E2F-1 Up-Regulates c-Myc and p14ARF and Induces Apoptosis in Colon Cancer Cells

Mary Jane Elliott, Yan Bin Dong, Hailiang Yang, and Kelly M. McMasters

Department of Surgery, University of Louisville, James Graham Brown Cancer Center, Louisville, Kentucky, 40202

ABSTRACT

Although overexpression of E2F-1 can induce apoptosis in a variety of tumor cell lines, the mechanisms by which E2F-1 induces apoptosis remain ambiguous. In this study, we examine the ability of E2F-1 to induce apoptosis in colon cancer and the molecular mechanisms underlying E2F-1-mediated apoptosis. HT-29 and SW-620 colon adenocarcinoma cells (both mutant p53) were treated by mock infection or adenoviral vectors Ad5CMV (empty vector), Ad5CMVLacZ (β-galactosidase), and Ad5CMVE2F-1 (E2F-1) at multiplicity of infection of 100. Western blot analysis confirmed marked overexpression of E2F-1 in both cell lines. By 5 days after infection, E2F-1 overexpression resulted in >25-fold reduction in cell growth and >90% loss of cell viability in both cell lines. Cell cycle analysis of Ad-E2F-1-infected cells revealed an increase in G2/M and sub-G1 populations. By in situ terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling analysis, evidence of apoptosis was observed including internucleosomal DNA fragmentation and the formation of apoptotic bodies. In addition, caspase-3 and poly(ADP-ribose) polymerase apoptotic fragments were detected by 48 h after treatment with Ad-E2F-1. Of mechanistic importance, overexpression of E2F-1 caused a G2/M arrest followed by increased levels of c-Myc and p14ARF proteins. Additionally, expression of the antiapoptotic Bcl-2 family member Mcl-1 was down-regulated in E2F-1-overexpressing cells. In conclusion, E2F-1 overexpression initiates apoptosis and suppresses growth in HT-29 and SW620 colon adenocarcinoma cells. Overexpression of E2F-1 triggers apoptosis and is associated with up-regulation of c-Myc and p14ARF proteins and down-regulation of Mcl-1. Therefore, E2F-1 is a potentially active gene therapy agent for the treatment of colon cancer.

INTRODUCTION

Essentially all of the anticancer drugs that are currently available as well as UV irradiation have been shown to kill cancer cells by triggering apoptosis (1). Defective apoptotic mechanisms are considered to play a role in both the development of malignancy and resistance to a wide range of chemotherapeutic drugs (1, 2). Studies have shown that mutations in the genes involved in apoptosis are responsible for >70% of the poly-p-tumor progression of colorectal cancer (3–5) and may contribute to the multidrug resistant phenotype of these cancers (6). Therefore, the development of new treatments that can trigger apoptosis in these extremely resistant cell types is a topic of intense investigation.

The process of apoptotic cell death is very distinct and involves unique morphological alterations, which include cell shrinkage, plasma and nuclear membrane blebbing, chromatin condensation, and the formation of apoptotic bodies (7). There are two distinct phases during apoptotic cell death: commitment and execution. The Bcl-2 family members appear to regulate the cellular commitment to survive or die when challenged with various apoptotic stimuli (8, 9). The execution phase of apoptosis is controlled by a sequential cascade of activation by proteolytic cleavage of the caspase family of proteins (10–12). Apoptotic cell death is induced by a number of stimuli. DNA-damaging treatments such as UV irradiation and a variety of chemotherapeutic drugs have been shown to induce apoptosis, which can involve both p53-dependent and -independent mechanisms (13–15).

Recent evidence points to an alternative apoptotic pathway that results in the induction of the tumor suppressor protein p14ARF. p14ARF is produced from the transactivation of an alternate reading frame found in the ink4a tumor suppressor gene (16, 17). This novel apoptotic pathway involving p14ARF is stimulated under hyperproliferative or oncogenic conditions but is not stimulated after DNA damage (18, 19). Therefore, p14ARF-associated apoptosis is believed to constitute a separate pathway to programmed cell death. Paradoxically, the same oncogenes and growth factors that can induce cellular proliferation can also cause apoptosis and growth arrest. To date, overexpression of the transcription factors E2F-1 and c-Myc as well as activated oncogenic Ras (H-Ras) and an adenoviral protein, E1a, have been shown to induce increased expression of p14ARF followed by either grow arrest and/or apoptosis (20–23). However, thus far, only c-Myc-like E box motifs and a potential E2F-1 binding site (GCGGGAAA) have been identified in the specific promoter for p14ARF (20, 23).

