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p53 gene transfer does not enhance E2F-1-mediated apoptosis in human colon cancer cells

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Abbreviations: SE, standard error; PBS, phosphate-buffered saline; PARP, poly-ADP ribose polymerase; CDK, cyclin-dependent kinase

Abstract

E2F-1 and p53 are sequence specific transcription factors that are intimately involved in the regulation of the cell cycle. In addition to their role in cell cycle control, both E2F-1 and p53 have been identified as tumor suppressors and mediators of apoptosis. We have shown previously that adenoviral-mediated E2F-1 overexpression induces efficient apoptosis in colon adenocarcinoma cells. Previous reports have suggested that E2F-1 and p53 cooperate to mediate apoptosis and therefore, in this study, we examined the efficacy of combination gene therapy using adenovirus vectors expressing E2F-1 and p53 in human colon adenocarcinoma cell lines, HT-29 and SW620 (both mutant p53). Cells were treated by mock infection or infection with adenoviral vectors expressing b-galactosidase (LacZ), E2F-1, p53 or a combination of E2F-1 and p53. IC_{25} concentrations of each virus were estimated and used for each treatment in order to detect any synergistic or cooperative effects on tumor cell death in the combination therapy. By 5 days post infection, E2F-1-overexpressing cells exhibited growth inhibition and approximately 40-50% cell death in both cell lines. Co-expression of p53 with E2F-1 abrogated E2F-1-mediated growth inhibition and cell death. Cell cycle analysis revealed that overexpression of E2F-1 resulted in an accumulation of cells in G2/M phase, while overexpression of p53 resulted in a G1 phase accumulation. However, co-expression of E2F-1 and p53 counteracted each other as fewer cells accumulated in G1 and G2/M when compared to either p53 or E2F-1 alone. Furthermore, co-expression of p53 with E2F-1 resulted in decreased levels of E2F-1 protein expression. Mechanistically, upregulation of the CDK inhibitory protein, $p21^{WAF1/CIP1}$, was demonstrated in HT-29 cells following overexpression of either E2F-1, p53 or the combination E2F-1/p53 therapy. However, in SW620 cells, only the cells infected with Ad-p53 alone or in combination resulted in upregulation of $p21^{WAF1/CIP1}$. These results suggest that p53 and $p21^{WAF1/CIP1}$ may cooperate to inhibit the expression and activity of E2F-1. In conclusion, combination adenoviral vector-mediated E2F-1 and p53 gene transfer was not therapeutically advantageous in this *in vitro* model of human colon adenocarcinoma.

Keywords: E2F-1, p53, p21, gene therapy, apoptosis, adenovirus, colon cancer, cell cycle

Introduction

E2F-1 is a member of the E2F family of transcription factors that regulates cell cycle progression from G1 to S phase. E2F-1 forms a heterodimer with a member of the DP family (DP-1 or DP-2) and binds to DNA in a sequence specific manner with high affinity (Dyson, 1998). It has been well established that the gene products of E2F-1 transcriptional activity can promote cells to enter S phase of the cell cycle (Helin, 1998). Although early studies with E2F-1 suggested that it functions as an oncogene (Johnson *et al.*, 1993), recent studies suggest that E2F-1 may also paradoxically function as a tumor suppressor gene, based on its ability to induce apoptosis under conditions of deregulated expression in several normal and malignant cell types (Hunt *et al.*, 1997; Liu *et al.*, 1999; Fueyo *et al.*, 1998). In fact, E2F-1 has been linked to two very distinct apoptotic pathways. First, E2F-1 protein levels increase in a variety of cell lines following DNA damage-induced apoptosis and induction of apoptosis occurs independent of pRb or p53 status (Helin, 1998; Meng *et al.*, 1999). Second, that overexpression of E2F-1 results in $p14^{ARF}$-associated apoptosis in both p53-wild-type and p53-null cells. (Bates *et al.*, 1998; Elliott and McMasters, unpublished data).

The p53 tumor suppressor protein is a negative regulator of cell growth. After a cell has incurred DNA damage, the induction of p53 serves either as a key regulator of a G1 cell cycle checkpoint or as an inducer of apoptotic cell death (Ko and Prives, 1996). The expression of p53 following genotoxic stress has long been associated with the transactivation of the $p21^{WAF1/CIP1}$ gene (Kastan

et al., 1991; El-Deiry *et al*., 1993; Nelson and Kastan, 1994). The $p21^{WAF1/CIP1}$ protein in turn inhibits the function of specific cyclin-dependent kinases responsible for G1/S transition (El-Deiry *et al*., 1993; Harper *et al*., 1993). By inhibiting these kinases, $p21^{WAF1/CIP1}$ induction results in a G1 cell cycle arrest. Alternatively, the stimulation of apoptosis by p53 may result, at least in part, from transcriptional upregulation of the death-promoting gene Bax, which has been shown to contain sequence specific p53-binding sites in its promoter region and from the downregulation of the survival factor Bcl-2 and related Bcl-2 family members (Miyashita *et al*., 1994; Miyashita and Reed, 1995; Kernohan and Cox, 1996). Importantly, the mechanism by which p53 dictates the choice between G1 arrest and apoptotic pathways remain ambiguous.

Although E2F-1 is capable of independently inducing apoptosis in certain mammalian cells (Hunt *et al*., 1997; Dong *et al*., 1999; Yang *et al*., 1999), much attention has been given to the role that wild-type p53 may play in that process. Previous studies have shown that E2F-1 and p53 cooperate to mediate apoptosis (Qin *et al*., 1994; Wu and Levine, 1994; Kowalik *et al*., 1995; Sladek, 1996; Phillips *et al*., 1997; Hsieh *et al*., 1997; Kowalik *et al*., 1998). In some tumor cells, wild-type p53 has been shown to enhance apoptosis with E2F-1 when both were overexpressed. For example, E2F-1-induced apoptosis in fibroblasts is potentiated by high levels of endogenous wild-type p53 (Kowalik *et al*., 1995; Hsieh *et al*., 1997). There is also evidence that overexpression of E2F-1 induces the accumulation of p53, again implicating p53 in E2F-1 mediated apoptosis (Hiebert *et al*., 1995; Kowalik *et al*., 1998).

