors, melanoma cells were treated with Ad-E2F-1 plus topoisomerase II inhibitors (etoposide or Adriamycin), followed by treatments with cycloheximide or actinomycin D. As shown in Fig. 8, cycloheximide had a cytoprotective effect on cell death induced by a combination of Ad-E2F-1 and topoisomerase II inhibitors. A significant difference in cell viability was observed between Ad-E2F-1/topoisomerase II inhibitor treatment and Ad-E2F-1/topoisomerase II inhibitor/cycloheximide treatment (P < 0.02), but no such effect was evident after the addition of actinomycin D (data not shown). Furthermore, cell cycle analysis revealed that the addition of cycloheximide to the Ad-E2F-1/topoisomerase II inhibitor combination-treated cells inhibited apoptotic DNA fragmentation (data not shown). Therefore, these data suggest that new protein synthesis is required in this cell death process.

p21 WAF1/CIP1 Can Block Cell Death Induced by Combination of Ad-E2F-1 and Topoisomerase II Inhibitors and Does Not Require Its Ability to Bind PCNA. Several studies have shown that p21 WAF1/CIP1 protects against drug-mediated apoptosis in certain cell types (29, 30). p21 WAF1/CIP1 can also inhibit the transcriptional activity of E2F-1 by a Rb-dependent and Rb-independent pathway (31). We next wished to examine the effect of p21 WAF1/CIP1 overexpression on the cellular response to combined treatment with Ad-E2F-1 and topoisomerase II inhibitors (etoposide or Adriamycin). For these experiments, melanoma cells were coinfected with Ad-E2F-1 (MOI = 1) and Ad-WAF1 (MOI = 10). At this concentration of Ad-WAF1, full-length p21 protein was overexpressed in both cell lines, but no toxicity occurred. Viral infection was followed by treatment with topoisomerase II inhibitors (etoposide or Adriamycin). As shown in Fig. 9A, overexpression of p21 WAF1/CIP1 efficiently blocked cell death induced by combined treatment with Ad-E2F-1 and topoisomerase II inhibitors; this effect was statistically significant (P < 0.02). Furthermore, cell cycle analysis (data not shown) and PARP cleavage assays confirmed that this protective effect is attributable to blockage of apoptosis (Fig. 9B). As a cell cycle regulator, p21 WAF1/CIP1 has not only been shown to block S-phase entry by inhibiting cyclin-Cdk activity, but also by inhibiting DNA synthesis by binding to PCNA (32). To determine whether both p21 WAF1/CIP1 binding activities are necessary for this protective effect on cytotoxicity, SK-MEL-28 cells were infected with Ad-E2F-1 together with Ad-WAF1–341 (expresses a COOH-terminal-deleted p21 WAF1/CIP1 protein deficient in binding to PCNA), after treatment with etoposide or Adriamycin. As shown in Fig. 9C, Ad-WAF1–341 was sufficient to block apoptotic cell death induced by Ad-E2F-1 and topoisomerase II inhibitors.
inhibitors \( (P < 0.005) \), these data indicate that the PCNA-binding activity of p21\( \text{WAF1/CIP1} \) is not necessary to facilitate the observed cytotoxicity-protective effect.

**In Vivo Antitumor Effect of the Combination Chemogene Therapy with Adenovirus E2F-1 and Topoisomerase II Inhibitors.** s.c. tumors in bilateral flanks of mice were randomized and treated as described with local injections of 0.9\% saline (control), LacZ adenovirus (vehicles), or E2F-1 adenovirus, followed by an i.p. injection of Adriamycin, etoposide, or 0.9\% saline. Athymic nude mice (BALB/c) were given a total of four chemogene therapy treatments over a period of 21 days. The total virus dose for each injection was \( \times 10^9 \) pfu and was administered as indicated in “Materials and Methods.” Use of E2F-1 adenovirus alone reduced mouse tumor size by \( \sim 37\% \) by day 21, which was not statistically significant \( (P > 0.05) \). The drugs (Adriamycin or etoposide) alone or combined with LacZ reduced tumor burden \( \sim 43\% \) and \( 30\% \), respectively. However, when combined with Adriamycin or etoposide, E2F-1 adenovirus caused \( \sim 87\% \) and \( 91\% \) decreases in tumor size, respectively, compared with controls, and this decrease was statistically significant \( (P < 0.002; \text{Fig. } 10, \text{A and B}) \). Tumors were excised on day 21. Final tumor burdens in mice treated only with mock, LacZ, adenovirus-E2F-1, Adriamycin, LacZ plus Adriamycin, etoposide, and LacZ plus etoposide were \( 1.62 \pm 0.14, 1.53 \pm 0.15, 0.99 \pm 0.15, 0.68 \pm 0.07, \) and \( 1.03 \pm 0.34, 1.18 \pm 0.16, \) and \( 1.69 \pm 0.028 \) g, respectively. When both E2F-1 viruses and a topoisomerase II inhibitor (either Adriamycin or etoposide) were combined, the tumors weighed \( 0.30 \pm 0.03 \) and \( 0.33 \pm 0.04 \) g, respectively, and this difference was statistically significant compared with the control groups \( (P < 0.005; \text{Fig. } 10 \text{C}) \).

