Induction of Apoptosis in Human Esophageal Cancer Cells by Sequential Transfer of the Wild-Type p53 and E2F-1 Genes: Involvement of p53 Accumulation via ARF-mediated MDM2 Down-Regulation

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ABSTRACT

Transcriptional factor E2F-1 as well as tumor suppressor p53 have been shown to cause apoptosis independently in some types of human cancer cells when overexpressed. Here we report that sequential transfer of the wild-type p53 and E2F-1 genes efficiently induces apoptosis in human esophageal cancer cells and that E2F-1 overexpression directly activates expression of p14 (ARF), which inhibits MDM2-mediated p53 degradation, resulting in the stabilization of p53. Infection of human esophageal cancer cell lines T.Tn and TE8 with adenovirus vector-expressing E2F-1 (Ad-E2F-1) enhanced mRNA and protein expression of ARF and decreased MDM2 protein expression. Transfection of ARF plasmid decreased MDM2 protein expression, which in turn increased p53 protein expression. Infection of T.Tn and TE8 cells first with adenovirus-expressing wild-type p53 (Ad-p53) and then with Ad-E2F-1 resulted in rapid induction of apoptosis; in contrast, simultaneous infection with Ad-E2F-1 and Ad-p53 had no significant antitumor effect. As shown by Western blot analysis, infection with suboptimal concentrations of Ad-E2F-1 induced the accumulation of exogenous p53 transduced by suboptimal concentrations of Ad-p53. Moreover, Ad-E2F-1-mediated ARF expression inhibited the up-regulation of MDM2 by overexpressed p53 in TE8 cells. Thus, overexpression of ectopic E2F-1 protein may stabilize endogenous as well as ectopic p53 protein via the E2F-1/ARF/MDM2/p53 regulatory pathway and, in this way, render cells more sensitive to apoptosis, an outcome that has important implications for the treatment of human esophageal cancers.

INTRODUCTION

The E2F transcriptional factor family was first identified as a group of proteins with E1A-inducible cellular activity (1) and was later shown to transactivate their target genes by phosphorylating retinoblastoma protein (2). Expression of the E2F-1 product, which is the best characterized member of the E2F family, can promote cell-cycle progression through the G1 checkpoint by activating a series of genes that are critical for the G1 to S phase transition (3, 4). In addition, overexpression of E2F-1 can lead to transformation of an established rat embryo fibroblast cell line (5) or, in cooperation with an activated ras oncogene, to oncogenic transformation of rat embryo cells (6). These observations suggest that the E2F-1 gene is oncogenic; other evidence (i.e., E2F-1 knockout mice develop spontaneous tumors in several tissues) suggests that E2F-1 is also a tumor suppressor (7). Moreover, because the induction of S phase by E2F-1 is also accompanied by the characteristic effects of programmed cell death (8), it may also play a role not only in stimulating cell proliferation but also in coordinating apoptosis.

Overexpression of E2F-1 can activate CPP32, one of the most important apoptosis-inducing molecules, and can cause cleavage of the death substrate poly(ADP-ribose) polymerase, which suggests that activation of the caspase cascade may be a pivotal mechanism in E2F-1-mediated apoptosis (9). Moreover, this pathway seems separate from the p53-mediated pathway, because E2F-1 does not increase the expression level of bax protein (a putative p53-mediated apoptotic pathway; Ref. 9). However, in light of our previous finding that overexpression of the wt-p53 gene by recombinant, replication-deficient viral...
vectors induces apoptosis in a variety of human cancer cells of differing p53 status (10–14), we have hypothesized that the combined transfer of wt-p53 and E2F-1 genes into human cancer cells could be therapeutically advantageous because two different but major apoptotic pathways would be activated.

