Evaluation of Topical Gene Therapy for Head and Neck Squamous Cell Carcinoma in an Organotypic Model

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ABSTRACT

The organotypic (raft) culture system has been shown to be a useful model for examining the effects of biochemical manipulations on various epithelial cell types, using in vitro conditions that simulate the in vivo environment of the tissue of origin. To investigate this method as a model for topical gene therapy, we cultured the oral head and neck squamous cell carcinoma cell line TR146 on fibroblast-containing collagen gels at the air-medium interface and assessed the efficiency of transduction of a topically applied adenoviral vector containing β-galactosidase cDNA. Diffuse expression of β-galactosidase activity in multiple cell layers demonstrated effective penetration of the vector. Transduction efficiency and therapeutic activity of a replication-defective recombinant adenovirus containing wild-type p53 cDNA linked to a FLAG marker (AdCMV-p53-FLAG) were then assessed in TR146 organotypic cultures transduced by topical application. Twenty-four, 48, and 72 h after transduction, the cultures were harvested, and residual cell number and FLAG peptide expression were determined. The number of cells in p53 transduced cultures was significantly reduced in comparison to controls at all three time points (P < 0.001), which resulted from the induction of apoptosis as determined by in situ DNA end labeling. In addition, the FLAG peptide was expressed diffusely in the residual cells, further confirming effective transduction and expression of the exogenous gene products throughout multiple layers.

We conclude that the organotypic culture is an effective in vitro model for assessing the efficacy of topically applied gene therapy on head and neck squamous carcinomas and premalignancies.

INTRODUCTION

Alteration of gene expression is currently being evaluated as a method for prevention and treatment of HNSCC. Preliminary studies of gene therapy typically involve in vitro monolayer cell cultures. However, cells grown in monolayer are submerged, grown on a foreign surface, and do not stratify, characteristics that do not simulate either the environment or the behavior of normal and neoplastic upper aerodigestive tract epithelium. To overcome these shortcomings, animal models may be utilized, but the use of such models in preliminary investigations is frequently costly and time consuming.

Organotypic cultures have been used increasingly to examine the effects of biochemical manipulation on a variety of benign and malignant cells. This technique involves growing cell lines on fibroblast-containing collagen gels at the air-liquid interface, approximating the in vivo environment and allowing the cells to stratify and differentiate into a morphology that closely resembles the epithelium of origin. We have adapted this technique to analyze the growth and differentiation of human HNSCC cell lines and are studying this method as a model for topical gene therapy, a potential route of gene delivery for the treatment of upper-aerodigestive tract malignancies and premalignancies. The purpose of the current study was to determine whether genes can be transferred efficiently into organotypic cultures by means of a topically applied adenoviral vector and then to compare the effects of the transduced gene expression in this model to those seen in monolayer and murine models. p53 was chosen as the gene for analysis, because it is mutated frequently in HNSCCs and overexpression of exogenous wild-type p53 in HNSCC cell lines has been shown to induce cell loss through apoptotic mechanisms.

Because exogenous p53 protein overexpression cannot be distinguished from endogenous, mutated protein expression in situ, a p53-FLAG adenoviral vector was used. This vector contains a nucleotide sequence that is coexpressed with the p53 cDNA. The encoded FLAG peptide can be detected immunohistochemically and serves as a marker for identifying exogenous gene product expression.

MATERIALS AND METHODS

Cell Lines. The human HNSCC cell line TR146 (gift from Dr. Alfonse Balm, The Netherlands Cancer Institute, Amsterdam, the Netherlands) was derived from a cervical lymph node metastasis that originated from a well-differentiated buccal HNSCC. These cells have a homozygous mutation involving codon 248 in exon 7 of the p53 gene, as shown by single-stranded conformation polymorphism analysis and direct se-
quencing. This cell line was propagated as a monolayer culture in DMEM-F12 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Human fibroblasts were isolated from tissue explants during the establishment of primary HNSCC cell lines and maintained in the above medium.

