Bystander Effect in the Adenovirus-mediated Wild-Type p53 Gene Therapy Model of Human Squamous Cell Carcinoma of the Head and Neck

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

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

ABSTRACT

In models of human lung and head and neck cancer, there have been anecdotal reports of a bystander effect in wild-type p53 gene therapy, an apoptosis-inducing molecular intervention strategy. These reports do not definitively demonstrate the presence of a bystander effect, nor do they elucidate requirements for or characteristics of this phenomenon. We have investigated human squamous cell carcinoma of the head and neck for the presence and requirements of a bystander effect after wild-type p53 gene transduction. Recombinant adenovirus, Ad-p53, was used for wild-type p53 gene transfers. To investigate the role of intercellular contact between p53-transduced and nontransduced cells in mediating a growth inhibitory (bystander) effect on nontransduced cells, coculturing experiments were conducted on human squamous cell carcinoma of the head and neck cell lines TU138 and TU167. For TU138, 29% growth inhibition of nontransduced cells was demonstrated 3 days after p53-transduced and nontransduced cells were cocultured with intercellular contact. This growth inhibition was abolished when cells were cocultured without intercellular contact. TU167 did not demonstrate a bystander effect under any coculturing condition. Supernatant from p53-transduced cells demonstrated no growth-inhibitory effect on respective nontransduced cells. The bystander effect in the adenovirus-mediated wild-type p53 gene therapy model of squamous cell carcinoma of the head and neck, when present, requires intercellular contact. Possible mechanisms of the observed in vitro bystander effect are discussed.

INTRODUCTION

The phenomenon whereby cancer cells that have received a specific therapy are able to bring about growth inhibition in untreated cells is called a “bystander” effect. The existence of the bystander effect in the management of human malignancy has been demonstrated in HSV-tk\(^3\) gene therapy (1–6). Cancer cells expressing transfect ed HSV-tk are able to phosphorylate ganciclovir when this agent is administered to a cell population. Ganciclovir is toxic to transfected cancer cells, resulting in their death as well as the death of adjacent cells that do not express the HSV-tk gene. These adjoining bystander cells receive the toxic phosphorylated ganciclovir through gap junctional intercellular channels that connect them with adjacent HSV-tk-expressing cells (1, 3, 7–9). Clearly, any treatment strategy for human malignancy that results in a bystander effect, through whatever mechanism, offers tremendous therapeutic advantages.

Although apoptosis-inducing molecular therapies, such as those resulting in overexpression of the wild-type p53 tumor suppressor (10–15), are not toxin based; growth-inhibitory signals may, nonetheless, be communicated to bystander cells from apoptotic cells through a variety of possible mechanisms. Anecdotal reports of a bystander effect in wild-type p53 gene therapy models of human lung and head and neck cancer are consistent with this hypothesis (16–19). However, data definitively demonstrating a bystander effect in any wild-type p53 gene therapy model, along with its requirements and characteristics, are lacking.

Our interest in wild-type p53 gene transfer as a clinical tool in patients with SCCHN in the upper aerodigestive tract led to this present in vitro investigation. We set out to explore wild-type p53 gene therapy, an apoptosis-inducing molecular therapy, for the presence of a bystander effect in SCCHN in a controlled fashion, and in so doing elucidate its requirements and characteristics.

MATERIALS AND METHODS

Cell Lines. The p53-mutated SCCHN cell lines TU138 and TU167 were developed in the Department of Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Department of Head and Neck Surgery, 1515 Holcombe Boulevard, Box 69, Houston, Texas 77030. Phone: (713) 792-6920; Fax: (713) 794-4662.

Received 4/3/98; revised 6/8/98; accepted 7/17/98.

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.), American Society for Head and Neck Surgery Research Grant (to D. K. F.), National Institute of Dental Research 1-P50-DE11906 (93-9; to G. L. C.), National Institute of Health First Investigator Award R29 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 The University of Texas M. D. Anderson Cancer Center, Department of Head and Neck Surgery, 1515 Holcombe Boulevard, Box 69, Houston, Texas 77030.