Recent studies have identified physical interactions between E2F-1 and ARF (human p14ARF and mouse p19ARF; Refs. 15, 16), which shares a portion of the p16INK4a coding region and has a unique first exon (termed exon 1b) originating approximately 20 kb centromeric to p16INK4a exon 1 (termed exon 1a; Ref. 17). Potential E2F-1 binding sites have also been detected in the sequence of the ARF promoter region (18). A proto-oncogene MDM2, which can be induced by wt-p53 activity, binds to p53 and masks the p53 transcriptional activation domain (19). MDM2 targets p53 protein for degradation in the ubiquitin pathway, which results in abrogation of its antiproliferative and apoptosis-promoting effects (20). In addition, it has been shown that ARF binds to and induces the degradation of the proto-oncogene MDM2, which results in stabilization of p53 (21). Furthermore, cell-cycle arrest mediated by ARF can be abolished in cells lacking functional p53 (22, 23), which indicates that ARF may act upstream of p53.

In the present study, we demonstrated that: (a) E2F-1 gene transfer can down-regulate MDM2 protein levels through activation of ARF and thereby enhance apoptosis induced by exogenously transfected wt-p53; and (b) sequential transduction of p53 and E2F-1 promote apoptosis more efficiently than does simultaneous introduction of these genes. Together, our data indicate that this combination strategy may be useful in treating human squamous cell carcinoma of the esophagus.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The human esophageal cancer cell line T.Tn, which has a point mutation at codon 272 in p53 (mut-p53), was obtained from JCRB (Japanese Collection Research Bioreources, Japan) and maintained in DMEM/F-12 supplemented with 10% FCS, 100 units/ml penicillin, and 100 mg/ml streptomycin. The human esophageal cancer cell line TE8, which contains wt-p53, was kindly provided by Dr. Nishihira (Tohoku University, Sendai, Japan) and maintained in RPMI 1640 supplemented with 10% FCS, 25 mm HEPES, and penicillin/streptomycin. A p53-expressing subline of HeLa cells, HeLa-s3, was maintained in DMEM supplemented with 10% FCS and penicillin/streptomycin. The transformed embryonic kidney cell line 293 was grown in high-glucose (4.5 g/liter) DMEM supplemented with 10% FCS and penicillin/streptomycin.

Recombinant Adenoviruses. A recombinant, replication-deficient adenovirus vector that was capable of expressing either human p53 (24) or E2F-1 (25) was previously constructed and characterized. The resultant viruses were termed AdSCMVp53 (Ad-p53; Refs. 10–14) and AdSCMV E2F-1 (Ad-E2F-1; Ref. 25) and have been described herein. An E1A-deleted adenovirus vector lacking a cDNA insert (dl312) was used as a control vector.

RT-PCR. Total RNA was isolated using RNAZOL (Cinna/Bio Tecx, Friendswood, TX) in a single-step phenol-extraction method. One µg of total RNA was subjected to RT using an oligodeoxynucleotidylate primer and avian myeloblastosis virus polymerase (Promega) in a 40-µl reaction volume for 10 min at 23°C and then for 20 min at 42°C. Then, by using a PCR kit according to the manufacturer’s protocol (Perkin-Elmer Corp., Norwalk, CT), 2 µl of the RT reaction product was amplified by PCR in total volumes of 50 µl using the forward and reverse primer for exon 1b human p19ARF gene (p14ARF).

Western Blot Analysis. For Western blot analysis, attached cells collected by trypsinization and cells floating in the culture medium were combined in a 10-ml conical tube and were washed twice in cold PBS. Cells were then lysed in SDS solubilization buffer (0.5 M Tris (pH 6.8), 10% SDS, and glycercol). Equal amounts of proteins were boiled for 5 min and electrophoresed under reducing conditions on a 12.5% (w/v) polyacrylamide gel. Proteins were then electrophoretically transferred to a Hybond-polyvinylidene difluoride (PVDF) transfer membrane (Amersham, Arlington Heights, IL) and were incubated with primary antibodies against E2F-1 (KH95; Oncogene Science, Manhasset, NY), MDM2 (SMP14; Santa Cruz Biotechnology), and ARF (C-18; Santa Cruz Biotechnology) and with mouse antihuman actin monoclonal antibody (Amer- sham). An Amersham enhanced chemiluminescence chemilumi- minescent system was used to detect secondary probes.