Organotypic Cultures. Organotypic cultures were established using methods similar to those described by Kopan et al. (6). Collagen gels were prepared using type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA) to which 10% 10× high-glucose DMEM, 10% fetal bovine serum, and 5 μl/ml of 1 n NaOH were added. Fibroblasts were suspended in the collagen at a concentration of 1 × 10^5 cells/ml, and the collagen-fibroblast mixture was allowed to gel for a minimum of 4 h at 37°C in a 24-well cluster plate. Approximately 1 × 10^6 TR146 cells were layered onto the collagen gels and incubated overnight at 37°C until confluent. At this point, the gels were elevated to the air-liquid interface by first placing a collagen-coated, glutaraldehyde-fixed nylon disc onto a surgical stainless steel mesh platform and then placing the gel on the nylon disc. Medium was then added so that it reached the undersurface of the collagen gel but did not cover the cell layer. The cultures were incubated at 37°C for 7 days after elevation to the air-medium interface, at which time the transduction experiments were performed. The medium was changed every 3 days.

Recombinant Adenovirus Preparation. The replication-defective d312 adenovirus, the adenovirus harboring the β-galactosidase cDNA, and the adenovirus containing the p53-FLAG construct were prepared using procedures as described previously (7–9).

In Vitro Transduction with β-Galactosidase Adenovirus. Seven days after elevation to the air-medium interface, 10^6 plus of β-galactosidase adenovirus suspension (10^10 plus/ml) were diluted in 100 μl of PBS and placed on the surface of TR146 organotypic cultures. The virus was not allowed to contact the underlying media. The cultures were incubated with the virus for 60 min, washed once with media, and then incubated for an additional 24 h at 37°C. The cultures were then fixed with 0.5% glutaraldehyde for 5 min, washed with PBS, and stained for 24 h with 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]. After staining, the specimens were dehydrated in graded ethanol solutions and paraffin embedded. Five-μm sections were cut and deparaffinized but were not counterstained to avoid masking the β-galactosidase signal.

In Vitro Transduction of p53-FLAG Adenovirus. For the p53-FLAG adenovirus transduction, TR146 cells were grown in organotypic culture for 7 days. After this period, the cells were removed from two cultures with trypsin-EDTA and counted, and the average number of cells per organotypic culture was determined. One hundred plus cells of the virus were diluted in 100 μl of PBS and applied to the surface of the remaining cultures as described above. The same concentration of replication-defective adenovirus d312 was applied to control cultures, and PBS was used in mock-infected cultures. All experiments were performed in triplicate. After a 60-min incubation, the cultures were washed with medium and incubated for an additional 24, 48, or 72 h. After the appropriate incubation period, the cultures were fixed in 10% formalin and embedded in paraffin. Five-μm sections were then cut and counterstained with H&E.

Determination of Cell Number after p53-FLAG Adenovirus Infection. From H&E sections, the average number of remaining cells from multiple high-powered fields was determined at each time point for mock, d312, or p53-FLAG adenovirus-infected cultures. The significance of the differences in cell number between treatment groups was determined using Student’s t test with two-tail comparison.

Detection of Apoptosis. The induction of apoptosis was detected using an in situ DNA end labeling technique (10), as modified by Gregoire et al. (11). Briefly, the sections were deparaffinized in three changes of xylene and progressively hydrated in 100, 95, 80, and 50% ethanol solutions for 3 min each. Endogenous peroxidase activity was inhibited by exposure to 3% hydrogen peroxide in 100% methanol for 15 min. After this, the sections were washed with PBS, digested with pepsin reagent solution (Biomedia, Foster City, CA) for 5 min at 37°C, washed again with PBS, and incubated with the DNA end-labelling mixture for 1 h at 37°C. This mixture contained terminal deoxynucleotidyl transferase (0.45 enzyme units/μl; Life Technologies, Inc., Gaithersburg, MD), 5× terminal deoxynucleotidyl transferase buffer (Life Technologies, Inc.), 0.001 mM biotin-16-dUTP (Boehringer Mannheim), and double-distilled water to achieve the desired volume. The reaction was terminated by a 30-min exposure at room temperature to a buffer consisting of 300 mM sodium chloride and 30 mM sodium citrate. The sections were washed in PBS and incubated for 30 min with a horseradish peroxidase-avidin conjugate complicated with biotin (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) followed by incubation with 0.1% DAB (Sigma Chemical Co., St. Louis, MO) for 1 min to develop the peroxidase color reaction. The sections were then washed in PBS, counterstained with methyl green, dehydrated, and mounted in Permount (Fisher Scientific, Pittsburgh, PA).

