Combination E2F-1 and p53 Gene Transfer Does Not Enhance Growth Inhibition in Human Squamous Cell Carcinoma of the Head and Neck

Douglas K. Frank, Ta-Jen Liu, Mitchell J. Frederick, and Gary L. Clayman

Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Ample data exist contending that wild-type p53 and E2F-1 cooperate to mediate apoptosis, that E2F-1-mediated apoptosis is p53 dependent in some situations, and that E2F-1 can induce accumulation of p53 in mammalian cells. These data support the investigation of the biological consequences of combined wild-type p53 and E2F-1 overexpression in human squamous cell carcinoma of the head and neck (SCCHN) for the purpose of developing apoptosis-inducing molecular intervention strategies for the management of this devastating disease. The recombinant adenovirus (Ad) vectors Ad-p53 and Ad-E2F-1 were used for wild-type p53 and E2F-1 gene transfers, respectively, into SCCHN cell lines TU138 and TU167. SCCHN cells transduced with either p53, E2F-1, or both underwent in vitro growth analysis, which revealed that simultaneous p53 and E2F-1 gene transfer did not result in enhanced growth inhibition. To explain our growth assay findings on the basis of potential negative molecular interactions between E2F-1 and p53, Western and Northern blotting analyses were performed to investigate the differential expression of the downstream p53-transactivated genes, p21<sup>WAF1</sup> and BAX, under various p53 and E2F-1 gene transfer conditions. Whereas Western immunoblotting demonstrated that E2F-1 antagonized p53 induction of p21<sup>WAF1</sup> and BAX, Northern blotting revealed that this interference was pretranslationally regulated and p53 dependent. Coimmunoprecipitation assay confirmed that the wild-type p53 and E2F-1 gene products formed protein-protein complexes in our cell lines.

INTRODUCTION

The E2F family of genes (E2F-1 through E2F-5) encodes transcription factors involved in DNA replication and cell cycle progression (1–4). Not unexpectedly, overexpression of the E2F-1 family member has been shown to have oncogenic potential (5, 6). Interestingly, E2F-1 also has the ability to induce apoptosis when overexpressed in mammalian cells (2, 7–11), independent of its function as a positive regulator of DNA synthesis and cell cycle progression (2, 7, 9). Further evidence that E2F-1 may also function as a tumor suppressor has come from recent studies in E2F-1<sup>−/−</sup> mice, which developed tumors in various organ systems (12, 13). Additionally, we have established that in vitro and in vivo Ad<sup>q</sup>-mediated wild-type E2F-1 transduction of human upper aerodigestive tract SCCHN cell lines induces apoptosis (4).

Although E2F-1 appears to be capable of independently inducing apoptosis in mammalian cells (2, 7, 8), much attention has been given to the role that wild-type p53 appears to play in the process (8, 9, 11, 14, 15). In some cell systems, exogenously introduced E2F-1 leads to apoptosis only in the presence of endogenous wild-type p53 (11). Another study demonstrated that E2F-1 was able to overcome wild-type p53-mediated growth arrest to cause a massive loss of cell viability (15). Wild-type p53 has also been demonstrated to enhance apoptosis with E2F-1 when both were overexpressed in osteosarcoma cells (9). Additionally, it seems that wild-type E2F-1 was able to posttranscriptionally induce the accumulation of p53 in fibroblasts (14). In this latter study, Ad vectors were used to transfer wild-type E2F-1 and wild-type p53 into the cells of interest.

We have shown that wild-type p53 gene transfer mediated via Ad vectors induces apoptosis in vitro and in vivo in SCCHN (16, 17). Overexpressed wild-type p53 is also a potent inducer of apoptosis in other malignant mammalian cell types (18–21). The existing evidence that wild-type p53 and E2F-1 cooperate to mediate apoptosis in mammalian cells when simultaneously

Received 3/25/98; revised 5/29/98; accepted 6/9/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by an American Cancer Society Clinical Oncology Fellowship Grant (to D. K. F.), National Institute of Dental Research Grant 1-P50-DE11906 (93-9; to G. L. C.), National Institute of Health First Investigator Award R29 AD-DE11689-01A1 (to G. L. C.), and Training of the Academic Surgical Oncologist Grant T32 CA60374-03 (to G. L. C.).