E2F-1 has been shown to possess both oncogenic and apoptotic properties. As a transcription factor, E2F-1 is intimately involved in the regulation of the cell cycle. When un-
bound to the pRb, E2F-1 transactivates genes required for DNA synthesis and progression into S phase. Early studies with E2F-1 suggested that this protein can function as an oncogene when overexpressed (24–26). However, increasing E2F-1 activity by overexpression of E2F-1 has been shown to lead to apoptosis in many cell types (27–31). When E2F-1 is overexpressed, an unscheduled S phase entry occurs and is often followed by a G2/M arrest and apoptosis (27, 28, 32). Furthermore, recent studies demonstrate that the apoptotic function of E2F-1 is independent of its transcriptional function but is dependent on its DNA-binding capability (33, 34). In fact, E2F-1 has been linked to two very distinct apoptotic pathways: (a) E2F-1 protein levels increase in a variety of cell lines after DNA damage-induced apoptosis and induction occurs independent of pRb or p53 status (35); and (b) overexpression of E2F-1 has been shown to result in the up-regulation of p14ARF and apoptosis in both p53-wild-type and p53-null cells (20). Still, to date, little is known of the mechanism of E2F-1-mediated apoptosis.

In the present study, we investigate the effect of adenoviral-mediated gene transfer of E2F-1 on colon cancer cell growth. The results of this study demonstrate that adenoviral-mediated gene transfer is an efficient method for the delivery of genes to colon cancer cells, because E2F-1 was highly expressed after infection. The overexpression of E2F-1 induced the cells to accumulate in G2/M within 24 h after treatment with apoptotic DNA fragmentation in >50% of the cells by 12 h after treatment. Mechanistically, we found that p14ARF and c-Myc were up-regulated, and Mcl-1 expression was abrogated after E2F-1 gene transfer. These results suggest that p14ARF, c-Myc, and Mcl-1 play an important role in E2F-1-mediated apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human colon cancer cell lines HT-29 and SW620 (both mutant p53) were purchased from American Type Culture Collection (Rockville, MD). Both cell lines were cultured in McCoy’s 5A medium modified and supplemented with 10% heat-inactivated FBS, streptomycin/penicillin (100 units/ml). The transformed embryonic kidney cell line 293 was grown in minimum essential α-medium [medium with high glucose (4.6 g/l), supplemented with 10% FBS and 1% antibiotic/antimycotic. All of the 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.

Preparation of Adenoviral Vectors. A replication-defective recombinant adenoviral vector (Ad-5) that has had portions of the early region 1 deleted, and a constitutive CMV promoter inserted was used in these experiments. In addition to the empty vector (Ad5), two vectors containing either the transgene E2F-1 or LacZ were used. These vectors have been described previously (29, 36). The adenoviruses Ad5 and Ad-E2F-1 were generous gifts from Ta Jen Liu (University of Texas, MD Anderson Cancer Center, Houston, TX). Ad-LacZ was a generous gift from Dr. Brent French (University of Virginia, Charlottesville, VA). For preparation of large virus stocks, 293 cells were infected at MOI 1–5 and harvested after cytopathic effect became visible (24–48 h). Cells were harvested and lysed in 1 × PBS containing 1% sucrose, and virus aliquots were stored at −70°C. Titers were determined by either plaque assay on 293 cells for plaque forming units/ml or spectrometer reading at 260 nm for particles/ml (37) Ad5 (empty vector) and Ad-LacZ (expressing nuclear-localized β-gal) were used as control vectors.