Immunohistochemical Analysis for FLAG Peptide Expression. To detect the presence of the FLAG peptide within transduced cells, sections were deparaffinized in xylene, rehydrated in graded ethanol solutions (100–50%), and washed in PBS. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in 100% methanol. After they were washed in PBS, the sections were preincubated for 15 min at room temperature with normal horse serum (Sigma Chemical Co.) diluted 1:5 v/v in PBS and 0.1% BSA (Sigma Chemical Co.) to block nonspecific binding. The sections were then washed and incubated with mouse anti-FLAG M2 monoclonal antibody (International Biotechnologies, New Haven, CT) and diluted 1:500 in PBS and 0.1% BSA for 45 min at room temperature. The sections were again washed in PBS and exposed to biotinylated antimouse antibody (Vectastain ABC kit), 1:50 dilution, for 30 min. The slides were subsequently washed in PBS and incubated for 30 min at room temperature with avidin-biotin-peroxidase complexes (Vectastain ABC kit), followed by incubation with 0.1% DAB (Sigma Chemical Co.) for 1 min to develop the peroxidase color reaction. The sections were immersed in PBS, washed for 10 min under running tap water, and counterstained with hematoxylin. The DAB signal was then enhanced by exposure to 4% acetic acid followed by saturated
Fig. 1  A, transduction efficiency after a single, topical application of the β-galactosidase adenoviral vector to TR146 HNSCC cells grown in organotypic culture. Diffuse gene expression, as evidenced by blue nuclear and cytoplasmic staining, is present throughout all of the cell layers 24 h after transduction. No enzyme expression by the fibroblasts in the underlying stroma is seen. B-D, effect of topical transduction by p53-FLAG adenovirus. Extensive cell loss is seen 72 h after transduction by p53-FLAG adenovirus (C), in contrast to mock-infected (B) and replication-defective adenovirus dl312-infected (D) cultures.

lithium carbonate, and the sections were dehydrated and mounted in Permount.

**Immunohistochemical Analysis for p53 Protein Expression.** p53 protein expression in transduced and control cultures was determined using the avidin-biotin-peroxidase complex method (Vectastain ABC kit). Mouse antihuman p53 PAb1801 (Oncogene Science, Cambridge, MA) was used as the primary antibody.

**RESULTS**

**Organotypic Cultures.** The TR146 cell line formed a stratified epithelium resembling carcinoma in situ. In addition, the cultures had a well-differentiated phenotype similar to the histological appearance of well-differentiated oral cavity carcinomas in vivo, demonstrating keratinization in the suprabasal layers and forming keratin pearls. Invasion into the underlying collagen stroma was not seen, which likely reflects the high concentration and type of collagen used.

**Transduction Efficiency by Topical Adenoviral Application to Organotypic Cultures.** A single topical application of the adenoviral vector containing the β-galactosidase gene resulted in diffuse expression of enzyme activity throughout the cell layers of the TR146 organotypic cultures, penetrating all cell layers (Fig. 1A). Most, but not all, cells within the layers expressed enzyme activity, achieving a 74% transduction efficiency as determined by examination of sections from multiple cultures. No enzyme expression by the fibroblasts in the underlying collagen base was evident.