Flow Cytometric Analysis. For flow cytometric analysis, cells were collected, washed twice with cold PBS, and resuspended in PBS containing 0.1% Triton X-100 and 1 g/liter RNase for 5 min at room temperature. Samples were then stained with propidium iodide at 50 mg/ml and analyzed for DNA content in a cell sorter (FACScan; Becton Dickinson, Mountain View, CA).

Visualization of Apoptotic Nuclei. To visualize apoptotic nuclei, T.Tn and TE8 cells grown on coverslips were fixed with 1.0% glutaraldehyde and stained with DNA intercalating dye Hoechst 33342 (1 mM in PBS). Fluorescence was visualized using a fluorescence microscope (Zeiss, Oberkochen, Germany).

Plasmid Transfection. Cell transfections were carried out using the LIPOFECT ACE reagent according to the manufacturer’s instructions (Life Technologies, Inc.). Cells were plated on 6-well plates at a density of approximately 10⁶ cells/well and allowed to grow to 70% confluence (24 h after seeding), at which time they were transfected using LIPOFECT ACE in a total volume of 1 ml OPTI-MEM I (Life Technologies, Inc.). The cells were transiently or stably transfected with various amounts of ARF cDNA (pcDNA3-Myc-ARF; 21) as indicated in the figure legend for each experiment. After a 24-h incubation with the DNA/lipid mixture, the cells were replenished with growth media. Stably transfected T.Tn (T.Tn/ARF) and TE8 (TE8/ARF) cells were selected in medium supplemented with...
RESULTS

E2F-1 Overexpression Induces Endogenous ARF Expression and Promotes MDM2 Degradation. To examine whether overexpression of E2F-1 protein specifically affects the expression of other related genes, we first analyzed ARF expression by semiquantitative RT-PCR and Western blotting. Human esophageal cancer cell lines T.Tn, expressing mut-p53, and TE8, which contains wt-p53, were transduced with the E2F-1 gene by exposure to various concentrations of Ad-E2F-1. As revealed by the RT-PCR assay, ARF mRNA transcript expression was basal in parental T.Tn cells and very low in parental TE8 cells. However, when both cell lines were infected with Ad-E2F-1 (100 MOI), ARF mRNA expression was enhanced (Fig. 1A). HeLa-S3 cells, which constitutively express ARF (27), were used as a positive control. ARF and MDM2 protein levels were determined by Western blot analysis of proteins from Ad-E2F-1-infected T.Tn cells. Cells were infected with Ad-E2F-1 (50 MOI) or Ad-E2F-1 (10, 30, or 50 MOI). Equivalent amounts of extracts obtained from whole-cell lysates were loaded, probed with anti-E2F-1, anti-ARF, or anti-MDM2 antibody, and then visualized with an enhanced chemiluminescence detection system. Equal loading of samples was confirmed by stripping each blot and reprobing with antiactin antisera.

500 μg/ml of geneticin (Sigma Chemical Co., St. Louis, MO) for at least 30 days.

Effect of E2F-1 and p53 Overexpression on T.Tn and TE8 Cell Proliferation. A cell proliferation assay was performed to evaluate the effect of Ad-E2F-1 or Ad-p53 infection on T.Tn and TE8 cell growth in vitro. Cells were infected with either Ad-E2F-1 or Ad-p53 at various MOI; cells mock-infected...
with culture medium were used as a control. Cells that remained attached to the culture plates as a monolayer were assessed for viability by measuring their trypan blue uptake. Infection with Ad-E2F-1 and Ad-p53 significantly suppressed the growth of both T.Tn and TE8 cells in a dose-dependent manner, although TE8 cells were more sensitive to E2F-1 and wt-p53 than T.Tn cells in a dose-dependent manner. Infection with Ad-p53 and Ad-E2F-1 as a combination therapy did not enhance apoptosis (Fig. 4B).