2 To whom requests for reprints should be addressed, at Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 69, Houston, TX 77030. Phone: (713) 792-6920; Fax: (713) 794-4662.

3 The abbreviations used are: Ad, adenovirus; SCCHN, squamous cell carcinoma of the head and neck; β-gal, β-galactosidase; GFP, green fluorescence protein; MOI, multiplicity of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

overexpressed (9, 14, 15) indicated to us that their combined application in an Ad-mediated gene therapy model of SCCHN could be therapeutically advantageous. This is important because SCCHN is a devastating disease that has not seen a significant improvement in survival rates (50%) for several decades with standard surgery, radiotherapy, and chemotherapy (22, 23). The need to develop alternative molecular therapies led us to investigate the biological consequences of combination p53/E2F-1 Ad-mediated gene transfer into SCCHN.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The SCCHN cell lines TU138 and TU167 were developed in the Department of Head and Neck Surgery at the University of Texas M. D. Anderson Cancer Center. The p53 mutant TU138 line (24) contains a single base mutation in exon 5, leading to an amino acid substitution. The p53 mutant TU167 line contains a single base mutation at the exon 5-intron 5 splice site, leading to an abnormally processed transcript. These cell lines were chosen for the purposes of this study because of their high Ad transduction efficiency (see below; Ref. 24). Furthermore, TU138 (16) and TU167 undergo apoptosis upon Ad-mediated wild-type p53 gene transfer. Ad-mediated wild-type E2F-1 gene transfer also induces apoptosis in each of these cell lines. Cells were cultured in DMEM-Ham’s F-12 supplemented with 10% heat-inactivated fetal bovine serum.

Recombinant Ad and Infection Strategy. Wild-type p53 and wild-type E2F-1 gene delivery to our SCCHN cell lines was accomplished with recombinant Ad vectors. The recombinant wild-type p53 Ad vector (Ad-p53) used in our studies has been described in detail elsewhere (24). A similar vector containing wild-type E2F-1 cDNA was also used. The negative control Ad used, Ad-DE1, contains no therapeutic gene.

The Ad gene-transduction efficiency of our SCCHN cell lines is high, thus ensuring homogeneous delivery and subsequent overexpression of genes of interest. Detection of expression of the β-gal marker gene in our cell lines after infection with the recombinant β-gal Ad (Ad-β-gal) revealed that 60% of infected cells are transduced at 100 MOI (24). β-gal expression is detected via an enzymatic reaction that turns transduced cells blue (detectable by direct visualization). A much more sensitive Ad transduction efficiency assay using Ad-GFP, a recombinant Ad containing the GFP marker gene (detectable by flow cytometry of infected cell populations) has revealed that in fact nearly 100% of our infected SCCHN cells are transduced at only 20 MOI.

TU138 and TU167 were treated under five infection conditions for the purposes of conducting cell growth assays, acquiring protein lysates for Western immunoblotting and coimmunoprecipitation, and obtaining RNA for Northern blotting. These five infection conditions were as follows: Ad-p53 infection; Ad-E2F-1 infection; Ad-p53 plus Ad-E2F-1 infection (simultaneous combined infection); mock infection (negative control); and Ad-DE1 infection (negative control). The amount of Ad used for gene transfer was 20 MOI for Ad-p53 and 50 MOI for Ad-E2F-1 (in individual and combination infections for both vectors). The MOIs of Ad-p53 and Ad-E2F-1 were carefully chosen (via separate viral dose/percentage of growth inhibition assays) so that neither vector alone would result in death of the entire cell population, allowing for determination of combined transduction effects in cellular growth assays. The total amount of Ad used under each infection condition was controlled at 70 MOI, because this was the amount used in combination Ad-p53/Ad-E2F-1 infections. Thus, in Ad-p53 (20 MOI) or Ad-E2F-1 (50 MOI) individual infections, the balance of infecting Ad was achieved with Ad-DE1.