Virus Infection. For infections, 2.5 × 105 cells were plated in 75-mm tissue culture flasks and allowed to incubate for 48 h. Cells were then infected by adding the adenoviral vectors in 1800 μl of McCoy’s 5A medium at MOI of 100 plaque forming units/cell. Mock infection was performed by treatment of cells with vehicle (medium) only. After a 2-h incubation at 37°C, 28.2 ml of fresh McCoy’s 5A medium with 5% FBS was added. Cells were trypsinized at 24, 48, 72, and 120-h time points, counted via a Coulter counter, and prepared for various testing as outlined below. Optimal concentrations of viral vectors causing expression in 80–100% of the cells without visible toxic effect were determined by infection of cells with the β-gal expressing virus at different doses and subsequent staining with X-gal (French).

β-Gal Assay. The cell lines infected with Ad-LacZ were assayed for β-gal expression by the X-gal staining method as described previously (38). Briefly, 48 h after infection, the tumor cells were washed with PBS and fixed in 2% (vol/vol) formaldehyde and 0.2% (vol/vol) glutaraldehyde in PBS (pH 7.4) for 5 min at 4°C. The cells were then washed and stained with X-gal solution [1 mg/ml 5-bromo-4-chloro-3-indolyl-b-galactopyranoside, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2 in PBS (pH 6.5)] for 12–18 h at 37°C. Blue staining of cell nuclei identified transduced cells. Mock-infected cells and cells transduced with other adenoviral vectors served as controls.

Immunoblotting Procedures. Cells were harvested at selected time points and lysed in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxylcholate and 0.1% SDS) or in PBS containing 1% NP-40. A protease inhibitor cocktail [4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, transpeoxysuccinyl-l-leucylamido(4-guanidino)butane(E-64), bestatin, leupeptin, and aprotinin] was added to each lysis buffer. Protein concentration was determined by Bio-RAD DC protein assay (BIO-RAD, Hercules, CA). For the detection of Bcl-2, Bax, Bak, Bcl-x, Mcl-1, and caspase-3 proteins, 30 μg of protein were loaded in each lane of a 12% SDS-Page gel. A 10% SDS-Page gel was used for the detection of p-Myc, PARP, and E2F-1 and a 15% SDS-Page gel for the detection of p14ARF. Protein was transferred to a polyvinylidene difluoride membrane (Bio-Rad). Proteins were detected using antihuman Bcl-2, Bax, Bak, Bcl-x, Mcl-1, c-Myc (PharMingen), E2F-1, p14ARF (Santa Cruz Biotechnology), PARP (Calbiochem), and Caspase-3 (Transduction Laboratories, Lexington, KY). α-Actin polyclonal antibody (Sigma Chemical Co., St. Louis, MO) was used as control to demonstrate equal loading and transfer.
Enhanced chemiluminescence reagents were used to detect the signals according to the manufacturer’s instructions (Amer sham, Arlington Heights, IL). Intensity of each protein band was quantified using NIH image software after scanning of the image.

**Cell Growth and Viability Assays.** Cells were harvested at indicated time points, and cell viability was estimated as the proportion of cells that excluded 0.2% trypan blue. Cell growth (suspension/ml) was estimated by counting 100 μl of cells with a Coulter counter ZM at each time point.

**Cell Cycle Analysis.** Adherent and nonadherent cells were harvested, washed once with PBS, fixed in 70% (vol/vol) ethanol, and stored at 4°C for up to 1 week. Cells were pelleted, washed once with phosphate buffered saline, and resuspended in propidium iodide solution [50 μg/ml PI and 0.5mg/ml in PBS (pH 7.4)] for 30 min in the dark. Cells were analyzed for DNA content by fluorescence-activated cell sorting (FACScan; Becton Dickinson). Data were analyzed using a CellFit Cell-Cycle Analysis program (version 2.01.2). The subdiploid population was calculated as an estimate of the apoptotic cell population. The proportion of G1, S, G2-M and subG1 phase cells was estimated each as a percentage of the total (10,000) events.

