Advances in Brief

In Vivo Molecular Therapy with p53 Adenovirus for Microscopic Residual Head and Neck Squamous Carcinoma

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Abstract

Developing gene therapy strategies may allow contemporary medicine to reassess its management of solid malignancies. We have demonstrated previously that the wild-type p53 adenovirus (Ad5CMV-p53) suppressed the growth of established tumors of the head and neck. In this paper we develop a microscopic residual model which mimics the postsurgical environment of head and neck cancer patients with advanced disease. Using this squamous cell carcinoma of the head and neck model, we prevented the establishment of tumors in nude mice in which tumor cells had been s.c. implanted by transiently introducing exogenous wild-type p53 via an adenoviral vector 2 days following tumor cell implantation. These effects were vector dose dependent but independent on the endogenous wild-type or mutated p53 status of the cells. Importantly, karyotypically normal and nontumorigenic fibroblast cell lines are inert to the p53 adenovirus treatment. These results pave the ground work for further development of molecular therapy for head and neck cancer and other solid malignancies.

Introduction

Patients with SCCHN are afflicted with a disease process that often has profound effects upon speech, swallowing, and cosmesis. Furthermore, the overall rate of survival among these patients, approximately 50%, has remained unchanged for the nearly 30 years since contemporary surgery and radiation therapy were instituted (1). Recurrences among these patients remain predominantly local and regional; approximately only 10% of patients die of distant metastasis alone (2, 3). In patients with SCCHN, the pathological findings of extracapsular invasion, neurotropism, and microscopic residual disease necessitate adjunctive therapy and predict aggressive local-regional disease. Moreover, these factors can usually be predicted prior to surgical intervention.

In head and neck cancer, direct gene transfer to microscopic residual carcinoma may not be technically difficult. When the primary tumor is removed, the tumor milieu is readily accessible for molecular therapy and is the most likely pathway of lymphatic spread when the regional lymphatic dissection is performed. Therefore, novel means of addressing assumed microscopic residual disease using direct transfer of genes that encode toxic products, specific tumor suppressor genes, or genes that induce products that specifically promote tumor cell death and spare nonmalignant cells may provide desperately needed improvement in local-regional control among these patients and thus be an important approach to cessating these malignancies. In addition, several other solid malignancies possess the same dilemma, and therefore the model of SCCHN may provide insight into cancers of several other organ systems.

We believe that promising new therapies for SCCHN are interventions at the molecular level, and adenovirus-mediated gene transfer is our clinical method of choice for such intervention. Adenoviruses have a known tropism for the epithelium of the aerodigestive tract and are linked only to minor disease in humans (4). Moreover, in contrast to retroviruses, they are capable of transferring genes to nonproliferating cells, which appears preferable because of the heterogeneity of cell cycling within the tumor microenvironment (5). Finally, the transient nature of gene expression after adenoviral gene delivery allows selection of a molecular intervention that will provide the desired outcome (tumor cell death in cancer) without long-term integration of the recombinant molecular therapy into bystander cells and the potential ramifications thereof.

Materials and Methods

Cell Lines and Culture Conditions. Human SCCHN cell lines Tu-138, Tu-177, MDA 686-LN, and MDA 886 were all established at the Department of Head and Neck Surgery, M. D. Anderson Cancer Center, and had been characterized previously (6, 7). These cells were grown in DMEM (DMEM/ Ham’s F-12) supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin.

Recombinant Adenovirus Preparation and Infection, Cell Growth Assay, and Western Blot Analysis. All the procedures have been described previously (8). Cell growth assays were all performed in triplicate.

In Vivo Transduction with ß-Galactosidase Adenovirus. X-Gal staining of tissue specimens were performed on ornithine carbamyl transferase [Tissue Tek O. C. T. Compound® (Miles, Elkhart, IN)] frozen tissue sections to determine transduction efficiency. Eight-μm-thick specimens were washed in cold PBS and fixed in 0.5% glutaraldehyde at room temperature for 5 min. Slides were then washed twice with 4°C PBS and incubated for 4 h in X-Gal solution [1.3 mM MgCl₂, 15 mM NaCl, 44 mM Hepes buffer (pH 7.4); 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 2% X-Gal in N,N-dimethylformamide]. Slides were counterstained with H & E.