3 The abbreviations used are: HSV-tk, herpes simplex virus thymidine kinase; SCCHN, squamous cell carcinoma of the head and neck; GFP, green fluorescence protein; MOI, multiplicity of infection; CMTMR, (5-(and-6)-((4-chloromethyl)benzoyl)amino)-tetramethyl-rhodamine; FACS, fluorescence-activated cell sorter; GJIC, gap junctional intercellular communication; AGA, 18-o-glycyrrhetinic acid.
Neck Surgery at The University of Texas M. D. Anderson Cancer Center. TU138 (12) and TU167Δ underwent apoptosis upon adenovirus-mediated wild-type p53 gene transfer and subsequent overexpression of the wild-type p53 gene product. Cells were cultured in DMEM-Ham’s F-12 supplemented with 10% heat-inactivated fetal bovine serum unless otherwise specified.

**Recombinant Adenovirus.** The recombinant adenovirus, Ad-p53, was used for all wild-type p53 gene transfers. This vector has been described in detail elsewhere (20). An identically prepared vector containing the GFP gene (in place of the wild-type p53 gene), Ad-GFP, was also used in certain experiments. The GFP gene product confers a green fluorescence emission peak, 509 nm) to transduced cells (21). The control adenovirus, Ad-DE1, contains no therapeutic gene and does not result in significant SCCHN growth inhibition (10, 12, 20).

**Bystander Supernatant Studies.** For TU138 and TU167, respectively, the growth of 2.5 × 10^5 cells cultured with the supernatant derived from either 2.5 × 10^5 Ad-p53-infected cells, 2.5 × 10^5 Ad-DE1-infected cells, or 2.5 × 10^5 mock-infected cells was compared with the growth of 2.5 × 10^4 Ad-p53 monolayer-infected cells. All supernatants were harvested 2 days after infection and spun in Centricon 500 (pore size of 0.5 µm) centrifuge/filter tubes (Amicon, Inc., Beverly, MA) to remove potentially extraneous Ad-p53 while preserving macromolecular constituents. Cells were counted on a Coulter Counter (Coulter Corp., Miami, FL) at 1, 3, 5, and 6 days after supernatant exposure. Experiments were performed in triplicate for each time point. TU138 and TU167 were infected with Ad-p53 and Ad-DE1 at 100 MOI.

**Bystander Coculturing Studies.** For TU138 and TU167, respectively, 2.5 × 10^5 Ad-p53 suspension-infected cells were cocultured with an equal number of uninfected (bystander) cells in six-well tissue culture dishes. In coculturing experiments, 5 × 10^5 total cells (high cell plating density) yielded at least a 70% confluent monolayer in the 35-mm well of a six-well tissue culture plate for both cell lines, ensuring intercellular contact. The growth of the bystander cells relative to uninfected negative control cells (2.5 × 10^5) and Ad-p53-infected positive control cells (2.5 × 10^5) was studied by counting viable cells on a Coulter Counter each day for 3 days subsequent to Gene transfer and coculturing. All experiments were performed in triplicate for each time point. An additional growth assay was conducted on TU138 whereby 2.5 × 10^4 infected cells were cocultured with 2.5 × 10^4 bystander cells (low cell plating density). Cells were not in contact at this plating density. To remove extraneous virus subsequent to all infections and before coculturing, cell suspensions were washed and centrifuged twice before plating. All growth assays were repeated a minimum of two times. TU138 was always infected with Ad-p53 at 30 MOI, whereas TU167 was infected at 200 MOI. Preliminary studies revealed that these MOIs induced nearly complete death of respective TU138 and TU167 cell populations.