We have shown previously that adenoviral-mediated E2F-1 overexpression at high concentrations [multiplicity of infection (MOI) of 100] induces a G2/M arrest and efficient apoptosis in a variety of cancer cell types, including colon adenocarcinoma cells (Dong *et al*., 1999; and Yang *et al*., 1999; unpublished data). This study was designed to investigate the potential synergistic effects of expressing E2F-1 and p53 on colon cancer cell death. The results of this study demonstrate that adenovirus-mediated p53 gene transfer is not associated with an increased susceptibility of colon cancer cells to E2F-1-mediated apoptosis. In fact, concomitant adenovirus-mediated overexpression of p53 resulted in a marked inhibition of E2F-1 expression and activity, corresponding with induction of $p21^{WAF1/CIP1}$. These findings suggest that p53 and p21 may cooperate to inhibit the expression and apoptotic activity of E2F-1.

Materials and Methods

Cells and culture conditions

Human colon adenocarcinoma cell lines, HT-29 and SW620 (both mutant p53), were purchased from ATCC (Rockville, MD). Both cell lines were grown as monolayers in McCoy's 5A medium, supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (100 units/ml). The transformed embryonic kidney cell line, 293, was grown in Minimum Essential Media [medium with high glucose (4.6 g/l)], supplemented with 10% FBS and 1% antibiotic/antimycotic. Cells were cultured in a 5% CO_2 incubator at 37°C and subcultured every 3 days. All cell culture reagents were obtained from Gibco/BRL (Bethesda, MD).

Preparation of recombinant adenovirus

A replication-defective recombinant adenoviral vector (Ad-5), with portions of the early region (E1) deleted and a constitutive CMV promoter inserted, was used in these experiments. The adenovirus Ad5CMV-E2F-1, Ad5CMV-LacZ, and Ad5CMV-p53 (here in called Ad-E2F-1, Ad-LacZ and Ad-p53) were generous gifts from Drs. Ta-Jen Liu (M.D. Anderson Cancer Center), Brent French (University of Virginia), and Bert Vogelstein (Johns Hopkins University). For preparation of large virus stocks, 293 cells were infected at MOI 1-5 and harvested after the CPE (cytopathic effect) became visible (24-48 h). Cells were harvested and lysed in 1X PBS^{+2} containing 1% sucrose, and virus aliquots were stored at -70°C. Titers were determined by plaque assay on 293 cells. Ad-LacZ (expressing nuclear-localized β-galactosidase) was used as a control vector and contained no therapeutic gene.

Dose response curves

HT-29 and SW 620 cell lines were seeded in 6-well plates at 2×10^5 total cells and incubated for 24 h at 37°C. One 6-well plate was infected with 200 μl of media (mock). Separate 6-well plates were infected with Ad-LacZ, Ad-E2F-1 and Ad-p53 at increasing MOIs (10, 25, 50, 100, and 150) for a period of 2 h. Four ml of media supplemented with 5% FBS and 1% penicillin/streptomycin were added to the cells and allowed to incubate for 5 days. Cells were then harvested with trypsin-EDTA. A cell count was obtained with a Coulter counter, and Trypan Blue assays were performed to determine cell viability. Total viable cells were calculated (% viability X cell count).

Recombinant virus infection

HT-29 and SW620 cell lines were seeded at 2.5×10^4 cells/ml in 75 mm flasks and allowed to incubate for 24 hours at which time the cells were 35% confluent. Cells were treated with replication defective recombinant adenovirus vectors for two hours, and then fresh medium was added with 5% FBS. Both cell lines were treated under five infection conditions as follows: Ad-E2F-1 infection, Ad-p53 infection, Ad-E2F-1 plus Ad-p53 infection (simultaneous infection), mock infection (control)

and Ad-LacZ infection (control). Cells were treated with replication defective recombinant adenovirus vectors at IC_{25} concentrations (Ad-E2F-1 MOI 20 and Ad-p53 MOI 20) for 2 h and then fresh medium was added. Non-specific viral toxicity was controlled by using LacZ adenovirus thereby ensuring equivalent viral concentrations. Cells were trypsinized at 24, 72 and 120 h time points, counted via a coulter counter and prepared for various testing as outlined below.

Cell growth and cell viability

Cell growth (cells/ml) was estimated by counting 100 µl of cells with a Coulter Counter at each time point. Cell number provides an estimate of the effect of therapy on the inhibition of cellular proliferation, which includes both cell cycle arrest and cell death. Viability was estimated as the proportion of cells that excluded 0.2% trypan blue. Cells were stained for 5 min, loaded onto a hemocytometer and scored as either alive or dead using a 10 square count method.

Western blot analysis

Cells were harvested, centrifuged and washed twice with cold 1X PBS. The pellets were lysed in lysis buffer (50 mM Tris.Cl, 150 mM NaCl, 1%NP-40, 0.5% Sodium Deoxycholate and 0.1% SDS) with a protease inhibitor cocktail [4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), pepstatin A, transepoxysuccinyl-L-leucylamido(4-guanidino)butane(E-64), bestatin, leupeptin and aprotinin]. The protein concentration was determined by BIO-RAD protein assay (BIO-RAD, Hercules, CL). For the detection of E2F-1, p53 and actin, 30 µg of protein was loaded in separate lanes of a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. A 12% SDS-polyacrylamide gel was used for the detection of p21. Proteins were transferred overnight to a polyvinylidone difluoride (PVDF) membrane (BIO-RAD). Proteins were detected using antihuman E2F-1, p53, p21 and actin polyclonal antisera (Sigma). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Amersham) were utilized. Enhanced chemiluminescence (ECL) reagents were used to detect the signals according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Cell cycle analysis

For cell cycle analysis, cell suspensions were washed twice with ice-cold PBS, fixed in 70% cold ethanol and stored at 4°C until all time points were collected. Samples were then washed twice with ice-cold PBS and resuspended in 500 µl of PBS containing 5 µg/ml propidium iodide and 500 µg/ml RNase A. Following a 20-min incubation at room temperature in the dark, flow cytometric analysis was performed using a FAC-scan (Becton-Dickinson). Data was 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.