**Confirmation of Apoptosis.** Several methods were used to confirm apoptotic cell death. First, simultaneous evaluation of morphological and biochemical changes attributable to apoptosis was performed via in situ TUNEL. The TUNEL assay identifies internucleosomal DNA strand breaks as well as morphological changes characteristic of apoptosis. Cells were fixed in 4% formaldehide in PBS (pH 7.4) for 15 min at room temperature. After centrifugation, cells were resuspended in 80% ethanol and stored at 4°C for up to 1 week. A TdT-FragEL DNA fragmentation detection kit (Calbiochem, Oncogene Research Products, Cambridge, MA) was used to detect apoptosis according to instructions provided by the manufacturer. The detection of caspase 3 and PARP cleavage has been shown to be a sensitive method for confirmation of apoptosis (39). Cleavage assays were performed using a monoclonal mouse anti-PARP antibody (Calbiochem, Oncogene Research Products, Cambridge, MA) at a dilution of 1:100 and anti-Caspase-3 (Transduction Laboratories, Lexington, KY) at a dilution of 1:1000.

**Statistics.** Unless stated otherwise, numerical data represents mean values for at least two experiments. SE, unless shown, were <10% of the value of the respective data point. Student’s t test was used to evaluate whether differences between data sets were statistically significant. Only Ps <0.05 were considered significant.

**RESULTS**

**Transduction Efficiency of Adenoviral Vectors.** To estimate the transduction efficiency of adenoviral vectors, the two human colon cell lines were infected with Ad-LacZ at MOI ranging from 25–150. At MOI of ≥5, 85% of cells demonstrated nuclear staining for β-gal. Increased intensity of β-gal staining was demonstrated with increased MOI up to 150 (data not shown). No significant cytotoxicity was observed with MOI up to 150. All of the subsequent experiments were performed at MOI of 100.

**Overexpression of E2F-1.** Western blot analysis confirmed marked overexpression after infection with the Ad-E2F-1 vector in both the HT-29 and SW620 colon cancer cell lines (Fig. 1). The presence of multiple bands of E2F-1 likely indicates various species of hyper- and hypophosphorylated E2F-1 as described previously (40). In contrast, baseline expression of E2F-1 in mock-infected, Ad-5, or Ad-LacZ-infected control cells was barely detected.

**Effects of Adenoviral Vector-mediated E2F-1 Expression on Cell Growth.** At 24 h after infection, there was no apparent growth inhibition in E2F-1-overexpressing cells compared with mock-infected, Ad5, or Ad-LacZ-infected cells (Fig. 2, A and B). By 48 h, however, cells infected with the Ad-E2F-1 vector exhibited marked growth inhibition, which was even more pronounced at 72 h and 120 h. The effects were similar in both HT-29 and SW620 (both mutant p53) cell lines. A dramatic decline in cell viability was also detected in the E2F-1-overexpressing cells, as assessed by trypan blue exclusion (Fig. 3, A and B). These data indicate that overexpression of E2F-1 effectively inhibits cell growth and induces cell death in colon cancer.

**Effects of Ad-E2F-1 Infection on Cell Cycle.** To additionally investigate the mechanism of E2F-1-induced colon cancer cell death, cell cycle distribution after the Ad-E2F-1 was analyzed. As shown in Fig. 4 and Tables 1, α and β. E2F-1 overexpression was associated with a decrease in G1 phase cells and a concomitant increase in sub-G1 and G2-M populations by 24 h in both cell lines. By 5 days after infection, the subdiploid population predominated. Mock-infected, Ad5, and Ad-LacZ-infected cells showed little alteration of normal cell cycle profiles and few subdiploid cells in both cell lines. Quantitation of cell cycle data are shown in Table 1, α and β. These data suggest that E2F-1 overexpression is associated with premature S phase entry, G2-M arrest, and DNA fragmentation in HT-29 and SW620 colon cancer cells.

