The Role of Adenovirus-mediated Retinoblastoma 94 in the Treatment of Head and Neck Cancer

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ABSTRACT
A truncated retinoblastoma (RB) protein of ~94 kDa (RB94), lacking the NH2-terminal 112 amino acid residues of the full-length RB, has been found to have great efficacy in tumor suppression. This study investigated the role of adenovirus-mediated RB94 (Ad-RB94) gene therapy for human head and neck squamous cell carcinoma (HNSCC) and explored the cellular and molecular mechanism of tumor inhibition after Ad-RB94 gene transfer. Randomized controlled studies in vitro and in vivo were performed to assess antitumor responses of Ad-RB94 gene transfer against human HNSCC. Human HNSCC cell lines, JHU006 and JHU012, were used in this study. Tumors originated from the HNSCC cell lines were propagated as xenografts in nude mice. Ad-RB94 gene transfer was performed both in vitro and in vivo with replicative-defective virus (DL312) and no treatment as controls. Transgene expression, cell viability, and tumor growth were evaluated in transfected cells and tumor implants. To determine the mechanism behind the observed antitumor action, cell cycle analysis was performed, and telomerase activity was examined. Tumors were evaluated for RB94-induced apoptosis. Transgene expression of RB94 was detected by Western blot analysis, real-time quantification reverse transcription-PCR, and immunohistochemistry. RB94 expression led to flattening of cell growth curves and caused tumor regression. Animals treated with Ad-RB94 were seen to have a significant reduction in tumor size when compared with DL312 (P = 0.02, both cell lines) and to no treatment groups (P = 0.01, both cell lines). Cell cycle arrest in the G2-M phase and increased levels of apoptosis occurred in tumor cells treated with Ad-RB94. In addition, telomerase activity decreased significantly and specifically after Ad-RB94 treatment. This study demonstrates that Ad-RB94 gene transfer effectively inhibits HNSCC tumor cell growth in vitro and in vivo. The unique property of Ad-RB94 gene transfer to arrest HNSCC tumor cells in the G2-M phase of the cell cycle makes it a good candidate for adjuvant therapy with radiation or chemotherapy, as tumor cells are most sensitive to radiation or cytotoxic drug in this cell cycle phase.

INTRODUCTION
HNSCC1 is an important cause of cancer morbidity and mortality. It will comprise ~40,000 new cancer cases and an estimated 11,000 deaths in the year 2000 in the United States alone. Despite recent advances in surgery, radiation, and chemotherapy, there has been no improvement in overall survival in >30 years.2 As traditional treatments have not curbed this menace, many innovative approaches are being looked at for a cure. The local-regional biology of HNSCC and the persistent poor treatment outcomes make this disease an ideal target for tumor suppressor gene delivery.

The effective administration of therapeutic genes to target tumor cells requires an efficient delivery vehicle. Replication-defective adenovirus vector possesses several important advantages among the available vehicles for directly delivering foreign genes into mammalian cells and has been proven effective at gene delivery (1).

The RB gene is a tumor suppressor gene that encodes a protein that plays a key role in regulating cell cycle progression (2). The RB gene product is a 928 amino acid, 110-kDa nuclear phosphoprotein in which the normal function is to suppress cell growth by repressing DNA transcription and preventing cell division (2–4). Recent studies have shown that gene therapy using wild-type RB causes cessation of tumor cell growth in vitro as well as in vivo (3, 5). Re-expression of the RB protein in tumor cells has been found to cause cell cycle arrest in the G2-M phase of the cell cycle (6).