Confirmation of apoptosis

In addition to DNA fragmentation estimated from the subG1 population, apoptosis was confirmed morphologically by spinning 15,000 cells onto a microscope slide and staining with Wright/Giemsa stains. One thousand cells were counted and scored as either normal, or apoptotic (condensed, intense chromatin staining). In addition, Poly (ADP-ribose) polymerase (PARP) cleavage assays were performed using a monoclonal mouse anti-PARP antibody (Calbiochem, Oncogene Research Products) at a dilution of 1:100.

Statistics

Unless stated otherwise, numerical data presented are the means of at least two experiments. SEs are shown and Student's t-test was used to evaluate whether differences between data sets were statistically significant. Only P-values <0.05 were considered significant.

Results

Dose response assays

Cell count and viability data were collected 5 days after infection of HT-29 and SW620 cell lines with increasing amounts of Ad-E2F-1 and Ad-p53 viral vectors. Dose-dependent inhibition of total viable cell growth (% viability x cell count) was demonstrated following infection with both E2F-1 and p53 viral vectors (Figure 1). Transduction with control virus Ad-LacZ did not result in significant nonspecific viral toxicity at an MOI ≤ 100. IC_{25} (concentration that inhibited total viable cell growth by 25%) values were calculated based on total viable cells and used for all subsequent experiments. Approximate IC_{25} values were calculated and found to be MOI 20 for Ad-E2F-1 and MOI 20 for Ad-p53. This sub-lethal amount of virus was selected for this study so that possible cooperative effects of simultaneous E2F-1/p53 overexpression could be identified.

E2F-1 and p53 Overexpression

Western blot analysis of HT-29 and SW620 protein lysates revealed significant baseline expression of the mutant p53 endogenous protein in both cell lines (Figure 2). Mutant p53 in the HT-29 cell line has a lower molecular weight than the adenoviral wild-type p53 but a higher molecular weight than the virally-expressed p53 in SW620 cells. In addition, previous data indicate that the p53 gene mutations found in these two colon cancer cell lines do not result in dominant negative p53 molecules (Goldsmith *et al.*, 1995; Merchant *et al.*, 1996). Baseline expression of exogenous E2F-1 was

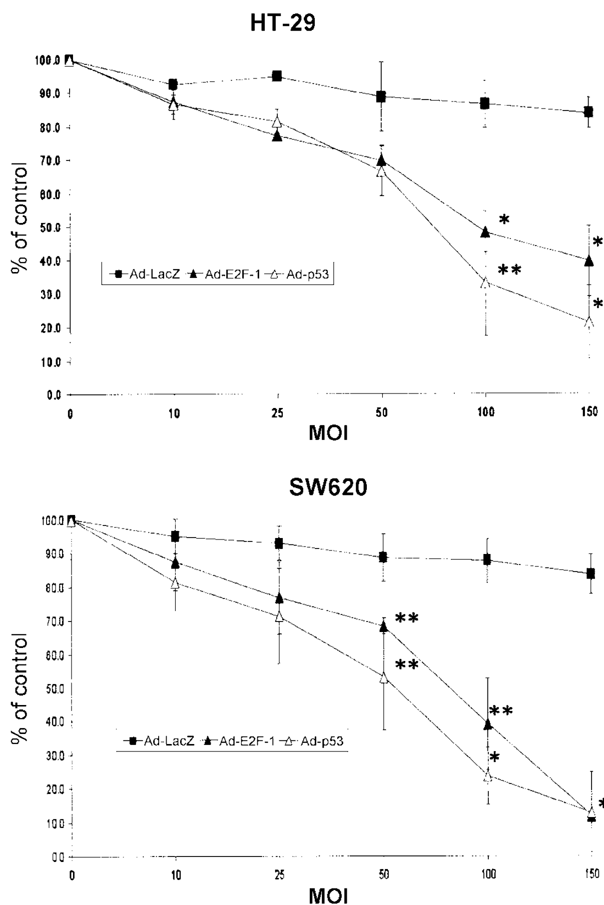

Figure 1. The effect of Ad-E2F-1 and Ad-p53 on viable cell growth of colon cancer cells. Total viable cells were determined by trypan blue exclusion assay and cell counting. Dose response curves demonstrating dose-dependent reduction in total viable cells with increasing multiplicity of infection (MOI) from 10 to 150 with Ad-E2F-1 and Ad-p53 and minimal nonspecific viral toxicity with Ad-LacZ control at an MOI of ≤150. Results are expressed as a percent of mock control. Each data point represents the mean ± SE of three separate experiments. *P*-values vs Ad-LacZ control are *≤.005 and **≤.05.