To confirm the induction of apoptosis of TE8 cells by sequential infection, cells were harvested 72 h after the Ad-E2F-1 infection and then were analyzed for DNA content by flow cytometric analysis. Ad-p53/Ad-E2F-1-infected TE8 cells exhibited a less-than-expected DNA content in the diploid G0-G1 peak, indicating apoptosis-specific nuclear fragmentation; Ad-E2F-1/Ad-p53 infection, however, did not enhance apoptosis (Fig. 4C).

Blockage of MDM2 Up-Regulation and Induction of p53 Accumulation by Sequential Transfer of wt-p53 and E2F-1 Genes. To further study the mechanism of apoptosis induced by sequential infection with Ad-p53 and Ad-E2F-1, we determined the expression levels of various regulatory proteins after transfer of p53 and/or E2F-1 genes. We used higher MOI of Ad-p53 and Ad-E2F-1 for these experiments, because detection of suboptimal p53 and E2F-1 proteins was difficult, presumably because of the low sensitivity of the assay. When T.Tn and TE8 cells were infected with Ad-p53 at 50 MOI, a substantial increase in the level of exogenous p53 was observed; however, when the cells were coinfected instead with Ad-p53 and Ad-E2F-1 or sequentially infected with Ad-E2F-1 first and Ad-p53 second, a decrease in the level of exogenous p53 gene product was seen. In contrast, the level of p53 was clearly enhanced by sequential infection with Ad-p53 first and Ad-E2F-1 second (Fig. 5A and B), a result presumably attributable to a dramatic reduction in Ad-p53-induced MDM2 protein expression (Fig. 5A).

Finally, to explore further the dependence of rapid MDM2 degradation on E2F-1, TE8 cells were infected first with Ad-p53 at 50 MOI and then with Ad-E2F-1 at various MOI. As shown in Fig. 5C, ectopic overexpression of E2F-1 activated endogenous ARF expression in a dose-dependent manner, resulting in markedly decreased expression of MDM2, which was up-regulated by the feedback loop of p53.

DISCUSSION

The tumor suppressor protein p53 functions as a transcriptional factor that regulates cell-cycle arrest and apoptosis in normal as well as in cancer cells (29). Transfer of the wt-p53 gene by an adenovirus vector has proved effective in inducing apoptosis in some types of human cancer cells (10–13), and its therapeutic efficacy is being assessed in Phase I/II clinical trials in human non-small cell lung cancer (30) and SCCHN (31).
Adenovirus vectors made replication-defective have been used successfully for efficient gene transfer into various types of human tissues. However, because high doses of adenovirus alone may also affect the normal cells surrounding the tumor, the amount of the vector must be minimized. Thus, strategies designed to effectively induce apoptosis in cancer cells may be critical to improving the therapeutic potential of p53 gene therapy. In the present study, we demonstrated that sequential transfer of the wt-p53 gene first and the E2F-1 gene second effectively caused the apoptotic death of human esophageal cancer cells.

Normally, steady-state levels of p53 protein are regulated by complexing the proteins with MDM2, which in turn promotes the rapid degradation of p53. Thus, the cellular concentration of p53 protein is kept low by MDM2, even in the absence of genotoxic stress. Interestingly, the MDM2 gene, a proto-oncogene that is amplified in 30–40% of human sarcomas, is known to be a transcriptional target of p53, and the stress-induced up-regulation of endogenous p53 protein is known to enhance MDM2 protein expression through a feedback regulatory loop (32). Consistent with these observations, we observed that adenovirus-mediated overexpression of exogenous wt-p53 induced MDM2 protein accumulation (Fig. 5, A and C), which in turn may have inhibited the ability of p53 to induce apoptosis.