**DISCUSSION**

Combination chemotherapy has been shown to be more effective than single-agent therapy in many types of cancer. However, none of the present chemotherapy combinations are any more effective against metastatic melanoma than single-agent therapy \( (2–4, 33) \).

Chemogene therapy is emerging as a viable alternative to conven-
tional chemotherapy combinations (34, 35). In theory, when the tumor
cells are sensitized by overexpression of a proapoptotic protein,
such as E2F-1, lower doses of chemotherapeutic drugs may be needed,
therefore reducing systemic toxicity. The present work demonstrates
that transduction of the E2F-1 gene in melanoma cells markedly
increases cell sensitivity to some chemotherapeutic agents, especially
to topoisomerase II inhibitors, thereby producing a synergistic effect
on apoptotic cell death. Topoisomerase II inhibitors also cooperated
with Ad-E2F-1 to enhance antitumor activity in an in vivo nude mouse
model.

Type II topoisomerases are ATP-dependent enzymes that catalyze
topological changes in DNA. DNA topoisomerases II plays an important
role in DNA metabolism (36). At present, a number of anticancer drugs
used in the clinic act by targeting this enzyme (37, 38). However, these
chemotherapeutic agents yield low response rates and have not been
shown to prolong survival in melanoma patients (3, 39, 40).

The mechanisms of resistance of melanoma to topoisomerase II
inhibitors remain unclear. A previous report indicated that the multi-
drug resistance-associated protein may play a role in chemoresistance
of human melanoma cells (41). Recent studies have suggested that
E2F-1 may play a role in the mediation of cytotoxicity of some drugs
in tumor cells (4–7). In the present study, adenovirus-mediated E2F-1
gene transfer markedly sensitized melanoma cells to etoposide and
Adriamycin, but not to 5-FU, cisplatin, or cycloheximide. These
results are consistent with other studies (4–7).

Although the mechanism of the synergistic effect provided by the
combination of Ad-E2F-1 and topoisomerase II inhibitors is unclear,
several studies may provide clues regarding this effect. First, E2F-1 has
been identified as a tumor suppressor gene based on its ability to induce
apoptosis (15–21). E2F-1 potentiates cell death by down-regulating
TRAF2 protein levels and blocking antiapoptotic signaling pathways
(42). In this way, E2F-1 expression can lead to the sensitization of cells
to apoptosis by a number of agents (42). This is confirmed by the study
of Meng et al. (4), who found that the E2F-1 protein level increased in
some tumor cell lines after treatment with topoisomerase II inhibitors
(Adriamycin and etoposide). Furthermore, the induction of E2F-1 expres-
sion in tumor cells correlated with their sensitivity to these drugs, and loss
of E2F-1 expression contributed to drug resistance. This finding suggests
that induced expression of E2F-1 in some tumor cell lines after treatment
with topoisomerase II inhibitors may play a role in mediating apoptosis.

In the present study, etoposide and Adriamycin did not induce the
expression of endogenous E2F-1 in either of the melanoma cell lines
tested (Fig. 3). Therefore, our observations suggest that this apoptotic
pathway may be defective in human melanoma cell lines and that this
may contribute to the relative resistance of melanoma cells to topoi-
somerase II agents. Introduction of exogenous E2F-1 in melanoma

Fig. 8. Protective effect of cycloheximide (CHX) on melanoma cell death induced by
the combination of topoisomerase II inhibitors and Ad-E2F-1. Melanoma cell lines were
infected with Ad-E2F-1, at a MOI of 1, in the presence of topoisomerase II inhibitors with
or without cycloheximide at indicated concentrations. Etoposide (Etop), 2 μM for SK-
MEL-2, 5 μM for SK-MEL-28; Adriamycin (Adr), 100 nM for both cell lines. Cell viability
was assessed by trypan blue exclusion. Each point represents the mean of three independ-
ent experiments ± SD (bars).

Fig. 9. Overexpression of p21 can block Ad-E2F-1/topoisomerase II inhibitor-induced
cell death. Cells were infected with adenovirus vectors (Ad-E2F-1 at MOI of 1, Ad-p21
and Ad-p21–341 at MOI of 10) alone or in combination. One h later, cells were treated
with topoisomerase II inhibitors at indicated doses. A, cell viability assay shows a
protective effect of p21 on Ad-E2F-1/topoisomerase II inhibitor-induced cell death. Etoposide
(Etop), 2 μM for SK-MEL-2, 5 μM for SK-MEL-28; Adriamycin (Adr), 100 nM for both cell lines. Cell viability
was assessed by trypan blue exclusion. Each point represents the mean of three independent experiments ± SD (bars).
cells may reconstitute this pathway, thus allowing for efficient induction of apoptosis. In addition, the enhanced sensitivity of human melanoma cells to topoisomerase II inhibitors by adenovirus E2F-1 gene transfer may be the result of molecular events during the late S and G2 phases. It has been demonstrated that mutations in E2F-1 that block cyclin A binding trigger an S-phase cell cycle checkpoint that leads to G2 arrest, apoptosis, and sensitivity to DNA-damaging agents (43, 44). In an analogous manner, topoisomerase II inhibition coupled with DNA damage and high levels of E2F-1 may also trigger this S-phase checkpoint, arrest the cell cycle at G2 phase, and then induce apoptosis. In fact, our previous studies showed that overexpression E2F-1 resulted in premature S-phase entry and G2 arrest followed by apoptotic cell death (19). In this report, we show that topoisomerase II inhibitors also caused G2 arrest in both melanoma cell lines. Therefore, it is possible that the combination of Ad-E2F-1 and topoisomerase II inhibitors can accelerate the triggering of the S-phase checkpoint and that increased levels of E2F-1 allow for the induction of apoptosis.