A truncated RB94, lacking the NH2-terminal 112 amino acid residues of the full-length RB, has been found to have even greater efficacy than wild-type RB in tumor suppression (3, 5). Pulse-chase labeling of the RB94 protein expressed in transiently-transfected RB−/− tumor cells indicated that the half-life of the protein was ~12 h, about three times longer than that for wild-type RB in the same cell line. Western blot analysis of the transfectants indicated that the RB94 protein was hypophosphorylated, in contrast to the hyperphosphorylated state of the 110-kDa RB protein in similar studies (3, 5). Studies comparing the effects of RB94 and wild-type RB on a series of RB−/− tumor cell lines with diverse tissue/organ origins demonstrated that RB94 was more potent than the full-length RB in reducing DNA replication. Even more striking was that expression of RB94, but not wild-type RB, significantly reduced colony formation by tumor cell lines expressing endogenous wild-type RB. Thus, it appears that RB94 is a more potent tumor-suppressing agent than full-length, wild-type RB (3, 5).

To survive, tumor cells must be able to maintain telomeres (7). Telomeres are the caps on chromosomes that protect gene-containing DNA and prevent errors during mitosis. They are usually diminished after each cellular division because DNA polymerase cannot fully replicate the lagging strand. Greater than 90% of cancers achieve telomere maintenance through the reactivation of telomerase, a ribonucleoprotein that uses its RNA component to add on nucleotides to extend the ends of DNA. This enzyme is inactive in most normal cells other than germ cells but has been found to be present in most cancers (6–8). Interestingly enough, a significant reduction in telomerase activity has been observed in RB−/− breast carcinoma cells after transfection with wild-type RB (6).

The findings of RB94 as a potent tumor suppression agent led us to investigate the effect and mechanism of the Ad-RB94 gene transfer on human HNSCC cells in vitro and in vivo in an HNSCC xenograft nude mouse model.

MATERIALS AND METHODS
Cell Lines
The human squamous cell carcinoma cell lines JHU006 and JHU012 (originally derived at the Johns Hopkins University Head and Neck Laboratories, Baltimore, MD) were used in all experiments. These cell lines were

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2 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; RB, retinoblastoma; RB94, RB protein of ~94 kDa; Ad-RB94, adenovirus-mediated RB94; MOI, multiplicity of transfection; TBS-T, Tris-buffered saline with 0.1% Tween; RT-PCR, reverse transcription-PCR; TRAP, telomerase repeat amplification protocol.


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derived from human tumor explants and have been well characterized and are known to express wild-type RB (data not published; Ref. 1). Cells were propagated in RPMI 1640 with 10% FBS and 1% Penicillin and Streptomycin at 37°C in 5% CO₂.

Animals
Animal experiments were performed on nude mice (athymic, BALB/c nu/nu). The care and use of all animals were in accordance with the guidelines of the animal welfare committee of University of Maryland School of Medicine.

Construction of Recombinant Adenoviral Vectors
Construction of recombinant adenoviral vectors with a tetracycline-inducible promoter have been described previously (9, 10). The Ad-RB94 vector was constructed by Dr. Hong-Ji Xu (Department of Molecular Oncology and Department of Hematology, Division of Medicine, The University of Texas M. D. Anderson Cancer Center). Briefly, the desired human RB cDNA fragments encoding the NH₂-terminal-truncated pRB94 protein (from nucleotide +322 to +3548, the A of the first in-frame AUG codon in the RB coding sequence was designated +1) was first inserted into the plasmid vector EC1214A. The modified pTA expression cassette (10) and the tetracycline-responsive pRB94 expression cassette were inserted, respectively, into a master plasmid, pBHG11 (Microbix Biosystems, Inc.), which contains the Ad5 E1/E3 deletion mutant genome, and into a shuttle plasmid, pDIEps1A. The resultant plasmids, pHG11.pTA and pE1.RB94, were then cotransfected into 293 cells (ATCC CRL1573) to produce infectious virions by in vivo coinfection. Presence of recombinant adenoviruses in the transfected 293 cells were initially identified by cytopathic effect. Cell culture supernatants were collected from the transfected 293 cells in which cytopathic effect had occurred. Recombinant viruses were then isolated by screening adenovirus plaques from 293 monolayers after infection with the virus supernatants and additionally characterized by restriction enzyme digestion mapping and by pRB94 protein expression in virus-infected RB-defective tumor cells. The recombinant adenoviruses containing the pRB94 expression cassette were subjected to at least three rounds of plaque purification and designated AdVtTA.RB94 (Ad-RB94). Purified Ad-RB94 was prepared as a sterile suspension containing 2–5 × 10¹¹ virus particles/ml in PBS with 10% glycerol. Control adenovirus DL312 with E1a region deletion was obtained from Dr. Tom Shenk (Princeton University, Princeton, NJ). Viruses were amplified and plaque purified. Titers were determined by standard plaque assay with the addition of 1% doxycycline to the cell media before infection and in the agarose overlay after infection in the case of Ad-RB94 (1). Doxycycline was only used in Ad-RB94 plaque assays and in the manufacture of the virus to shut down RB94 gene expression because RB94, being more potent than wild-type RB, could impact on cell growth and virus production. No other applications of Ad-RB94 required the addition of any doxycycline, and no doxycycline was present in the viral stocks used.