Fig. 3  Effects of Ad-E2F-1 and Ad-p53 on the growth of T.Tn (A) and TE8 (B) human esophageal cancer cells. Cells were plated in triplicate in 24-well culture plates and infected with either Ad-E2F-1 or Ad-p53 at indicated MOI. Mock-infected cells were used as a control. Cell viability was determined by trypan blue staining on a daily basis. Each point, the mean ± SD of triplicate experiments.
Fig. 4 Induction of apoptosis by sequential infection with Ad-p53 first and Ad-E2F-1 second. In A, T.Tn and TE8 cells were cultured as monolayers in triplicate in 24-well or 6-well culture plates and then infected with Ad-E2F-1 (20 MOI for T.Tn; 5 MOI for TE8) or Ad-p53 (10 MOI for T.Tn; 5 MOI for TE8); they were further exposed to Ad-p53 (10 MOI for T.Tn, 5 MOI for TE8) or Ad-E2F-1 (20 MOI for T.Tn, 5 MOI for TE8) 24 h after the first infection and were finally assessed for cell viability. ■, mock-infected; □, Ad-E2F-1/Ad-p53-infected; □, Ad-p53/Ad-E2F-1-infected. Results were expressed as the mean ± SD of triplicate experiments. Student’s t test was used to compare viability between the treatment groups. * statistical significance (P < 0.05).

B, phase-contrast photomicrographs of T.Tn cells infected with indicated vectors (Ad-E2F-1, 20 MOI; Ad-p53, 10 MOI). Cell morphology was evaluated 48 h after the second infection. Bottom row, right. T.Tn cells infected first with Ad-p53 at 10 MOI and then with Ad-E2F-1 at 20 MOI were stained with Hoechst 33342 and analyzed under a fluorescence microscope. C, TE8 cells exposed first to Ad-p53 at 10 MOI and then to Ad-E2F-1 at 20 MOI were harvested at 72 h posttreatment and then were assayed for DNA content by propidium iodine staining and subsequent flow cytometric analysis. Induction of apoptosis in these cells and cells infected with the vectors in the reverse order was then compared.
Indeed, it has been reported that p53 degradation is accelerated when MDM2 cDNA is transfected into human cells (33). Together, these results suggest that disruption of the feedback regulatory loop between p53 and MDM2 may enhance the cytocidal effect of wt-p53 gene transfer.

In seeking a strategy to specifically inhibit p53-mediated MDM2 up-regulation, we focused on ARF, a protein that can interact directly with the MDM2 protein (21). ARF reportedly inhibits the ubiquitin ligase activity of MDM2 (33) and promotes MDM2 degradation (21). Although the biochemical mechanism underlying ARF-mediated MDM2 degradation remains unclear, our present data show that transfection of human ARF...

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**Fig. 5** Sequential infection of Ad-E2F-1 and resulting stability of exogenous p53 transduced by prior Ad-p53 infection via ARF up-regulation. T.Tn cells (A) and TE8 cells (B) were infected with indicated vectors (Ad-p53, 50 MOI; Ad-E2F-1, 50 MOI; or dl312, 50 MOI) with a 24-h interval between first and second infection. E2F-1, p53, and MDM2 expression were assessed by Western blot analysis 48 h after the second infection. Actin was used as a control to demonstrate equal loading and transfer. C. Western blot analysis of E2F-1, ARF, and MDM2 expression in TE8 cells sequentially infected with 50 MOI of Ad-p53 and with 5, 10, or 50 MOI of Ad-E2F-1.
esophageal cancer cells with a plasmid designed to express an entire sequence of human ARF reduced the levels of constitutive MDM2 expression (Fig. 2A) and induced p53 accumulation (Fig. 2B). The mutant form of p53 in these cells was also stabilized by ectopic ARF expression, consistent with a previous report that a mutation of p53 did not affect the association with MDM2 (34); mut-p53, however, could not activate the transcription, which indicated that ARF function is dependent on the status of the endogenous p53. Recently, it was shown that the ARF promoter is highly responsive to E2F-1 overexpression and that E2F-1 directly activates expression of the human ARF in a p53-null human osteosarcoma cell line (16), which is similar to our finding in human esophageal cancer cells infected with Ad-E2F-1 of both the mRNA and protein levels (Fig. 1, A and B). In addition, we showed the existence of an intact autoregulatory loop between ARF, MDM2, and p53 in those same cells (Fig. 1B).