To further assess the role of intercellular contact in any observed bystander effect, 2.5 × 10^5 Ad-p53-infected TU138 cells were cultured in the same growth medium as 2.5 × 10^5 uninfected (bystander) TU138 cells but kept physically separate from them via use of transwell chambers (Corning-Costar Corp., Cambridge, MA) with a 0.4-µm pore size. Ad-p53-infected cells were plated in the upper reservoir of the transwell chamber, while bystander cells were plated in the lower. Solutes and nutrients in the medium can pass freely between the upper and lower reservoirs. Positive control assays were performed by culturing both the 2.5 × 10^5 Ad-p53-infected cells and 2.5 × 10^5 bystander cells in the lower reservoir, thus allowing intercellular contact (lower reservoir is the same size as the well of a six-well tissue culture plate). Negative control assays had either mock- or Ad-DE1-infected cells plated in the upper transwell reservoir, with bystander cells plated in the lower. The bystander cells in all experiments, performed in triplicate, were counted on a Coulter Counter at 72 h after plating. All Ad-p53 and Ad-DE1 infections were carried out at 30 MOI.
Assaying for Extraneous Adenovirus in Bystander Coculturing Studies. Cellular and adenoviral fluorescent markers were used for determining whether bystander cells were being infected with extraneous adenovirus carried over from infected cell populations (despite washes) after coculturing. An adenovirus vector encoding the detectable green fluorescent marker GFP (Ad-GFP) was used in place of Ad-p53. A total of 2.5 × 10^5 TU138 cells tagged with the red fluorescent molecule CMTMR (10 μM in serum-free medium; Molecular Probes, Inc., Eugene, OR) were subsequently infected with Ad-GFP. This 100% dual red-green fluorescent cell population was cocultured (in six-well tissue culture plates) with 2.5 × 10^5 untagged bystander TU138 cells subsequent to centrifugation and washing. GFP, like CMTMR (emission peak, 575 nm), can be distinguished by FACS scanning after excitation at 488 nm. The percentage of cells expressing green fluorescence only (untagged bystander cells infected with extraneous Ad-GFP) in the cocultured population was determined by FACS scanning with a Coulter Epics Profile Flow Cytometer (Coulter Corp.) at 1 and 2 days after infection and coculturing. To control for the number of directly Ad-GFP-infected cells that missed red CMTMR tagging in the above coculturing assays (false positives expressing green fluorescence only), FACS scanning was carried out on 2.5 × 10^5 red CMTMR-tagged, GFP-transduced TU138 cells (dual red-green fluorescent) at 1 and 2 days after infection. Experiments were performed in triplicate for each time point. All Ad-GFP infections were carried out at 30 MOI.

Scrape Loading and Dye Transfer Technique to Determine Gap Junctional Intercellular Communication Activity. TU138 and TU167 were subjected to a scrape loading and dye transfer assay to determine their GJIC activity. GJIC has been implicated in mediating bystander effects in other gene therapy systems (1, 3, 7–9) and plays an important role in the communication of growth-regulatory signals both during embryogene-
sis and between normal and transformed cells in culture (9, 22–24). The scrape loading and dye transfer technique was adapted from El-Fouly et al. (25). Briefly, cell lines were grown to 80% confluent monolayers in 35-mm tissue culture dishes. Monolayers were scraped with a sharp metal probe across the diameter of the plate subsequent to adding 2 ml of a 0.05% lucifer yellow (Sigma Chemical Co., St. Louis, MO)/rhodamine dextran (Molecular Probes, Inc.) fluorescent dye mixture (dissolved in PBS). Lucifer yellow is a small fluorescent molecular dye (0.5 kDa) that cannot pass through intact plasma membranes, but it can pass between adjoining cells through gap junctions (25). Its fluorescence upon excitation is distinct from that of rhodamine dextran, which is too large (10 kDa) to pass through gap junctions. Damaged cells adjacent to a scrape take up both dyes; however, only cells with GJIC activity will pass lucifer yellow beyond the margins of the scrape to adjacent contacting cells. All monolayers were viewed with a Wild Leitz Laborlux S fluorescence microscope (Solms, Germany) fitted with FITC and rhodamine filters for the detection of lucifer yellow and rhodamine dextran, respectively, subsequent to scrape loading and a 10-min incubation period.