Evaluation of RB94 Expression after Ad-RB94 Gene Transfer
Western Blotting. RB94 expression was confirmed by Western blotting after transfection with Ad-RB94. JHU006 or JHU012 cells were plated in 75-mm tissue culture flasks at a density of 1 × 10⁶ to allow adhering overnight. Transfection was performed at a MOI of 1:10 in 4 ml of media at 37°C for 4 h. An additional 16 ml of media were then added, and cells were incubated at 37°C for 24 h. Media were removed, and cells were trypsinized. Trypsin was neutralized with media; cells were pelleted by centrifugation at 3000 rpm for 5 min and rinsed thoroughly with PBS twice. PBS was aspirated and cells were resuspended in 100 µl of hot (85°C) SDS gel-loading buffer [50 mM Tris-CI (pH 6.8) 100 mM DTT, 2% SDS, and 0.1% bromphenol]. Supernatant representing 1 × 10⁶ cells was run on 8% SDS-PAGE gel, then electrophoretically transferred onto Hybond enhanced chemiluminescence nitrocellulose membrane as described previously (10). Nonspecific binding sites were blocked using TBS-T and 5% nonfat dried milk for 1 h at room temperature. Samples were incubated 1 h at room temperature with primary antibody mouse-antihuman RB (PharMingen, San Diego, CA), which recognizes both full-length RB and NH₂-terminal-truncated RB94 proteins, at a concentration of 1:1000 in TBS-T. Filters were rinsed with TBS-T, and a secondary antibody goat antimouse conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ) was applied for 1 h at room temperature. Filters were again rinsed and then incubated with chemiluminescence substrates of ECL Western Blotting Detection System (Amersham Pharmacia Biotech). The blotting results were recorded by exposing to a Hyperfilm chemiluminescence film (Amersham Pharmacia Biotech). The bound antibodies on the filters were removed by submerging the filters in stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCI (pH 6.7)] and incubating at 50°C for 30 min with occasional agitation. The filters were washed twice using large volumes of TBS-T buffer. β-Actin was then detected by using monoclonal anti-β-Actin (Sigma, St. Louis, MO) as the primary antibody with the methods aforementioned.