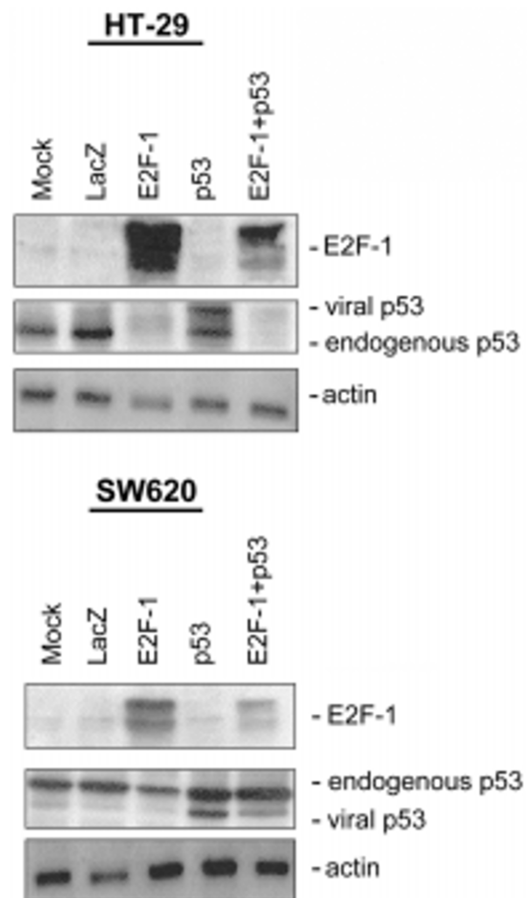

Figure 2. Overexpression of E2F-1 and p53 through adenoviral gene transfer in human colon adenocarcinoma cell lines. The HT-29 and SW620 cell lines were treated by mock infection or infection with adenoviral vectors expressing-galactosidase (LacZ), E2F-1 or p53 in 75mm flasks. Blot represents cells harvested at 5 days and lysed in RIPA lysis buffer. Blot is representative of at least three separate experiments. Actin is shown to demonstrate similar levels of protein loading for each of the samples.

undetectable in control groups treated with mock infection and Ad-LacZ. Substantial overexpression of E2F-1 and p53 was evident after Ad-E2F-1 and Ad-p53 infections, respectively. However, E2F-1 expression was greatly reduced in the E2F-1/p53 combination treatment. Furthermore, both endogenous and viral p53 expression were inhibited following co-infection with the E2F-1 and p53 adenoviruses. These findings suggest negative regulation between E2F-1 and p53 in these colon cancer cells.

Effects on cell growth

A Coulter Counter ZM was used to perform *in vitro* growth analysis of our colon cancer cell lines under various infection conditions. After one day, cells infected with Ad-E2F-1, Ad-p53 and Ad-E2F-1/Ad-p53 displayed no apparent growth inhibition as compared to mock-infected and Ad-LacZ-infected control groups (Figure 3). By three days, cells expressing E2F-1 displayed marked growth inhibition compared to controls. This inhibition became even more pronounced after five days. Cells expressing p53 displayed a similar, although somewhat lower, growth inhibition after three and five days. On day five, cells overexpressing E2F-1 exhibited a 3.8 to 6 fold reduction in cell growth versus control cells, and cell growth was reduced 2.5 to 2.7 fold in the p53-overexpressing cells. Cells co-expressing E2F-1/p53 revealed a 3.0 to 4.3 fold reduction in growth by 5 days post infection when compared to control groups which was between the level of cell growth reduction demonstrated in the cells expressing either E2F-1 or p53 only. The apparent inhibition of growth demonstrated following infection with Ad-E2F-1, Ad-p53 or a combination thereof is most likely due to a combination of cell cycle growth arrest (Figure 5) and cell death (Figure 4). These

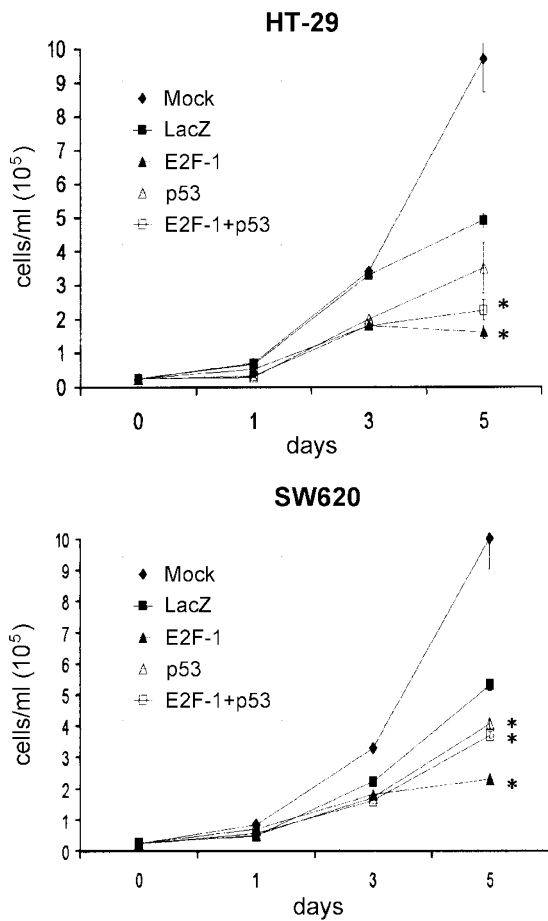

Figure 3. Cell growth analysis of HT-29 and SW620 cells under various gene transfer conditions. Cell growth was determined by counting with a Coulter counter ZM, and results are expressed as cells/ml at time points 1, 3, 5 days post infection. SEs are shown. **P*-values *vs* Ad-LacZ control < 0.05.