Immunohistochemical Analysis. Formalin-fixed paraffin-embedded in vivo animal experimental tissues were cut at 4–5 μm, dried at 60°C, deparaffinized, and hydrated with distilled water. Sections were then treated with 0.5% saponin in distilled water and rinsed in several changes of distilled water; endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol, followed by rinsing in several changes of distilled water. Sections were microwave-irradiated in distilled water for 3 min using a Sharp Model R9H81 microwave oven operating at a frequency of 2450 MHz at 700 W. After cooling, sections were washed in several changes of distilled water and placed in PBS; immunohistochemical studies were performed by using the avidin-biotin-peroxidase complex method of Hsu et al. (9) in the following manner: sections were blocked with normal horse serum and incubated overnight at 4°C with...
rabbit antihuman p53 polyclonal antibody, clone OM-1, 1:80 (Signet Laboratories, Denham, MA). An anti-rabbit IgG Elite kit (Vector Laboratories, Burlingame, CA) was then used to apply biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complexes which were incubated for 45 min each. The immunostaining reaction was visualized by using 0.5% dimethylaminoazobenzene in PBS containing 0.01% hydrogen peroxide (pH 7.6), counterstained with 0.01% toluidine blue, dehydrated, cleared, and mounted in Permount. To verify the specificity of the immunostaining reaction, immunoperoxidase staining was performed (using the same method as on test samples) on a known positive cytospin of a tissue culture of a squamous carcinoma cell line as well as on a negative rabbit monoclonal antibody control.

**Inhibition of Tumor Growth in Vivo.** The effect of Ad5CMV-p53 on a microscopic disease model of SCCHN was determined in nude mice in a defined pathogen-free environment. Experiments were reviewed and approved by institutional committees for both animal care and utilization and the Biosafety Committee for recombinant DNA research. Briefly, nude female mice (aged 4–7 weeks) were anesthetized with i.p. ketamine/acepromazine (70 mg/kg of body weight) (Parke-Davis, Morris Plains, NJ). After their bodies were prepared with alcohol wipes, incisions were made in the dorsal flanks and three s.c. flaps were elevated with sharp dissection. Sterile pipette dispensers were used to introduce the desired number of tumor cells in 100 µl of culture medium into the flap, which was sealed with a horizontal mattress suture. Forty-eight h following tumor-cell delivery, the animals were reanesthetized and the sutures removed. The flap was infected with Ad5CMV-p53, dl312, β-galactosidase adenovirus, or PBS alone (mock infection) by pipetting in 100-µl increments; the flap was then resealed with a horizontal mattress suture. The PFU of the inoculant was increased in log increments with animals serving as their own controls as well as single-flap models on animals. The animals were observed daily for tumor development and killed in cases of excessive tumor burden or after 12 weeks of observation. All surgical sites were evaluated pathologically as well as by necropsy analysis for systemic toxicity.

**Results**

**Effect of Exogenous p53 on SCCHN Cell Growth in Vitro.** We described previously the in vitro inhibition of cell growth by Ad5CMV-p53 in SCCHN cell lines with endogenously mutated p53 (8). We therefore sought to determine whether SCCHN cell lines with endogenous wild-type p53 would be similarly affected. We also investigated the effect of Ad5CMV-p53 on nonmalignant fibroblasts.

Four human SCCHN cell lines were chosen for this study: Tu-138 and Tu-177, which possess a mutated p53 gene, and MDA 686-LN and MDA 886, which are homozygous for the wild-type p53 gene.4 A fibroblast cell line derived from normal fibroblast outgrowth, which is karyotypically normal and nontumorigenic, was used as a nonmalignant control cell line. Cells infected with the control virus, dl312, had karyotypically normal and nontumorigenic, was used as a nonmalignant control cell line. Cells infected with the replication-defective adenovirus, dl312; Lane 3, Fibroblast-138 cells infected with the replication-defective adenovirus, dl312; Lane 4, Fibroblast-138 cells infected with the Ad5CMV-p53. ß, Growth curve of normal fibroblast cell line. Mock infected cells (○), dl312 infected cells (△), and Ad5CMV-p53-infected cells (●).

**Results**

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* R. G. Clayman, unpublished data.
Expression of Exogenous p53 Protein in Adenovirus Infected Normal Fibroblasts and its Effect on Growth Rate. Additionally, we investigated the effect of the Ad5CMV-p53 on karyotypically normal and nontumorigenic fibroblast cell lines. These cells were isolated during the establishment of primary tumor cell lines. Twenty-four h after infection, Western blot analysis was performed to compare the levels of protein produced by the different infected cell types. A p53 band, recognized by the monospecific anti-p53 antibody, PAb1801, was observed in cellular extracts isolated from all samples infected with the Ad5CMV-p53 (Fig. 2A; Lanes 1 and 4). As has been shown previously (8), cell line Tu-138 infected with the p53 adenovirus showed high levels of p53 protein following transduction and served as a control (Fig. 2A; Lane 1). The level of p53 expression remained similar in both mock-infected and Ad312-infected cells (Fig. 2A; Lanes 2 and 3). The Ad5CMV-p53-infected fibroblasts showed higher levels of p53 protein than did the control cells (Fig. 2A; Lane 4). This result indicates that the p53 gene is efficiently translated into normal fibroblasts infected with Ad5CMV-p53 as evidenced by production of immunoreactive p53 protein. The protein expression and transduction efficiency of cytospins of Ad5CMV-p53 infected fibroblasts were verified by immunohistochemical analysis (data not shown). This fibroblast cell line exhibited normal growth rate and morphology independent of the intervention (mock, replication-defective virus, or Ad5CMV-p53) (Fig. 2B). These experiments were repeated twice and also verified in other normal human fibroblast cell lines.