RESULTS

Supernatant Derived from Wild-Type p53-transduced Cells Has No Effect on the Growth of Nontransduced Cells. Supernatant harvested 2 days (and 1 day for TU138, data not shown) after Ad-p53 infection and subsequently used as the culture medium for fresh, uninfected cells did not inhibit growth in either TU138 (Fig. 1A) or TU167 (Fig. 1B) relative to controls. These data refuted a role of some factor(s) secreted into the supernatant of wild-type p53 transduced cells in mediating growth inhibition in nontransduced (bystander) cells.

Growth Inhibition of TU138 Bystander Cells Occurs When There Is Contact with TU138 Cells Overexpressing Wild-Type p53. In TU138 coculturing growth assays performed with intercellular contact (high cell plating density), 25% (P = 0.034) and 29% (P = 0.020) growth inhibition of bystander cells were demonstrated at 2 and 3 days, respectively, relative to the growth of negative control cells (Fig. 2A). Growth inhibition of bystander cells was not demonstrated when TU138 coculturing growth assays were performed at low cell plating density (no intercellular contact; Fig. 2B). These data suggested
a role for intercellular contact in the observed TU138 bystander effect. No growth inhibition of TU167 bystander cells was demonstrated in coculturing growth assays performed with intercellular contact (high cell plating density; Fig. 2C).

When \(2.5 \times 10^5\) wild-type p53-transduced and \(2.5 \times 10^5\) nontransduced (bystander) TU138 cells were cultured in the same medium but kept separate in transwell chambers, there was no growth inhibition of bystander cells (at 72 h) relative to bystander cells in negative control transwell chamber assays (see Fig. 5). In the positive control assay where the \(2.5 \times 10^5\) wild-type p53-transduced and \(2.5 \times 10^5\) nontransduced (bystander) cells were not physically separated (i.e., cocultured in the lower reservoir), there was a 30% growth inhibition (\(P = 0.002\)) of bystander cells relative to the growth of the bystander cells that were cultured with but kept separate from Ad-p53-transduced cells (Fig. 3). These data reinforced the importance of intercellular contact in the observed bystander effect for TU138. Furthermore, these data refuted a role for media deconditioning in the observed bystander effect.

Growth inhibition of TU138 bystander cells induced by nonspecific intercellular contact (contact growth inhibition) with Ad-p53-transduced TU138 cells was ruled out as an etiology of the observed bystander effect. There was no growth inhibition in \(2.5 \times 10^5\) TU138 bystander cells (relative to noncocultured controls) when cocultured with an equal number of red CMTMR-tagged TU138 cells (data not shown). The percentage of bystander cells in counted cell populations after coculturing was determined by FACS scanning.

**Extraneous Adenovirus Is Not Causing the Bystander Effect in TU138 Coculturing Studies.** It was possible that extraneous Ad-p53 (carried over even after infected cell populations were washed) was introduced into TU138 bystander cells after coculturing with directly infected cells. To address this concern, a highly sensitive assay using fluorescent markers was designed to detect residual adenovirus. When \(2.5 \times 10^5\) red CMTMR-tagged, GFP-transduced TU138 cells (dual red-green fluorescent) were cocultured with an equal number of untagged bystander TU138 cells, 3.2% of the resultant cell population expressed green fluorescence only as determined by FACS scanning at 2 days (Fig. 4A) with nearly identical results at 1 day (data not shown). These green-only expressing cells could have represented bystander cells that were infected with extraneous Ad-GFP or directly infected cells that missed red CMTMR tagging. Indeed, in the separate assay where only \(2.5 \times 10^5\) red CMTMR-tagged, GFP-transduced cells were subjected to FACS scanning, 2% of the cell population expressed green fluorescence only at 2 days (Fig. 4B) with nearly identical results at 1 day (data not shown). This indicated that only a minority of the green-only cells in the coculturing assay were bystander cells infected with extraneous adenovirus. In titration assays (data not shown), it was determined that 1 MOI of Ad-GFP transduces 29% of a TU138 cell population, and this equivalent MOI of Ad-p53 has no growth inhibitory effect on directly infected cells. Thus, given that our bystander cells were being infected with much less than 1 MOI of extraneous adenovirus, it was highly unlikely that their growth inhibition in coculturing experiments was mediated by residual Ad-p53.