**E2F-1-mediated Cell Death Is Attributable to Apoptosis.** In addition to internucleosomal DNA fragmentation and the cellular morphological markers seen during apoptosis, a cascade of molecular events can serve as early biochemical
markers, including activation of the caspase family of proteins and PARP cleavage. Cleavage of the nuclear enzyme PARP by caspase-3 has been proposed as one of the earliest events in the execution phase of apoptosis (9, 10). On activation, caspase-3 is cleaved forming a Mr 17,000 active fragment (41, 42). Caspase-3 preferentially cleaves on the carboxyl side of the tetrapeptide sequence DEVD, a sequence present in PARP (42). Proteolysis of the Mr 113,000 PARP protein by caspase-3 generates 89 and Mr 24,000 cleavage products (39). Cleavage of caspase-3 and PARP that is consistent with apoptosis was detected in the E2F-1-overexpressing cells (Fig. 5, A and B) by 48 h. Additionally, in the HT-29 cell line, low levels of caspase-3 are evident in mock- and LacZ-infected cells (data not shown; Fig. 5A); however, after infection with E2F-1, levels of caspase-3 increase dramatically. It is unclear why this induction of caspase-3 protein occurs in HT-29 cells. Mock-infected, Ad5, or Ad-LacZ-infected cells demonstrated none of these morphological or biochemical changes. Therefore, these data confirm the involvemen of the caspase cascade in E2F-1-induced apoptosis of these cell lines.

Using in situ TUNEL assay, it is possible to simultaneously observe morphological changes and DNA fragmentation consistent with apoptosis. Five days after infection with Ad-E2F-1, apoptotic evidence was observed including internucleosomal DNA fragmentation and the formation of apoptotic bodies by TUNEL assay in the HT-29 and SW620 cells (data not shown).

**Intracellular Levels of Bcl-2 Family Members.** Because the Bcl-2 family of proteins are believed to interact to control the cellular commitment to programmed cell death, immunoblots were performed to detect any measurable changes in the levels of Bak, Bax, Bcl-xl, Bcl-xs, Bcl-2, and Mcl-1 (Fig. 6, A and B). Results confirmed that no changes in intracellular levels of Bak, Bax, or Bcl-xl (Bcl-xs and Bcl-2 were not detected in either cell line) occurred by 3 days after infection in any of the samples. However, expression of the antiapoptotic protein Mcl-1 disappeared in the cells infected with E2F-1 in both HT-29 and SW620 cells by 72 h after infection. These results suggest that the cleavage or down-regulation of Mcl-1 might be a controlling factor in E2F-1-induced apoptosis. Cleavage of the proapoptotic protein Bax, was also detected in the E2F-1-overexpressing cells, which demonstrates the activation of calpains and caspases (43, 44). However, a number of studies show that inhibition of calpains inhibits Bax cleavage but does not inhibit PARP cleavage or apoptotic death (43, 44). Therefore, cleavage of Bax is not expected to be involved in the cellular commitment to apoptosis but rather a result of apoptosis.

**Intracellular Levels of c-Myc and p14ARF.** Because overexpression of E2F-1 is known to result in unscheduled S phase entry and, therefore, a potential hyperproliferative signal, we suspected that the p14ARF apoptotic pathway might be involved. As shown in Fig. 7, up-regulation of p14ARF was detected in both cell lines 72 h after infection with Ad-E2F-1. To additionally characterize the mechanisms underlying E2F-1-

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**Fig. 2** The effect of Ad-E2F-1 on colon cancer cell growth. A, HT-29 cells; B, SW620 cells. Cell growth was determined by cell counting with a Coulter counter ZM. Results are expressed as cells/ml at time points 24, 72, and 120 h after infection. Each data point represents the mean ± SE of three separate experiments. SEs are shown only if >10% of the respective value.

**Fig. 3** Loss of cell viability after infection with Ad-E2F-1. A, HT-29 cells; B, SW620 cells. At 1, 3, and 5 days after mock infection, or infection with Ad-5 (empty vector), Ad-LacZ (LacZ), or Ad-E2F-1 (E2F-1), both floating and adherent cells were harvested, counted, and scored for viability by the trypan blue exclusion method. Results are expressed as the percentage of viable cells (live cells/total cells) and represent the mean of three independent experiments. SEs are shown only if >10% of the respective viability value.
mediated apoptosis, we probed for c-Myc, which has been shown to up-regulate p14ARF when overexpressed and induce apoptosis under hyperproliferative conditions (22). A dramatic increase in c-Myc protein levels was observed in the E2F-1-overexpressing cells by 48 h after infection in both cell lines (Fig. 7). In the E2F-1-overexpressing cells, p14ARF expression was detected only after c-Myc was induced, and a large majority of the cells were apoptotic. The protein expression timeline seen in these results suggests that E2F-1 may induce up-regulation of p14ARF with the cooperation of c-Myc in these cell lines.