In Vivo Transduction Efficiency. To measure the efficiency of gene transfer in vivo, we resected the s.c. flap site 72 h following molecular or control intervention. Dose-response experiments with the adenovirus β-galactosidase-marker vector demonstrate dose-response transduction efficiency in this model (Fig. 3). This was confirmed with immunohistochemical analysis 4 days following infection with Ad5CMV-p53 (Fig. 4). Both groups of experiments exhibited an in vivo dose response which had been described previously in vitro by us and others (8). In no instances did doses of virus exceeding 10^{10}-PFU effect expression of p53 in other organ systems including brain, liver, lung, heart, abdominal visceral organs, and skin (data not shown). These experiments illustrated a dose-response relationship between viral titer and transduction efficiency as well as the possibility of achieving extensive transient expression of the transduced gene within the desired surgical model field.

Suppression of Tumor Growth in Vivo. We designed our initial experiments to determine whether in vivo Ad5CMV-p53 mediated gene transfer would affect the establishment or growth of SCCHN cells implanted into a s.c. flap. We created a microscopic residual disease model. In this model, three s.c. flaps were elevated on athymic nude female mice, and 2.5 × 10^{6} of tumor cells were seeded by pipetting. Instead of allowing the tumor cells to form nodules...
Fig. 4. A, Suppression of tumor establishment in a microscopic residual disease model of SCCHN. Nude female mice implanted with Tu-138 cell lines and delivered 48 h later with 100 µl of Ad5CMV-p53 (10⁶ PFU) in the right anterior flap d1312 (replication defective adenovirus) 10⁸ PFU in the right posterior flap and transport medium (PBS) alone in the left posterior flap. Animals were observed daily and sacrificed at 12 weeks or earlier if excessive tumor burden developed. No gross or histological evidence of tumor in the Ad5CMV-p53 flap site was found. Photograph taken at 8 weeks. B, In vivo infectivity of Ad5CMV-p53 in the microscopic residual disease flap model. The Ad5CMV-p53 was pipetted into the s.c. flap 48 h following tumor cell line delivery. A representative experiment of the wild-type p53 SCCHN cell line MDA 686-LN is shown. Panel a, mock infection showing lack of immunostaining in the wild-type p53 cell line; Panel b, 10⁷ PFU of Ad5CMV-p53; Panel c, 10⁸ PFU of Ad5CMV-p53; Panel d, 10⁹ PFU of Ad5CMV-p53. Immunostaining was performed using the polyclonal rabbit antihuman antibody OM1 (Signet Laboratories) using the avidin-biotin method. A clear dose-response relationship is shown. No viable tumor was found in multiple histological sections of 10⁶ or 10⁷ PFU Ad5CMV-p53. Magnification 100x.
(generally occurring in 4 days), we delivered our single dose of molecular intervention at 48 h following tumor cells seeding. In this manner, although no gross tumors were present, microscopic tumor cells were within the surgical site mimicking the clinical dilemma of surgical excision of all gross tumor. The development of tumors was directly related to the number of tumor cells, the time allotted for implantation, and the dose of Ad5CMV-p53. Of the mice which received microscopically implanted tumor cells (2.5 × 10⁶) and were treated with Ad5CMV-p53 at 10⁶ PFU or greater, only two mice developed tumors, both of which were implanted with the wild-type p53 cell line (MDA 886-LN). All other cell lines exhibited absence of tumor development (Table 1). These experiments clearly indicate that the growth of microscopic tumor cells can be effectively suppressed in vivo if exposed to the Ad5CMV-p53 (Fig. 4A). Tumor formation was evaluated at the end of a 12-week period (earlier animal sacrifice in circumstances of excessive tumor burden) by gross and histological analysis of the surgical sites. The data of tumor establishment is summarized in Table 1, and a representative experiment is shown in Fig. 4A.

Immunohistochemical analysis was performed on the tumor sections of experimental animals. A representative experiment of the SCCHN cell line MDA 686-LN is shown (Fig. 4B). This cell line possesses the wild-type endogenous p53 gene. Lack of significant basal immunostaining with the viable tumor of MDA 686-LN (mock-infection) is seen in Panel A. Panel B (Ad5CMV-p53 at 10⁷ PFU) shows peripheral tumor necrosis with immunostaining in the more central portion of the tumor. Panel C (Ad5CMV-p53 at 10⁸ PFU) reveals total necrosis of the tumor with immunostaining found in the entire surgical pocket with multiple layers expressing protein, including stroma and superficial muscular layers. Panel D (Ad5CMV-p53 at 10⁹ PFU) shows similar results to that of Panel C, however increased exogenous p53 expression throughout the surgical site and edema are prominent.