E2F-1 activity can be regulated by several cell cycle regulator proteins, such as pRb and p21WAF1/CIP1. Underphosphorylated pRb binds to and inactivates E2F-1. After pRb phosphorylation in late G1 phase, E2F-1 is released, permitting the formation of active E2F-1/DP-1 complexes, thus allowing activation of E2F-1 target genes needed for DNA synthesis (9, 45, 46). p21WAF1/CIP1 protein, a cyclin-dependent protein kinase inhibitor, can negatively regulate E2F-1 activity by inhibiting pRb phosphorylation. Moreover, recent reports have indicated that p21WAF1/CIP1 can also inhibit E2F-1 activity by suppressing E2F-1-responsive promoters, which result in inhibition of DNA synthesis and cell cycle progression (31). Recently, the role of p21WAF1/CIP1 in cell growth suppression and its ability to bind and inhibit Cdk and PCNA has gained much attention; however, little is known of its function in inhibiting cytotoxic effects and apoptosis. In some cases, up-regulation of p21WAF1/CIP1 was observed during p53-dependent apoptosis (47), whereas in other cases p21WAF1/CIP1 was found to protect cells from apoptosis or drug cytotoxicity (29, 30). In this study, the effect of p21WAF1/CIP1 on cytotoxicity induced by the combination treatment of Ad-E2F-1 and topoisomerase II inhibitors was evaluated. Our observations revealed that overexpression of p21WAF1/CIP1 can block cell death induced by Ad-E2F-1 and topoisomerase II inhibitors. Moreover, infection of melanoma cells with Ad-WAF1–341 (COOH-terminal deletion p21 mutant protein defective in its ability to bind PCNA) suggests that the PCNA-binding activity of p21WAF1/CIP1 is not necessary to facilitate protection against apoptosis induced by the chemogene combination of topoisomerase II inhibitors and E2F-1.

Our previous study showed that adenovirus-mediated E2F-1 gene transfer alone at high doses (MOI = 100) effectively induced apoptosis in melanoma cells, which was associated with activation of CPP32 and down-regulation of an antiapoptotic Bcl-2 family protein, Mcl-1 (19). To investigate whether the same apoptotic pathway was involved in the cell death induced by combinations of Ad-E2F-1 and topoisomerase II inhibitors, we examined the expression of CPP32 and Mcl-1 after the chemogene combination treatments; however, no significant changes in CPP32 or Mcl-1 expression were evident in either melanoma cell line (data not shown). These observations suggest that the mechanism of loss of viability and apoptotic cell death induced by the combination of Ad-E2F-1 at low concentrations (MOI = 1) and drugs may be different from the apoptotic pathway induced by higher concentrations of Ad-E2F-1 (MOI = 100) alone. That PARP cleavage was observed in the E2F-1/topoisomerase II inhibitor-treated cells despite significant changes in CPP32 or Mcl-1 expression suggests that cleavage of PARP may be carried out by caspases other than CPP32.

Several reports have shown that cycloheximide, a protein synthesis inhibitor, can block topoisomerase II inhibitor-induced apoptosis (26–28). In this study, the same protective effect was evident in melanoma cells treated with a combination of Ad-E2F-1 and topoisomerase II inhibitors, suggesting that new protein synthesis is required in this cytotoxic process.

In summary, the present results show that expression of E2F-1 as a result of adenovirus-mediated gene transfer in melanoma cells can mark-
edly enhance the cell’s sensitivity to topoisomerase II inhibitors in vitro and in vivo. A synergistic effect on cell death was observed after combination therapy with Ad-E2F-1 and topoisomerase II inhibitors and was attributable to the swift induction of apoptosis. Therapeutically, this study provides new evidence that when melanoma cells are sensitized with a low concentration of Ad-E2F-1 (MOI = 1), topoisomerase II inhibitors can efficiently induce apoptosis at a greatly reduced concentration. Because only a low level of exogenous E2F-1 expression is required for this effect, it may be possible to achieve this level of E2F-1 expression for clinical use. The combined chemogene therapy of topoisomerase II inhibitors and Ad-E2F-1 provides a promising strategy for the effective treatment of melanoma with a potential reduction in systemic toxicity. Future studies will focus on achieving targeted expression of E2F-1 in melanoma cells, which may make this chemogene therapy strategy more clinically feasible.

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Adenovirus-mediated $E2F-1$ Gene Transfer Sensitizes Melanoma Cells to Apoptosis Induced by Topoisomerase II Inhibitors

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