**TU138 Demonstrates GJIC But TU167 Does Not.**

Given the importance of intercellular contact in the observed bystander effect, as well as role that GJIC plays in the communication of growth-regulatory signals both during embryogenesis and between normal and transformed cells in culture (9, 22-24), we conducted preliminary experiments to explore the potential role of GJIC in our findings. After scrape loading with the lucifer yellow/rhodamine dextran dye mixture, TU138 demonstrated transmission of the lucifer yellow to progressive rows of cells beyond the border of the scrape. Rhodamine dextran remained confined to the border of the scrape in TU138 (Fig. 5). This result was consistent with the presence of GJIC in this cell line. Recall that a bystander effect was observed in TU138 in coculturing studies performed with intercellular contact. TU167, unlike TU138, demonstrated uptake of both fluorescent dyes at the border of the scrape only. Lucifer yellow was not transmitted beyond the bordering cells (Fig. 6). This result was consistent with a lack of GJIC in this cell line. Recall that a bystander effect was not observed in TU167 in coculturing studies.

To further investigate the role of GJIC in our findings, we explored whether we could block bystander cell growth inhibition in TU138 coculturing assays (performed with intercellular contact) with the reversible inhibitor of GJIC, AGA (Sigma...
Bystander Effect in Wild-Type p53 Gene Therapy

Apoptosis results from overexpression of p53, as well (11). Overexpression of the wild-type p53 gene product is effective as an apoptosis-inducing molecular therapy in other malignancies as well (11, 13-15). Anecdotal reports that wild-type p53 gene therapy may also result in a bystander effect in some malignancies (16-19) led to this present investigation into establishing the definitive presence and thus potential requirements of this phenomenon in SCCHN. Any proposed cancer treatment that results in a bystander effect has the unique therapeutic advantage of being able to “miss” some of the cells in a tumor under treatment yet still affect these cells.

Our data have demonstrated that wild-type p53 overexpressing TU138 SCCHN cells can bring about growth inhibition in nontransduced cells. These data have shown that this bystander effect required intercellular contact between the wild-type p53-transduced and bystander cell populations. Media conditioning resulting from coculturing the transduced and nontransduced cells, nonspecific contact growth inhibition between cocultured cell populations, as well as extraneous Ad-p53 factors secreted into the supernatant of wild-type p53-transduced cells were also ruled out as contributing to a bystander effect in both TU138 and TU167.

There are several potential mechanisms by which a bystander effect can be mediated between apoptotic (wild-type p53-transduced) and nontransduced cell populations that are in physical contact. These may include receptor-ligand signaling (such as CD95-Fas ligand), bystander cell endocytosis of “apoptotic vesicles” from transduced cells, or mechanisms whereby “apoptotic signals” from wild-type p53-transduced cells are communicated to nontransduced bystander cells via GJIC between the two cell populations. An understanding of the genotypic and phenotypic differences between TU138 and TU167 may help to elucidate the mechanism of the observed bystander effect because only TU138 demonstrated this phenomenon under intercellular contact conditions.