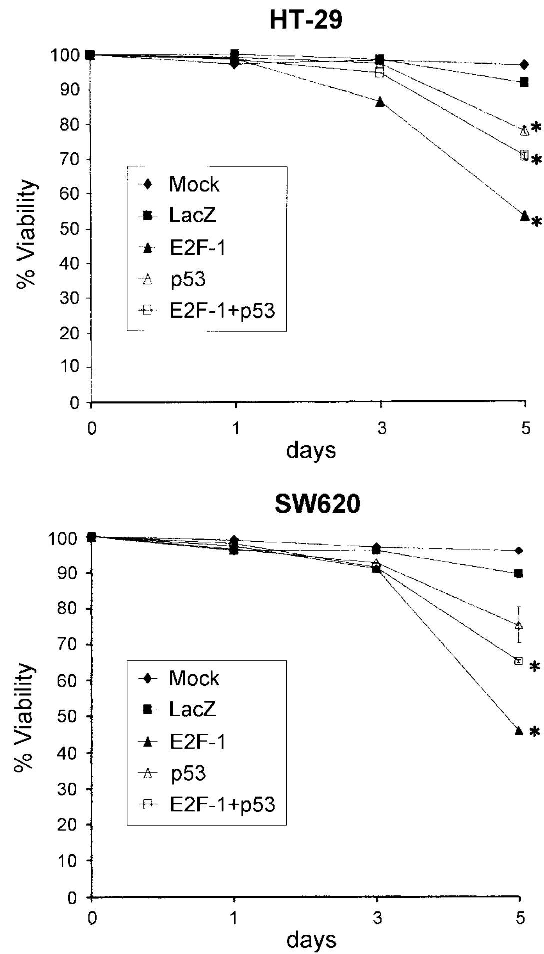

Figure 4. Loss of cell viability after infection with adenoviral vectors expressing E2F-1, p53 or a combination of each. At 1, 3 and 5 days after mock infection, or infection with adenovirus expressing LacZ, E2F-1, p53 or combination, both floating and adherent cells were harvested, counted and scored for viability by the trypan blue exclusion method. Results are expressed as the percentage of viable cells (live cells/total cells) mean ± SE. **P*-values *vs* Ad-LacZ control <0.01.

findings indicate that E2F-1 and p53 do not cooperate to enhance growth inhibition.

Effects on cell viability

The effects of E2F-1 and p53 gene overexpression on cell viability *in vitro* were assessed by trypan blue exclusion. Cells treated with mock infection or Ad-LacZ infection showed little reduction in cell viability after 5 days (Figure 4). Cells infected with Ad-E2F-1, Ad-p53 or Ad-E2F-1/Ad-p53 displayed viability similar to controls after 1 and 3 days. However, by 5 days post infection, only 45.9% to 53.2% of the Ad-E2F-1-treated cells were viable, while 75.2% to 78.0% of the Ad-p53 treated cells remained viable. The viability of cells co-expressing E2F-1 and p53 was 70.7% and 65.2%. Thus, the combination E2F-1/p53 treatment did not result in a cooperative effect on colon cancer cell death.

Cell cycle analysis

To further assess the effects of E2F-1 and p53 co-expression, cell cycle distribution was analyzed. Infections using Ad-E2F-1 alone at an MOI of 100 was associated with a decrease in G1 population and a notable increase in the G2/M population of cells (Ad-E2F-1 = 42 to 60% *vs.* controls = 25-33%) by one day following treatment (data not shown). Ad-E2F-1, at the reduced MOI of 20, still caused a reduction in G1 populations and an increase in the G2/M population by one day in both cell lines, albeit to a lesser degree (Figure 5). By five days, a subdiploid population indicative of apoptosis of 16.9% was evident in the HT-29 cells and 9.8% in the SW620 cells (Figures 6A and B). The p53-expressing cells accumulated in G1 phase by one day following infection in the HT-29 cells but little alteration occurred in SW620 cells. Five days post Ad-p53 infection, approximately 5% were now subdiploid in both cell lines. The co-infection of Ad-E2F-1 and Ad-p53

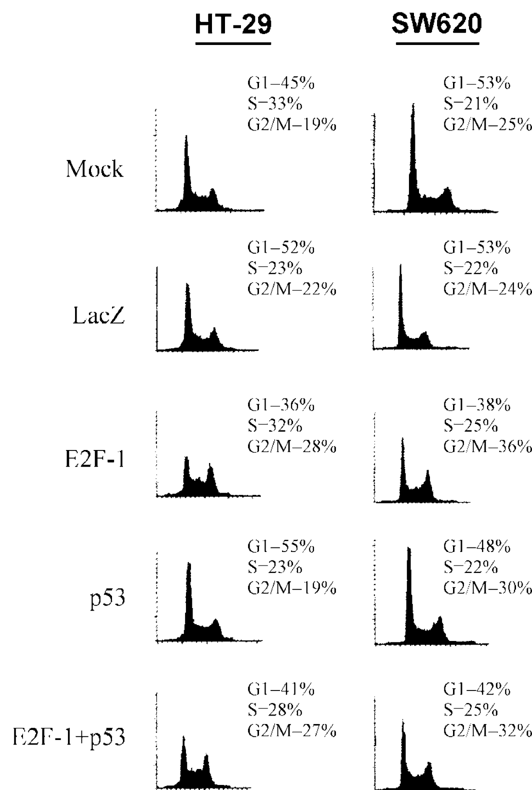

Figure 5. FACS cell cycle analysis. The flow cytometry profiles are shown here for the HT-29 and SW620 cell lines 1 day following infection. Histograms are representative of three separate experiments and percentages are the mean of three separate experiments. Ad-E2F-1 infection alone results in an increase in G2/M phases in both cell lines, whereas p53 induces a G1 accumulation in HT-29 cells but does not alter SW620 cells to any significant degree at the one day time point. Coexpression of p53 with E2F-1 inhibits E2F-1-induced G2/M accumulation in both cell lines. Remainder of cells is found in subG1 populations.