Together, these results suggest that the possible interaction of p53 and E2F-1 may enhance growth inhibition and/or apoptosis induction, although our finding that simultaneous infection with suboptimal concentrations of Ad-p53 and Ad-E2F-1 could not lead to cell death (Fig. 4B), suggesting that this effect is not so unambiguous. In fact, our finding is consistent with the finding by Frank et al. that coexpression of wt-p53 and E2F-1 did not enhance the growth-inhibitory effect in human SCCHN (35). It is likely that the physical interaction of the p53 and E2F-1 proteins suppresses the antitumor effect of coinfection of Ad-p53 and Ad-E2F-1 in SCCHN. In addition, as our data suggest, sequential infection with Ad-E2F-1 followed by Ad-p53 infection apparently induces no apoptotic cell death in human esophageal cancer cells (Fig. 4B), whereas suboptimal Ad-E2F-1 infection 24 h after suboptimal Ad-p53 infection induces an accumulation of p53 protein (Fig. 5, A and B), and the subsequent rapid induction of apoptotic cell death (Fig. 4). Thus, our data clearly suggest that the order of gene transfer may be important for mediating p53 accumulation as well as for causing apoptosis in human esophageal cancer cell lines.

Although the mechanisms by which Ad-p53/Ad-E2F-1 infection selectively induces apoptosis are not completely understood, the possible existence of a regulatory feedback cycle controlling the level of each protein has important implications. Although Ad-E2F-1 infection promotes endogenous ARF upregulation, the amount of constitutive MDM2 is very low in the absence of exogenous wt-p53; thus, the level of ARF may be rapidly suppressed through the feedback mechanism. In contrast, when Ad-p53 infection precedes Ad-E2F-1 infection, Ad-p53 activates the transcription of MDM2, which leads to the enhanced synthesis of MDM2 protein, which might in turn be effectively degraded by ARF protein induced by the later Ad-E2F-1 infection. Alternatively, the localization of each protein in the subnuclear compartments may be essential for the responses (36). When Ad-E2F-1 infection precedes Ad-p53 infection, Ad-E2F-1 can induce expression of ARF, which may in turn sequester constitutive MDM2 in the nucleus; however, the increased amounts of MDM2 resulting from the Ad-p53 infection may be sequestered in the nucleolus because of the localization of antagonistic ARF in the nucleus, thereby preventing the transactivation in the nucleolus. But as our results show that this is not the case. When Ad-p53-infected cells were subsequently transduced with Ad-E2F-1, up-regulated nucleoplasmic MDM2 protein could be moved to the nucleolus by E2F-1-induced ARF, thus allowing the activation of p53 in the nucleolus. The precise mechanisms that mediate this cooperation, however, remain to be elucidated.

In light of our data, we conclude that wt-p53 and E2F-1 genes, sequentially transferred into human esophageal cancer cells, can cooperate to efficiently induce apoptosis by stabilizing p53 via ARF-mediated MDM2 degradation. We also conclude that, because combination treatment with suboptimal doses of adenovirus vectors is sufficient to achieve antitumor effects, this strategy may also minimize the risk of adenovirus toxicity. Thus, the sequential combination of Ad-p53 followed by Ad-E2F-1 as gene therapy may lead to the development of molecular therapies for human esophageal carcinoma.

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