GJIC appears to be an important mechanism for the intercellular exchange of chemical information and may play a role in cellular nutrition, growth, growth regulation, and differentiation (22, 24, 27-30). The loss of GJIC activity is a common (although not universal) phenotype in human malignancy (24). It is theorized that the loss of GJIC in cancer cells allows them to escape the potential growth-regulatory controls of adjoining cancer cells and normal tissues (24, 27). This information led us to conduct preliminary experiments

Chemical Co., St. Louis, MO) (8, 26). Fig. 7 shows that with increasing concentrations of AGA in the growth medium, there was a decrease in the percentage of bystander cell growth inhibition relative to the growth of uninfected negative control cells at 2 and 3 days. This data roughly correlated with a reduction in lucifer yellow dye transfer seen with increasing concentrations of AGA in the growth medium (data not shown). It should be pointed out, however, that AGA slowed all cellular growth in general (it was modestly toxic to control and experimental cell populations with increasing doses). Furthermore, the percentage of decreases in bystander cell growth inhibition with increasing AGA concentrations did not achieve statistical significance. Thus, although the trend of this data are intriguing, it is inconclusive and warrants further investigation.

DISCUSSION

Previous work in our laboratory has shown that Ad-p53 transduces SCCHN cell lines with the wild-type p53 gene with high efficiency (20). Apoptosis results from overexpression of the wild-type p53 gene product in transduced cells (10, 12). Overexpression of the wild-type p53 gene product is effective as an apoptosis-inducing molecular therapy in other malignancies as well (11, 13-15). Anecdotal reports that wild-type p53 gene

Fig. 6 Medium-power photomicrographs of scrape loading of TU167 with 0.05% lucifer yellow/rhodamine dextran dye mixture to assay for GJIC. A. lucifer yellow dye confined to border (thin arrow) of the scrape. B. rhodamine dextran dye confined to border (thin arrow) of the scrape. Direction of the scrape is demarcated with a thick arrow.

Fig. 7 Percentage of bystander cell growth inhibition at 2 and 3 days in TU138 coculturing assays performed with intercellular contact (high cell plating density) and increasing concentrations of AGA (0, 50, 70, and 80 μM) in the growth medium. Percentage of bystander cell growth inhibition is relative to the growth of uninfected negative control cells.
explore the role of GJIC in the observed bystander effect. Although our data from these studies is intriguing (see Figs. 5–7), more definitive experiments are being conducted to elucidate the role that GJIC may be playing in the observed bystander effect.

In conclusion, we have demonstrated that wild-type p53 gene therapy, an apoptosis-inducing molecular therapy, is capable of producing a bystander effect in SCCCHN in vitro, and that this phenomenon requires intercellular contact between wild-type p53 transduced and nontransduced (bystander) cell populations when present. Our data support anecdotal reports that wild-type p53 gene therapy can produce a bystander effect, and in vivo experiments are presently underway to further explore our important in vitro findings. Other therapies associated with apoptosis may also demonstrate bystander effects. Such therapies may include standard modalities that are associated with apoptosis through wild-type p53-dependent (gamma irradiation and cytotoxic chemotherapy) or -independent pathways and apoptosis-inducing molecular therapies (either p53-dependent or -independent). Important potential directions in therapeutic intervention strategies are implied by our findings. Mammalian cells have the ability to communicate with one another through various mechanisms. These mechanisms no doubt extend to cancer cells, albeit with some phenotypic modification, depending upon the tumor type and state of differentiation. An understanding of how (and whether) cancer cells communicate with each other in a given tumor may direct the use of a particular treatment strategy that takes advantage of that communication phenotype. In this manner, increased therapeutic efficacy and efficiency, as well as prognostic information, may be achieved.

ACKNOWLEDGMENTS

We thank Karen Ramirez for assistance with FACS scanning, Dr. Dianna Roberts for assistance with statistical analysis, and Mary T. Wang for technical assistance.

REFERENCES


Bystander effect in the adenovirus-mediated wild-type p53 gene therapy model of human squamous cell carcinoma of the head and neck.

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


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/4/10/2521