Using animals, which served as their own internal controls, implants of 4.0 × 10⁶ or more cells significantly increased the establishment of s.c. implants as compared to the tumor implantation of 2.5 × 10⁶ cells (P < 0.01), even when treated at the surgical site with Ad5CMV-p53 48 h after inoculation. Allowing implanted cells to establish for 72 or 96 h prior to the Ad5CMV-p53 intervention similarly increased tumor take (data not shown). Dose-response experiments established that 10⁶ and 10⁷ PFUs of the Ad5CMV-p53 were equally effective in inhibiting tumor burdens of 2.5 × 10⁶ cells implanted for 48 h (data not shown). Endogenous p53 status of implanted tumor cell lines (whether homozygous mutated or wild-type p53) had little impact on the effectiveness of the Ad5CMV-p53 in the cessation of tumor development.

Table 1 Effect of Ad5CMV-p53 on tumorigenicity in a microscopic residual disease model of SCCHN

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>PBS</th>
<th>d1312</th>
<th>Ad5CMV-p53</th>
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<td>Tu-138 (homozygous mutation p53)</td>
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<td>8/8</td>
<td>0/8</td>
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<tr>
<td>Tu-177 (homozygous mutation p53)</td>
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<tr>
<td>886 (homozygous wild-type p53)</td>
<td>6/6</td>
<td>6/6</td>
<td>2/6</td>
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Discussion

Tumor suppressor genes are only one of several groups of genes whose transfer might be useful for the local and regional treatment of cancer. Increasing immune surveillance by enhancing of MHC antigen expression or inducing local cytokines to activate the immune system can induce a local tumor effect. Another approach is introducing a gene that may induce apoptosis or that would make a tumor sensitive to particular chemotherapeutic agents. Furthermore, focusing on cell-cycle arrest in malignancies may prove beneficial.

At least two gene "suicide vectors" have already been described; the herpes simplex thymidine kinase gene allows infected cells to be treated with ganciclovir while noninfected cells are unaffected, and the bacterial enzyme cytosine deaminase gene allows infected cells to convert 6'-fluorocytosine to 6-fluorouracil. Unlike these agents, Ad5CMV-p53 has shown no significant toxic effect on untransformed cells and its expression is transient, lasting about 15 days (data not shown).

In vitro experiments have shown that Ad5CMV-p53 stops cell growth regardless of the endogenous p53 gene status of the tumor cells. The mechanism by which abundant overexpression of wild-type p53 protein induces this effect appears to be apoptosis, but this requires further investigation. It is important that normal fibroblasts transduced by this vector express the wild-type protein at levels similar to the SCCHN cell lines but without inhibition of cell growth or abnormalities in morphology. The unique molecular events occurring within these transformed tumor cell lines that dispose them to cell death following Ad5CMV-p53 transduction (while nonmalignant cells are spared) requires elucidation. Nevertheless, this sparing of normal cells further supports the potential for molecular therapy with this vector, since transduction of normal cells will be unavoidable in vivo human trials.

Clearly a pure viral effect was also noted on delivery to the s.c. pockets, however this did not appear to be tumoricidal in these experiments. No clinical or histological findings of inflammation or edema different from those at the control sites were noted when the mice were treated with replication-defective virus or adenovirus β-galactosidase at 10⁷ PFU or less. At 10⁸ PFU and higher doses, however, inflammatory polymorphonuclear leukocytes and edema were histopathologically evident in the pocket sites, although no soft-tissue compromise was clinically evident. These conditions were not seen in mice mock-infected with transport medium alone or with lower viral doses.

In vitro studies in our laboratory have shown approximately 70% adenovirus transduction among SCCHN cell lines in a single exposure under optimal conditions. Nevertheless, the in vivo studies clearly showed significant suppression of tumor development from tumor burdens of 2.5 × 10⁶ cells. Whether this reflects a need for a particular minimum tumor burden for tumors to develop, a "bystander" infectious environment, or an insensitivity of our assays to determine the effectiveness of molecular transfer has not been established.

These studies were designed to evaluate the efficacy and potential therapeutic role of in vivo gene transfer in a local-regional microscopic residual disease model. They revealed an effective in vivo mechanism for molecular therapy in head and neck cancer and suggest that this model delivery system may have a profound impact in the management of microscopic residual disease.

References

WILD-TYPE P53 ADENOVIRUS FOR MICROSCOPIC RESIDUAL DISEASE


In Vivo Molecular Therapy with p53 Adenovirus for Microscopic Residual Head and Neck Squamous Carcinoma


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