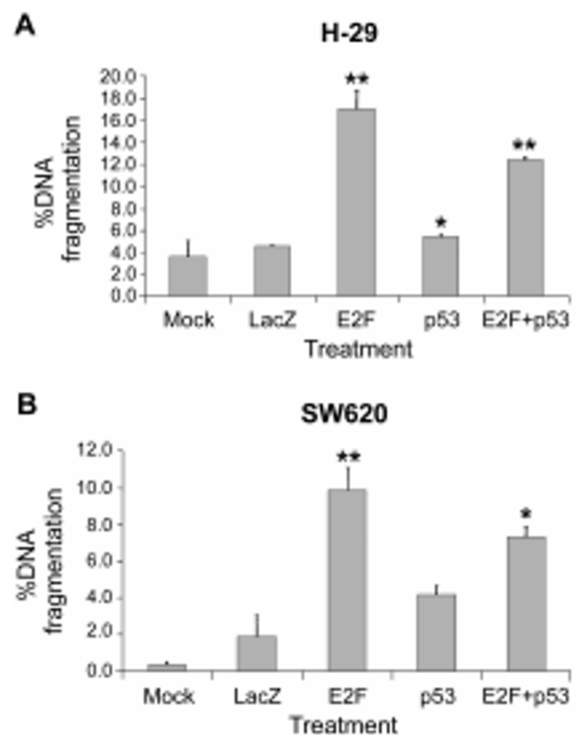

Figure 6. Effect of adenoviral gene transfer on DNA fragmentation (sub-G1 population) indicative of apoptosis. HT-29 and SW620 cells were treated by mock infection, infection with Ad-LacZ, Ad-E2F-1 or Ad-p53 alone or in combination. Samples were collected at the 5 day time point, DNA was stained with Propidium Iodide and DNA fragmentation was measured by flow cytometry. Each data point represents the mean ± SE of three separate experiments. *P*-values vs Ad-LacZ control are *≤0.05 and **≤.0.005.

was associated with fewer cells accumulating in G2/M phase and fewer apoptotic (sub-G1) cells in HT-29's (11.9%) and SW620's (7.3%) as compared to E2F-1-expressing cells only. Mock-infected and Ad-LacZ-infected cells showed little alteration of normal cell cycle profiles and few subdiploid cells. These data suggest that the coexpression of p53 inhibits E2F-1-induced G2/M accumulation and DNA fragmentation.

Confirmation of apoptosis

Cells were examined for morphological changes consistent with apoptosis including cytoplasmic blebbing, chromatin condensation, nuclear fragmentation, and the appearance of apoptotic bodies. Cells infected with Ad-E2F-1 or Ad-p53 alone or in combination induced morphological changes indicative of apoptosis within five days (data not shown). Such changes were not apparent in mock-infected or Ad-LacZ-infected controls. To estimate the percentage of apoptotic cells in each sample, one thousand cells were counted and scored as either normal, or apoptotic (condensed, intense chromatin staining and nuclear fragmentation). As shown in Figure 7, the percentage of apoptotic morphology in cells co-expressing E2F-1 and p53 was less than that seen in cells expressing only E2F-1. On the bases of these findings, co-expression of p53 with E2F-1 significantly inhibits E2F-1-mediated apoptosis in HT-29 and SW620 colon cancer cells.

To provide additional evidence consistent with apoptosis, we probed for cleavage of the nuclear enzyme PARP. PARP cleavage is thought to be one of the earliest events in the execution phase of apoptosis (Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994; Tewari *et al.*, 1995). Although down regulation of full-length PARP was evident in the E2F-1-overexpressing cells in both cell lines, we were unable to detect the 89 kD PARP fragment which is most associated with apoptosis (Figure 8). In the SW620 cells overexpressing both E2F and p53, we also detected a decrease in full-length PARP (113 kD) however, here again, we did not detect the 89 kD apoptotic fragment. Dual gene transfer of p53 with E2F-1 inhibited E2F-1-mediated downregulation of full-length PARP in the HT-29 cells but not in the SW620 cells. We have demonstrated previously that Ad-E2F-1 at MOI

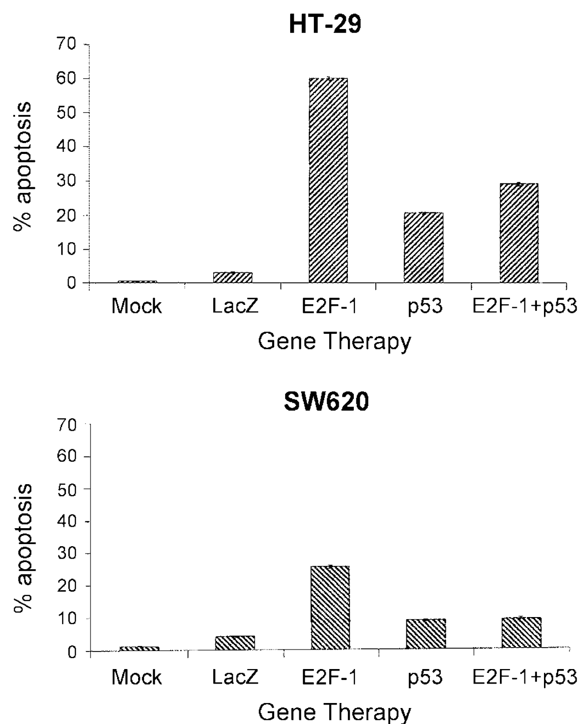

Figure 7. The proportion of morphologically apoptotic cells were estimated by examination of cell cytospins 5 days following mock infection and adenoviral gene transfer of E2F-1 or p53 alone or in combination. Each data point represents the mean ± SE of two separate experiments. *P*-values for treatment vs Ad-LacZ control are <.01.

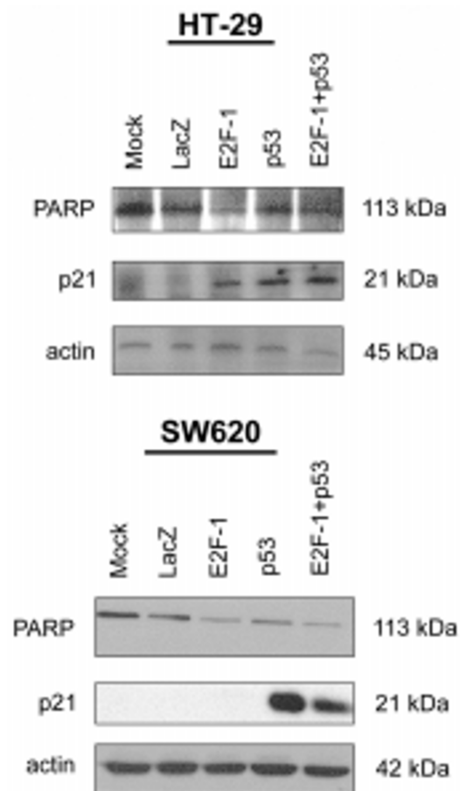

Figure 8. PARP and p21 expression at 5 days following treatment. Full-length (113 kD) PARP is down regulated in E2F-1-expressing cells in both cell lines. p53 inhibits E2F-1-mediated downregulation of PARP in HT-29 cells but not in SW620 cells. p21 upregulated following overexpression of E2F-1 and p53 in HT-29 cells but SW620 cells demonstrated an increase in p21 protein only in the WT p53-overexpressing cells. Actin is shown to demonstrate similar levels of protein loading for each of the samples.