**Effect of Topically Applied p53-FLAG Adenovirus on TR146 Organotypic Cultures.** Cultures treated with the p53-FLAG adenovirus (multiplicity of infection, 100) demonstrated marked cell loss when viewed microscopically (Fig. 1, B–D). The number of residual cells after topical treatment was approximately 3-fold less than the number remaining on dl312 or mock-infected cultures at all three time points ($P < 0.001$; Fig. 2). The majority of the cytotoxic effect was seen within the first 24 h after viral application, although a slight decrease in the residual cell number was seen at 48 h. No additional effect was noted after 72 h. Infection with dl312 at multiplicity of infection 100 appeared to have a slight cytotoxic effect on the cells compared to mock infection, but this was not statistically significant.
1662 Organotypic Model for Topical Gene Therapy

Fig. 2 Mean number of residual cells per high-powered field in TR146 organotypic cultures 24, 48, and 72 h after mock (○), dl312 adenovirus (□), or p53-FLAG adenovirus (△) infection. A substantial cytotoxic effect is seen at all three time points after p53-FLAG transduction (P < 0.001). Bars, SD.

Induction of Apoptosis by p53-FLAG Adenovirus Infection. Because the observed cell loss could have been due to either necrosis or apoptosis, the presence of apoptosis was determined by DNA end labeling, which detects the DNA fragmentation associated with the initiation of this process. After transduction of wild-type p53, diffuse end labeling was seen within the nuclei of the residual TR146 cells at 24, 48, and 72 h, implying that apoptosis was responsible for the cell loss in these cultures (Fig. 3A). No end labeling was seen in the dl312 or mock-infected controls (Fig. 3B).

Expression of the FLAG Peptide in p53-FLAG Transduced Organotypic Cultures. Because exogenous p53 protein expression could not be distinguished from endogenous expression of the mutated protein in this cell line (Fig. 4A and B), the FLAG peptide was utilized as a marker to verify transduction efficiency. Diffuse expression of the FLAG peptide was seen in a large proportion of the TR146 cells that remained after p53-FLAG adenoviral infection and confirmed the earlier β-galactosidase transduction efficiency results (Fig. 4C). This expression was also evident in the basal layer, implying full-thickness penetration of the virus. Although some FLAG expression could be detected within the cytoplasm, the signal was located primarily within the nuclei, corresponding to the region where p53 protein is typically expressed. No staining was seen in the control cultures (Fig. 4D). These findings further confirm that genetic information can be transduced successfully into organotypic cultures that contain multiple cell layers after a single topical application of an adenoviral vector.

DISCUSSION

Recently, an increasing number of genetic mutations in HNSCC have been identified. Mutations in the p53 tumor suppressor gene have been the focus of much attention (1, 2), although loss of heterozygosity and microsatellite analyses suggest that multiple genetic events occur during the progression toward malignancy (12, 13). Once the significance of these mutations and the mechanisms by which they result in carcinogenesis are elucidated fully, we can potentially correct these abnormalities through gene therapy. Alternative strategies for gene therapy are also being investigated for established HNSCCs, which involve the transfer of genes that produce substances that are toxic to malignant cells (14) or that induce apoptosis (3). Because of the accessibility of upper-aerodigestive tract epithelia, gene transfer through topical application is a potential method of gene delivery to neoplasms in this region and could also be employed in other organ systems, including lung, bladder, abdominal visceral organs, and uterus.

Our studies sought to determine whether a topically applied adenoviral vector containing specific genetic information would transduce carcinoma cells in an organotypic model. In contrast to monolayer cultures, the organotypic culture retains many characteristics of the parent tissue. It has been demonstrated previously that epidermal keratinocytes grown in this system stratify and differentiate, achieving a morphology that closely resembles the native epithelium (6, 15). In our investigations, HNSCC cells grown in organotypic culture also stratify and differentiate, occasionally forming keratin pearls. Histologically, these cultures resemble carcinoma in situ, because invasion of the underlying stroma is rarely seen. This may be secondary to the type and high concentration of collagen used. Because the three-dimensional cytoarchitecture and the accompanying cellular interactions are maintained in organotypic culture, the effects of molecular or other interventions may correlate more accurately with those that would be obtained in vivo. In addition, because the cells are grown at the air-liquid interface rather than submerged in medium, the conditions more closely approximate those of upper aerodigestive tract epithelium, which render them amenable to topical gene therapy.