Cell Growth Assays. Subsequent to monolayer infection with Ad-p53, Ad-E2F-1, or Ad-p53 plus Ad-E2F-1, cell growth assays were conducted on TU138 and TU167. Mock and Ad-DE1 infections were conducted as negative controls. Cells were counted on a Coulter Counter (Coulter Corp., Miami, FL) after trypsinization at days 1, 3, 5, and 7 subsequent to infection. Infections were carried out in triplicate for each time point. Experiments were repeated twice.

Western Immunoblotting. TU138 and TU167 cell lysates were obtained 24 h after monolayer infection with Ad-p53, Ad-E2F-1, or Ad-p53 plus Ad-E2F-1. Mock and Ad-DE1 infections were conducted as negative controls. Lysates were obtained by sonicating harvested cells in lysis buffer containing 5 mM EDTA, 50 mM Tris, 150 mM NaCl, 1% NP40, and 0.1% SDS supplemented with aprotinin, leupeptin, and phenylmethylsulfonyl fluoride. A total of 50 µg of each lysate was resolved by 10% SDS-PAGE, followed by transfer to Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membranes (Amer sham, Arlington Heights, IL). Membranes were incubated with the following primary antibodies: mouse DO-i antihuman p53 monoclonal (recognizes wild-type and mutant p53 forms) and mouse KH95 antihuman E2F-1 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit C-19 antihuman p21Waf1 monoclonal antibody (Boehringer Mannheim Corp., Indianapolis, IN) or goat anti-rabbit IgG conjugated to horseradish peroxidase (Boehringer Mannheim Corp., Indianapolis, IN) or goat anti-rabbit IgG conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) as appropriate. Blots were developed on radiographic film by the ECL method (Amer sham) as outlined by the manufacturer. Mouse AC40 anti-human actin monoclonal antibody (Sigma Corp., St. Louis, MO) was used to probe all blots as a loading control. Relative p21Waf1 and BAX message expression levels in all blots were quantitated by densitometry (Molecular Dynamics, Sunnyvale, CA) after normalization to loading controls.

Northern Blotting. Total RNA was isolated from TU138 and TU167 using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer’s instructions subsequent to monolayer infection with Ad-p53, Ad-E2F-1, or Ad-p53 plus Ad-E2F-1. Mock and Ad-DE1 infections were conducted as negative controls. Isolates were obtained 12 h after infection for TU138 and 20 h after infection for TU167. In separate kinetics studies investigating p53 gene transfer alone, maximal induction of p21Waf1 and BAX transcripts at these times points in these cell lines was demonstrated. Ten µg of each RNA isolate were fractionated by formala-
hyde-agarose gel electrophoresis and transferred overnight to Hybond-N+ nylon membranes (Amersham) by capillary blot technique using SSC. Membranes were hybridized with α-32p-labeled cDNA probes that were complimentary to either p21\textsuperscript{WAF1} (25), BAX (kind gift of Dr. S. J. Korsmeyer, Howard Hughes Medical Institute, Washington University, St. Louis, MO), or GAPDH (loading control). Probed blots were subsequently exposed to radiographic film at -80°C in the presence of an intensifying screen. Relative p21\textsuperscript{WAF1} and BAX transcript expression levels in all blots were quantitated by densitometry (Molecular Dynamics) after normalization to loading controls.

Coimmunoprecipitation Assays. TU138 cell lysates were obtained 24 h after monolayer infection with Ad-p53, Ad-E2F-1, or Ad-p53 plus Ad-E2F-1. Mock and Ad-DE1 infections were conducted as negative controls. Lysates were obtained by sonicating harvested cells in lysis buffer containing 25 mm Tris, 140 mm NaCl, 5 mm EDTA, 10 mm NaF, and 1% NP40 supplemented with leupeptin, aprotinin, and phenylmethylsulfonyl fluoride. Subsequent to preclearing lysates with protein A-agarose beads (Santa Cruz Biotechnology) pretreated with normal mouse IgG antibody (Dako, Glostrup, Denmark), lysates were treated with protein A-agarose beads pretreated with mouse DO-1 antihuman p53 monoclonal antibody. Immunoprecipitates were collected by centrifugation and subsequently resolved by 10% SDS-PAGE before transfer to Hybond ECL nitrocellulose membranes. For each experimental condition, 800 μg of protein were immunoprecipitated and resolved by SDS-PAGE in preparation for Western blotting. Membranes were incubated with rabbit C-20 antihuman E2F-1 polyclonal antibody. Secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase. Blots were developed on radiographic film by the ECL method as outlined by the manufacturer.