**DISCUSSION**

In the present study, we used an adenoviral vector system to transfer the E2F-1 gene to colon cancer cells and analyzed the ability of E2F-1 to trigger apoptosis. Here, for the first time, we demonstrate that treatment of colon cancer cells with adenoviruses expressing the E2F-1 gene can effectively induce apoptosis. Mechanistically, our data supports previous studies performed in other cell types in that overexpression of E2F-1 induces G2-M arrest and cell death (27–31). However, our report contributes the additional understanding that E2F-1-mediated apoptosis involves increased expression of p14ARF and c-Myc and down-regulation of Mcl-1. This data indicates that p14ARF, c-Myc, and Mcl-1 may play an important role in E2F-1-induced apoptosis in colon cancer.

A growing body of evidence indicates that oncogenes and growth factors can induce proliferation and promote cellular survival but when overexpressed can cause apoptosis and growth arrest (18, 45, 46). This mechanistic duality appears to
expression of p14ARF, a protein with tumor suppressor activity, induce an alternate apoptotic pathway that involves increased proliferation but in coordinating programmed cell death has been shown to induce apoptosis in several cell types, indicating that E2F-1 has a specific role in stimulating cell proliferation but in coordinating programmed cell death.

Recent evidence indicates that E2F-1 overexpression can induce an alternate apoptotic pathway that involves increased expression of p14ARF, a protein with tumor suppressor activity. The p14ARF-associated pathway is found to be stimulated by hyperproliferative signals and can result in a G1 or G2-M cell cycle arrest and apoptosis. Interestingly, p14ARF has been shown to induce apoptosis in several cell types, indicating that E2F-1 plays a role not only in stimulating cellular proliferation but in coordinating programmed cell death.

New evidence indicates that E2F-1 overexpression can induce an alternate apoptotic pathway that involves increased expression of p14ARF, a protein with tumor suppressor activity (20, 32). The p14ARF-associated pathway is found to be stimulated by hyperproliferative signals and can result in a G1 or G2-M cell cycle arrest and apoptosis (51). A substantial amount of data indicates that p14ARF facilitates a G1 arrest by neutralizing MDM-2 activity thereby causing stabilization, accumulation, and activation of p53 and ultimate transactivation of p21 (17, 51–53). However, virtually no data exists regarding the role of p14ARF during a G2-M arrest. In the current study of colon cancer, overexpression of E2F-1 resulted in G2-M arrest and apoptosis. As shown in Fig. 7, overexpression of E2F-1 in HT-29 and SW620 cells was followed by an increase in the level of p14ARF. These findings indicate that apoptosis induced by the overexpression of E2F-1 is associated with increased levels of p14ARF in colon cancer cells.

There is evidence that overexpression of E2F-1 induces the accumulation of p53, implicating p53 in E2F-1-mediated apoptosis (54). An increase in p14ARF expression may explain the accumulation of p53 seen after overexpression of E2F-1 in some wild-type p53 cell lines. Whereas it was originally believed that p53 was essential for E2F-1-mediated apoptosis, the results from a variety of experiments make it clear that active p53 is not always required (27–29, 33, 55). Although many studies show that a p14ARF-induced G1 arrest and subsequent apoptotic response is p53 dependent, a separate study showed that overexpression of E2F-1 in p53-null Saos-2 cells resulted in accelerated entry into DNA synthesis, a dramatic increase in p14ARF, and apoptotic cell death (20). Interestingly, p14ARF has been shown to be dispensable for cell proliferation and, therefore, is believed to have a specific role for priming or mediating cell death (22). In the present study, two colon cancer cell lines were examined: HT-29 and SW-620, both of which possess mutant p53. Overexpression of E2F-1 resulted in accumulation of cells in G2-M, increased levels of p14ARF, and apoptosis. Thus, although wild-type p53 may be required for p14ARF-associated G1 arrest, it appears that E2F-1/p14ARF-associated G2-M arrest and apoptosis can proceed without regard to the status of p53 in these cell lines.