100 activates the caspase cascade and induces cleavage of PARP (Yong *et al.*, Dong *et al.* and non-published data). Although we could not detect the 89 kD PARP fragment following infection with the reduced MOI of Ad-E2F-1 used in these experiments, apoptosis is believed to be the mode of death based on the apoptotic morphology observed.

Upregulation of p21$^{WAF1/CIP1}$

The cyclin-dependent kinase inhibitor, p21$^{WAF1/CIP1}$, is a transcriptional target of p53 and is known to cause cells to arrest in both G1 and G2/M phases of the cell cycle. Recently, E2F-1 has also been shown to upregulate p21 and subsequently, E2F-1 activity is inhibited, thus forming a negative feedback loop (Hiyama *et al.*, 1998). To further investigate the mechanisms of E2F-1 and p53 interactions, we probed for p21. We found that cells infected with Ad-E2F-1, Ad-p53 and Ad-E2F-1/Ad-p53 all exhibited increased expression of p21 in the HT-29 cell line, but only those SW620 cells infected with Ad-p53 either alone or in combination with Ad-E2F-1 showed upregulation of p21 (Figure 7). Interestingly, in the HT-29 cells, although p21 was upregulated to a similar degree in the E2F-1-expressing cells, only those cells co-expressing wild-type p53 were able to inhibit E2F-1-mediated G2/M accumulation and apoptosis (compare Figures 6 and 7). These data indicate that p53 may cooperate with p21 to inhibit E2F-1 activity. Additionally, although Ad-p53 induced p21 in SW620's, a G1 arrest was not achieved and may be due to other inherent mutations in this cell line.

Discussion

Considerable debate surrounds the role of p53 in the E2F-1-mediated apoptotic pathway. Early studies of E2F-1-mediated apoptosis suggest that the cooperation of wild-type p53 is necessary to induce cell death (Qin *et al.*, 1994; Wu and Levine, 1994; Kowalik *et al.*, 1995; Sladek, 1996; Phillips *et al.*, 1997; Hsieh *et al.*, 1997; Kowalik *et al.*, 1998), while subsequent studies report that E2F-1-mediated apoptosis can occur through a p53-independent pathway (Berry *et al.*, 1996; Hunt *et al.*, 1997; Dong *et al.*, 1999; Yang *et al.*, 1999; Phillips *et al.*, 1999). Combination E2F-1 and p53 gene therapy in a variety of tumor cell lines confirms the dual ability of

E2F-1 to induce apoptosis through p53-dependent and -independent pathways (Agah *et al.*, 1997; Frank *et al.*, 1998; Bates and Vousden, 1999). Taken together, these data imply that cell lineage and genetic alterations of particular tumor cells play an important role in whether or not p53 is required in E2F-1-mediated apoptosis.

The mechanism of E2F-1-induced apoptosis remains ambiguous. It has been suggested that apoptosis and cell cycle progression are separable functions of E2F-1 (Phillips *et al.*, 1997). Although DNA binding is required, transactivation is not necessary for the induction of apoptosis by E2F-1 (Hsieh *et al.*, 1997). It is believed that E2F-1 induces a p53-dependent apoptotic response by an accumulation of p53 (Hiebert *et al.*, 1995; Kowalik *et al.*, 1998) that involves the induction of human $p14^{ARF}$, which can then stabilize p53 levels by blocking MDM2-mediated degradation of p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Bates *et al.*, 1998). One p53-independent pathway has been proposed that suggests E2F-1 potentiates cell death by blocking anti-apoptotic signaling pathways thereby sensitizing cells to apoptosis by a number of factors other than p53 (Phillips *et al.*, 1999).

The mechanism by which p53 induces cell cycle arrest and apoptosis are better understood. In response to DNA damage, the p53 protein is stabilized by phosphorylation and transactivates genes that are associated with cell-cycle arrest ($p21^{WAF1/CIP1}$) or death (Bax) (Miyashita *et al.*, 1994; Ko and Prives, 1996; Cox, 1997; Sherr and Roberts, 1999). Variation in p53 induction is thought to play a key role in the choice between cell-cycle arrest and death (Chen *et al.*, 1996). Under conditions of modest genomic damage, the detection of lower levels of p53 correlates with p21 induction and cell-cycle arrest to halt cell growth and allow the initiation of DNA repair (Bates and Vousden, 1999). Whereas, more extensive DNA damage induces higher levels of p53, Bax induction and initiation of the apoptotic cascade (Bates and Vousden, 1999).