RESULTS

Simultaneous Overexpression of Wild-Type p53 and Wild-Type E2F-1 in SCCHN Does Not Enhance Growth Inhibition. Western immunoblotting for TU138 and TU167 demonstrated clear overexpression of the p53 and E2F-1 gene products after Ad-p53 and Ad-E2F-1 infections, respectively, as well as in infections where Ad-p53 and Ad-E2F-1 were introduced together (Fig. 1). Levels of p53 and E2F-1 expression were not altered in the combined infection relative to Ad-p53 and Ad-E2F-1 individual infections, suggesting no direct effects of the wild-type E2F-1 gene product on wild-type p53 gene product expression or vice versa.

Growth analysis of TU138 and TU167 cell populations infected with Ad-p53 plus Ad-E2F-1 revealed no enhancement (neither synergistic nor additive) in growth inhibition relative to the growth of respective cell populations infected with either vector alone (Fig. 2). These findings seemed to demonstrate that the interaction between p53 and E2F-1 was not cooperative. Furthermore, this data suggested that the p53 and E2F-1 gene products were not functioning independently of one another in SCCHN, and that they may be involved in negative molecular interactions.

Overexpression of Wild-Type E2F-1 Inhibits the Induction of p21\textsuperscript{WAF1} in Wild-Type p53-overexpressing SCCHN Cells. On Western immunoblots, when TU138 and TU167 were infected with Ad-p53 alone, we observed the expected induction of the gene product of the known wild-type p53 transactivated gene (26) that induces G1 cell cycle arrest (25), p21\textsuperscript{WAF1}. However, when wild-type E2F-1 was coconmitantly overexpressed with wild-type p53, we saw a 3.79- and 4.30-fold reduction in p21\textsuperscript{WAF1} gene product induction in TU138 and TU167, respectively (Fig. 3, A and C). Northern blotting showed a similar down-regulation of the p21\textsuperscript{WAF1} transcript in Ad-p53/Ad-E2F-1-infected TU138 cells (2.16-fold reduction) and
Combination E2F-1 and p53 Gene Transfer

TU167 cells (3.19-fold reduction) relative to the induction seen in Ad-p53-infected cells (Fig. 3, B and D). A pretranslational effect of the wild-type E2F-1 gene product on wild-type p53 induction of p21\textsuperscript{Waf1} was suggested by this latter finding.

**Overexpression of Wild-Type E2F-1 Inhibits the Induction of BAX in Wild-Type p53-overexpressing SCCHN Cells.**

On Western immunoblots, when TU138 was infected with Ad-p53 alone, we observed the expected induction of the gene product of the known wild-type p53 transactivated gene (27) that contributes to p53-mediated apoptosis (28–30), BAX. However, when wild-type E2F-1 was concomitantly overexpressed with wild-type p53, we saw an 8.25-fold reduction in BAX gene product induction (Fig. 4A). Similar to our observations of p21\textsuperscript{Waf1} transcript levels, Northern blotting showed a down-regulation of BAX transcript in Ad-p53/Ad-E2F-1-infected TU138 cells (1.3-fold reduction) and TU167 cells (2.24-fold reduction) relative to the induction seen in Ad-p53-infected cells (Fig. 4, B and C). A pretranslational effect of the wild-type E2F-1 gene product on wild-type p53 induction of BAX was suggested by this latter finding. Wild-type E2F-1 gene transfer alone did not reduce endogenous levels of BAX as seen on Western and Northern blots (Fig. 4) and quantitatively confirmed via densitometry. This demonstrated that the negative effect that wild-type E2F-1 was having on the expression of BAX (and probably p21\textsuperscript{Waf1}) was wild-type p53 dependent.