Like E2F-1, the transcription factor c-Myc can promote both cell survival and death and has been shown to have separable regulatory functions in both cell proliferation and death. Interestingly, p19ARF has been shown to be involved in c-Myc-induced apoptosis (22) and has been identified as a c-Myc target gene (23). A relationship between E2F-1 and c-Myc is indicated by data that show E2F transcription factors up-regulate c-Myc, and pRb-sequestered E2F induces the down-regulation of c-Myc (27–29, 33, 55). Although many studies show that a p14ARF-induced G1 arrest and subsequent apoptotic response is p53 dependent, a separate study showed that overexpression of E2F-1 in p53-null Saos-2 cells resulted in accelerated entry into DNA synthesis, a dramatic increase in p14ARF, and apoptotic cell death (20). Interestingly, p14ARF has been shown to be dispensable for cell proliferation and, therefore, is believed to have a specific role for priming or mediating cell death (22). In the present study, two colon cancer cell lines were examined: HT-29 and SW-620, both of which possess mutant p53. Overexpression of E2F-1 resulted in accumulation of cells in G2-M, increased levels of p14ARF, and apoptosis.
idence indicates that c-Myc and E2F-1 share specific apoptotic pathways involving p14ARF, but also take distinct routes to induce cell death (18, 23, 58). Recent studies using centrifugal elutriation demonstrated that high ectopic levels of E2F-1 in Rat1 cells induced apoptosis specifically from cells in G1, but overexpression of c-Myc triggered apoptosis from cells in G1 and G2-M phases (58). Interestingly, Rat1 cells accumulated in G2-M after overexpression of either oncogene; however, only c-Myc-overexpressing cells generated apoptosis from the G2-M population. As stated above, p14ARF is also associated with apoptosis after cell cycle arrests in both G1 and G2-M phases. In this study, 10–25% of the E2F-1-overexpressing cells had evidence of apoptosis by 24 h after treatment. At that time point, most cells were beginning to accumulate in G2-M; however, up-regulation of c-Myc or p14ARF had not yet occurred (compare Figs. 4 and 7 and Table 1, a and b). This initial wave of apoptosis seen at the 24 h time point is obviously not associated with c-Myc or p14ARF induction. However, apoptosis was triggered in ~40% of the cells after arrest in G2-M and up-regulation of c-Myc and p14ARF proteins (compare Figs. 4 and 7 and Tables 1, a and b). These findings suggest that E2F-1 has the ability to generate dual apoptotic signals at more than one stage of the cell cycle and may involve the cooperation of c-Myc and p14ARF after exit of G1 phase in these colon cancer cells.

One final observation is relevant to the mechanism of E2F-1-induced apoptosis in colon cancer. After infection with E2F-1, expression of the antiapoptotic protein Mcl-1 was completely abrogated. This is a novel finding and suggests that E2F-1-mediated growth inhibition and apoptosis may result, at least in part, from the inhibition of Mcl-1 activity. Little is known of this relatively new member of the Bcl-2 family; however, studies show that Mcl-1 has sequence and functional homology to Bcl-2 (59). Bcl-2 has been shown to inhibit apoptosis induced by both E2F-1 and c-Myc, and evidence shows that like Bcl-2, Mcl-1 inhibits apoptosis induced by c-Myc (33, 60, 61). The two colon cancer cell lines we tested in this report do not express Bcl-2; however, endogenous Mcl-1 expression is high (data not shown; Fig. 6). Because c-Myc appears to be important in E2F-1-induced apoptosis and their apoptotic pathways are similar, the elimination of Mcl-1 may be apoptotically advantageous.

In this report, we demonstrate that overexpression of E2F-1 by adenoviral-mediated gene transfer inhibits colon cancer cell growth by causing G2/M phase delay and activation of apoptosis. E2F-1-mediated cell death was associated with the induction of c-Myc and p14ARF, down-regulation of Mcl-1, and activation of the caspase cascade. Therefore, adenoviral-vector-mediated E2F-1 gene therapy is a promising approach for the treatment of advanced colon cancer. In vivo studies to evaluate the effectiveness of E2F-1 gene therapy are under way.

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