We have shown previously that infection with Ad-E2F-1 at an MOI of 100 triggers swift apoptosis in HT-29 and SW620 cells (unpublished data). For purposes of these studies, we used reduced levels of Ad-E2F-1 (MOI 20) in order to detect cooperative or inhibitory effects in the E2F-1/p53 combination therapy. HT-29 and SW620 cells are colon adenocarcinomas with mutant p53 genes; when infected with Ad-E2F-1 at MOI 20, these cells displayed a reduction in cell growth and viability (Figures 3, 4). Cell cycle analysis revealed an E2F-1-induced accumulation of cells in G2/M phase by 24 h (Figure 5) and a subsequent significant subdiploid population of cells by 5 days in both cell lines (Figure 6 A & B). Although we did not detect cleavage of PARP to the apoptotic 89 kD fragment, Wright/Giemsa staining displayed a significant number of cells with condensed morphology, intense chromatin staining and nuclear fragmentation consistent with death by apoptosis (Figure 7). Therefore, we found that even at the reduced levels used in these experiments, cells treated with Ad-E2F-1 still exhibited apoptosis as the mode of death. Furthermore, E2F-1 mediated apoptosis in these colon cancer cells is not dependent on the presence of wild-type p53.

Though E2F-1 mediated apoptosis is not p53 dependent in our *in vitro* colon cancer cell models, the effects of co-expressing wild-type p53 with E2F-1 transgene expression have not been clearly defined. It has been postulated that the re-introduction of wild-type p53 into transformed cells may sensitize cycling cells to chemotherapy by altering cell cycle dynamics and driving cells to a G1 cell cycle arrest or apoptosis (Clayman *et al.*, 1993; Liu *et al.*, 1994; Liu *et al.*, 1995). Alternatively, wild-type p53 may assume functionality by binding with E2F-1 and activating alternative proapoptotic mechanisms (Wu and Levine, 1994; Qin *et al.*, 1994; Sladek, 1996; Frank *et al.*, 1998; Kowalik *et al.*, 1998). Interestingly, Viashnav and Pant have reported that p53 specifically inhibits activated transcription of certain members of E2F family; however such inhibition was shown not to occur with E2F-1 (Vaishnav and Pant, 1999), supporting our hypothesis that E2F-1 and p53 might complement each other in the pathway to apoptosis. However, in our colon cancer cells, the simultaneous overexpression of the p53 transgene with the E2F-1 transgene was shown to antagonize E2F-1 mediated apoptosis. In fact, concomitant p53 expression counteracted E2F-1-mediated G2/M accumulations and reproducibly reduced its apoptotic effect. Additionally, the cells co-infected with Ad-E2F-1 and Ad-p53 demonstrated cell viability that was intermediate between the levels of cell viability associated with E2F-1 or p53 gene transfer alone (Figure 4). Morphological evidence of apoptosis in the co-infected cells was markedly less than that seen in cells infected only with Ad-E2F-1 (Figure 7). Furthermore, simultaneous infection of Ad-E2F-1 with Ad-p53 inhibited the expression of virally-expressed E2F-1 (Figure 2). We assume, this diminished expression of the E2F-1 protein was responsible, at least in part, for the reduced apoptosis seen in cells co-infected with Ad-E2F-1 and Ad-p53. Although we found no evidence of a cooperative effect at any dose levels tested, we cannot rule out the possibility that some combination of Ad-E2F-1 or Ad-p53 might produce a cooperative effect.

Interestingly, cells expressing the E2F-1 transgene, the p53 transgene, or both induced the expression of the CDK inhibitory protein, $p21^{WAF1/CIP1}$ in the HT-29 cells (Figure 8). It is well documented that factors other than p53 can induce p21 gene expression (Li *et al.*, 1995; Halevy *et al.*, 1995; Qiu *et al.*, 1996; Greenhalgh *et al.*, 1996; Billion *et al.*, 1996; Lee *et al.*, 1998). Our data suggest that E2F-1 may have a similar ability to induce p21 under certain circumstances and is in

agreement with a recent study where E2F-1 was found to transactivate p21 (Hiyama *et al*., 1998). Although one study reported that p21 growth arrest fails to protect gliomal cells from E2F-1-mediated apoptosis (Gomez-Manzano *et al*., 1999), a growing body of evidence now points to an opposing relationship between E2F-1 and p21 in both cell cycle control and induction of apoptosis (Chen *et al*., 1995; Shiyanov *et al*., 1996; Dimri *et al*., 1996; Hiyama *et al*., 1998; Delavaine and La Thangue, 1999). In the HT-29 cells, E2F-1-induced p21 did not prevent E2F-1-mediated apoptosis when expressed in the absence of wild-type p53. However, when p21 was expressed in the presence of wild-type p53, cell cycle analysis revealed a reduction in E2F-1-mediated S phase entry, G2/M accumulation and cell death by apoptosis. Therefore, these findings suggest that in the presence of wild-type p53, p21 acts as an antagonist to E2F-1-mediated apoptotic pathways.

One possible explanation for the inability of p21 to effectively inhibit E2F-1-mediated apoptosis in the absence of wild-type p53 is that the level of p21 seen in the Ad-E2F-1-infected cells may not be sufficient to prevent the activity of massively overexpressed E2F-1 mediating cell cycle progression. Co-expression of p53 with E2F-1 may provide additional cellular interactions that enhance the ability of p21 to successfully halt the cell cycle at the G1 checkpoint, rendering the cell resistant to E2F-1-associated G2/M accumulation and apoptosis.

We have demonstrated that adenovirus-mediated E2F-1 gene transfer is able to induce apoptosis in mutant p53 colon cancer cells. Combination E2F-1 and p53 gene transfer did not offer a therapeutic advantage in colon cancer cells *in vitro*; rather, the combined adenovirus-mediated wild type p53/E2F-1 gene transfer inhibited E2F-1 expression, E2F-1-mediated growth inhibition and E2F-1-induced apoptosis. We have also shown that the overexpression of E2F-1 was able to induce the expression of endogenous p21 in the HT-29 cell line but not in SW620 cells. Unlike the well-described induction of p21 by wild-type p53, E2F-1 mediated induction of p21 did not result in a G1 cell cycle arrest. Instead, E2F-1 mediated induction of p21 was associated with accumulation of cells in G2/M and cellular commitment to apoptosis. Further study into the role of p21 may provide a possible link to the actions of p53 and E2F-1 and their complex interplay in apoptosis and the regulation of the cell cycle.

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