**The Wild-Type p53 and E2F-1 Gene Products Form Protein-Protein Complexes in SCCHN.**

TU138 cells infected with Ad-p53 plus Ad-E2F-1 demonstrated that the induced wild-type E2F-1 gene product coprecipitated with wild-type p53 immunoprecipitates (Fig. 5). This confirmed that the wild-type p53 and E2F-1 gene products were capable of forming protein-protein complexes in our SCCHN cell lines. This finding also suggested that the ability of wild-type E2F-1 to suppress wild-type p53 induction of p21\textsuperscript{Waf1} and BAX was likely due to this protein interaction.

**DISCUSSION**

An understanding of the cell cycle events at the molecular level as well as an understanding of how the mediators of these events interact is one of the keys to developing effective molecular intervention strategies for the management of human malignancy. Our previous work with Ad-mediated wild-type p53 gene therapy in SCCHN has yielded promising results (16, 17, 24). However, observations of a cooperative p53/E2F-1 relationship in the mediation of apoptosis in mammalian cells (8, 9, 11, 14, 15) suggested that an even more effective molecular strategy for this devastating disease would use both of these genes simultaneously. The fact that E2F-1 was able to enhance the expression of p53 in fibroblasts added further support to this notion (14).

Despite induction of both the wild-type p53 and wild-type E2F-1 gene products in our SCCHN cell lines after simultaneous Ad-mediated gene transfer, enhanced growth inhibition was not seen (relative to individual p53 or E2F-1 gene transfer). This finding was a surprise and seemed contradictory with data indicating that p53 and E2F-1 cooperate to mediate apoptosis in mammalian cells, including experiments whereby enhanced growth inhibition was seen when both p53 and E2F-1 were simultaneously overexpressed (9). We do not feel that the mutant p53 genotypes of our SCCHN cell lines played a specific role in our growth assay and subsequent experimental findings because wild-type p53 gene transfer induces apoptosis in SCCHN cells regardless of their p53 status (17). Additionally, our study focused on the relationship between exogenously introduced wild-type E2F-1 and p53. Had our cell lines been wild-
Fig. 3 Western immunoblots (A and C) and Northern blots (B and D) of TU138 and TU167 for differential expression of the p21^{WAF1} gene product and transcript, respectively, under various Ad-p53 and Ad-E2F-1 infection conditions. For all blots, the total amount of Ad used under each infection condition was controlled at 70 MOI, because this was the amount used in the Ad-p53/Ad-E2F-1 combination infection. Thus, in Ad-p53 (20 MOI) and Ad-E2F-1 (50 MOI) individual infections, the balance of infecting Ad was achieved with the Ad-DE1 control vector.

Our data indicated that the process leading to a decrease in p21^{WAF1} and BAX induction by p53 in the presence of E2F-1 was a pretranslational event and was p53 dependent (see “Results”). Because the E2F-1 and p53 gene products form protein-protein complexes, it seemed plausible that E2F-1 was interfering with p53 induction of p21^{WAF1} and BAX through this interaction at the level of DNA transcription.

We entertained the possibility that our observations regarding the relationship of E2F-1 and p53 on p21^{WAF1} and BAX expression were nonspecific, given the fact that we were overexpressing each of these genes in the same TU138 and TU167 cells. However, combined infection of Ad-p53 (20 MOI) with Ad-GFP (100 MOI), an Ad vector carrying the gene encoding the GFP (no known interactions with p53), yielded no decrease of p21^{WAF1} or BAX transcript on Northern blotting relative to Ad-p53 infection alone (data not shown). These findings led us to conclude that the regulatory effect that wild-type E2F-1 was having on wild-type p53 was specific.