We have shown that when an adenovirus containing either the β-galactosidase or p53-FLAG construct was applied to the surface of organotypic cultures, the respective gene was transferred successfully to the majority of cells throughout the culture. Diffuse expression of the transduced gene products was apparent, and this expression was not confined to the superficial cell layer but involved multiple layers, including the basal layer. The transduction efficiency approximated 70%, which is consistent with that described previously in in vitro monolayer cultures after a single infection (8).

Our prior investigations in HNSCC cell lines have shown that significant overexpression of the p53 gene product after wild-type p53 adenovirus transduction inhibited growth (8) and induced apoptosis within in vitro monolayer cultures (3). These effects were seen in cell lines that had p53 gene mutations as well as those that were homozygous wild-type for this gene. In animal models, infection by the wild-type p53 adenovirus induced extensive apoptosis in HNSCC tumors, and atrophia intillation of this construct into s.c. flaps seeded with HNSCC cells significantly suppressed tumor development (3, 8, 16). In addition, we have shown that the p53-FLAG adenovirus and the p53 adenovirus have equal in vitro transduction efficiency as well as cytotoxicity (9). The FLAG marker simplifies the analysis of exogenous p53 protein expression after adenoviral trans-
Fig. 3 A and B, induction of apoptosis by p53-FLAG adenovirus infection. Using in situ DNA end labeling, several residual cells are shown to be undergoing apoptosis 48 h after p53-FLAG infection, indicated by the brown nuclear staining (A). No apoptosis is evident in dl312-infected cultures at the same time point (B).

Fig. 4 A and B, p53 protein immunohistochemistry. No difference in p53 protein expression by p53-FLAG-transduced (A) or dl312-transduced (B) cultures can be appreciated. C and D, p53-FLAG expression after p53-FLAG adenovirus infection. As evidenced by brown nuclear and cytoplasmic staining, multiple residual cells in p53-FLAG-infected cultures express the FLAG peptide 24 h after infection (C), implying effective transduction and expression of the transferred gene. No such expression is seen in the dl312-infected cultures (D).
duction, because its detection is not affected by mutant p53 protein expression, which, unlike the wild-type protein, is usually detectable to various degrees immunohistochemically.

Organotypic cultures similarly confirm induction of apoptosis after p53-FLAG adenovirus infection of HNSCC cells. However, in contrast to the results obtained in monolayer cultures and consistent with those seen in vivo, total cell loss did not occur in the organotypic cultures. This may reflect multiple factors, including the presence of multiple cell layers, the extent and distribution of adenovirus receptor expression, and the role of the bystander effect in the loss of nontransduced cells.

Although HNSCC cells can be transduced efficiently, previous animal studies suggest that normal, stratified epithelium of the oral cavity may be resistant to topically applied adenoviruses despite efforts to disrupt the integrity of the surface epithelium through abrasion, shocking, or dehydration (17). However, these cells can be transduced effectively by systemic injection of the adenovirus vector (17). This suggests that although nonneoplastic oral cavity epithelium can be transduced, the multiple steps that are required for carcinogenesis may facilitate transduction efficiency by topically applied adenoviral vectors.

In summary, the organotypic model provides an effective and rapid laboratory method of investigating strategies for gene therapy. It utilizes an environment that mimics that of the upper aerodigestive tract and histopathologically resembles carcinoma in situ. Additional progress in understanding the putative events required in the molecular cascade to head and neck carcinogenesis may allow us to target these events in prevention strategies.

REFERENCES

Evaluation of topical gene therapy for head and neck squamous cell carcinoma in an organotypic model.


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