Although some relate the ability of wild-type E2F-1 to possess the dual function of oncogene and tumor suppressor to absolute intercellular levels of the E2F-1 gene product (12),
Fig. 5 Western immunoblot of p53 immunoprecipitates for E2F-1. Immunoprecipitates were obtained from TU138 lysates under various Ad-p53 and Ad-E2F-1 infection conditions. The total amount of Ad used under each infection condition was controlled at 70 MOI, because this was the amount used in the Ad-p53/Ad-E2F-1 combination infection. Thus, in Ad-p53 (20 MOI) and Ad-E2F-1 (50 MOI) individual infections, the balance of infecting Ad was achieved with the Ad-DE1 control vector.

others feel that E2F-1 will direct the cell down an apoptosis pathway only under circumstances whereby cell cycle progression is abnormal or disturbed (2, 8–10). This latter point includes situations where the cell may prematurely enter S phase, as may be the case when E2F-1 is overexpressed. Nonetheless, it seems clear that the dual abilities of wild-type E2F-1 are separable, independent functions. Its role as a factor involved in cell cycle progression is governed by its ability to transcriptionally activate certain genes, whereas its ability to regulate apoptosis is thought to be governed by its ability to associate with the retinoblastoma protein and bind DNA in an inhibitory fashion (7, 9).

Our data clearly highlight the dual roles that wild-type E2F-1 plays in cell cycle events. A growth-inhibitory effect in both of our cell lines was demonstrated upon exogenous wild-type E2F-1 overexpression. This supports the role of E2F-1 as a tumor suppressor. Yet, E2F-1 down-regulated the induction of two genes known to be important in wild-type p53-mediated growth inhibition, p21\textsuperscript{waf1} and BAX (26, 28–30). This latter point supports the alternative role of E2F-1 as a positive regulator of cell cycle progression.

The negative effect that E2F-1 had on levels of p21\textsuperscript{waf1} and BAX should come as no surprise if we think of its primary
role as a transcription factor involved in cell cycle progression. In this sense, E2F-1 is participating in a feedback loop designed to perpetuate its positive regulatory effects on the passage of cells from the G1 through S phases of the cell cycle. Furthermore, the ability of the p53 and E2F-1 proteins to form physical complexes and the ability of E2F-1 to inhibit p53-driven transcription of p21\textsuperscript{Waf1} reporter constructs has been demonstrated previously (31). Thus, our data support the notion that the primary function of wild-type E2F-1, at least in SCCHN, is to drive cell cycle progression, like its other E2F family constituents. Our finding that E2F-1 also caused growth inhibition in TU138 and TU167 when overexpressed is not inconsistent with this hypothesis. The ability of wild-type E2F-1 to act as a tumor suppressor may occur, as has been suggested, in situations where cell cycle progression is abnormal (2, 8–10). In this sense, E2F-1 may be monitoring the cell for such activity. Exogenous overexpression of wild-type E2F-1 may be prematurely signaling our SCCHN cells to enter S phase, thereby triggering a signal for apoptosis. Furthermore, factors capable of inhibiting the clear apoptotic potential of E2F-1 and allowing E2F-1-mediated cell cycle progression may not be present in our cell lines.

In conclusion, despite evidence that wild-type p53 and E2F-1 cooperate to mediate apoptosis in mammalian cells (8, 9, 11, 14, 15), an enhanced growth-inhibitory effect of combined exogenous overexpression of these two genes was not seen in our Ad-mediated gene therapy model of SCCHN. Our growth assay findings may be explained by the negative molecular interactions we have described between the wild-type E2F-1 and wild-type p53 gene products. Combined Ad-mediated wild-type p53/E2F-1 gene transfer does not offer a therapeutic advantage in SCCHN in vitro. Our data suggest a relationship between p53 and E2F-1 that is quite complicated. Given the physical interaction that we and others (31) have demonstrated between the wild-type p53 and wild-type E2F-1 gene products, it is likely that E2F-1 directly influences either positively or negatively the transcription of other as yet unidentified p53-transactivated genes. It may also be possible that p53 influences either positively or negatively the transcription of E2F-1-transactivated genes. The investigation of such potential relationships will shed further light on the complex interplay between E2F-1 and p53 and help us understand the mitigating factors that may favor potential cooperative interactions.

ACKNOWLEDGMENTS

We thank Mary T. Wang for technical assistance and Dr. S. J. Korsmeyer for kindly providing BAX cDNA.

REFERENCES


Combination E2F-1 and p53 gene transfer does not enhance growth inhibition in human squamous cell carcinoma of the head and neck.

D K Frank, T J Liu, M J Frederick, et al.