Real-Time Quantification RT-PCR. Direct mRNA isolation from tumors in each of the studied groups was performed with an mRNA isolation kit (Roche Molecular Biochemicals) and quantified by a UV spectrophotometer. To detect specific RB94 transgene expression in vivo, two pairs of primers were designed. The first primer set was designed to span a 559-bp segment of the wild-type RB mRNA that is missing in the RB94 mRNA (sense primer 5’–CCCTCTGTCAGGCTTGTTG-3’ and antisense primer 5’–TTGAG-CACATGGAGGTAG-3’). The second primer pair set was designed to span a 719-bp segment that is common to both wild-type RB and RB94 mRNA (sense primer 5’–GACAGAGTGTCGACCGATAG-3’ and antisense primer 5’–GTGCCCATACCACTCCAG-3’). Quantification of wild-type RB and RB94 mRNA was performed with the LightCycler System according to the manufacturer’s instruction for the LightCycler-RNA Amplification Kit SYBR Green I and the LightCycler-Control Kit RNA (Roche Molecular Biochemicals). Briefly, designed primers were prepared at a final concentration of 0.3–1 µM each. A negative control (PCR grade water) was run with the samples. The master mix, including the LightCycler-RNA Amplification Kit SYBR Green I and the Control Kit RNA, was prepared and placed in the rotor of the LightCycler instrument. The cycle parameters were set up according to the kit’s instructions. The specificity of the amplified PCR products for wild-type RB and RB94 was assessed by performing a melting curve analysis with the LightCycler-Relative Quantification Software. The experiments were undertaken in triplicate, and the data were statistically analyzed with StatMost software.

Immunohistochemistry Studies. After tumor harvest, tumor samples were fixed in 4% paraformaldehyde and embedded in paraffin using standard procedures, and immunohistochemical analysis was performed using protocols from PharMingen. Tissue samples were cut in 7-µm sections and placed on superfrost glass slides. Sections were deparaffinized by washing in three changes of xylene each for 5 min, followed by two changes of absolute ethanol for 5 min each. The specimens were then washed with two changes of dH₂O for 2 min each, followed by a 10-min rinse in 3% H₂O₂ in methanol to quench endogenous peroxidase. They were rinsed twice in dH₂O and once in PBS. Slides were blocked with PBS with 5% goat serum and incubated for 30 min at room temperature and then incubated overnight at room temperature with primary antibody mouse-antihuman RB (1 mg/ml; PharMingen) at a 1:500 dilution in PBS. After this, slides were rinsed three times in PBS and the secondary antibody; goat antimouse conjugated to horseradish peroxidase (KPL Inc., Gaithersburg, MD) was applied for 2 h at room temperature. Slides were again rinsed, and protein detection was accomplished by incubation with diaminobenzidine in the standard fashion (10). Slides were then rinsed three times in dH₂O and counterstained by dipping twice in hematoxylin, rinsed in dH₂O, dipped twice in bluing reagent, and rinsed in dH₂O. Slides were then dehydrated with four changes of 100% ethanol, cleared in three changes of xylene, and coverslipped. A Nikon microscope digital image system was used to assess the immunohistochemical staining obtained. For analysis, four randomly selected high-powered fields on each slide were counted. The images were captured by a SPOT 2 cooled CCD color digital camera and saved in our image data storage system. Positive staining was quantified objectively using IPLab image analysis software. The calculated levels of staining in the no-treatment group provided a relative measure of baseline for endogenous wild-type RB expression and allowed assessment of additional RB94 production over and above endogenous RB after Ad-RB94 gene transfer in the HNSCC tumors. The experiments were repeated, and the resulting data were statistically analyzed by using StatMost software.
In Vitro Effects of Ad-RB94 Gene Transfer on Cell Growth and Survival

Triplicate samples of $3 \times 10^5$ log phase cells were plated in 96-well tissue culture plates and allowed to adhere overnight. The media were then removed, and cells were incubated with either Ad-RB94, or DL312 at an MOI of 1:10 or mock treated with PBS in 40 µl of media for 4 h, after which 160 µl of media were added. Cells were incubated at 37°C and evaluated daily for 5 consecutive days. Cell growth was determined by adding 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (5 µg/ml) to each well and incubating for an additional 4 h at 37°C. The supernatant was then discarded, and 150 µl of DMSO were added. The absorbance was determined by spectrophotometry using a wavelength of 570 nm with 630 nm as a reference.

In Vivo Ad-RB94 Treatment of Established Xenograft Tumors

For each experiment, 12 mice were randomized into 3 groups of 4 mice each for each cell line. Six-week-old female mice were anesthetized by i.p. injection of 6–10 mg of tribromoethanol with depth of anesthesia determined by toe pinch. Mice then received s.c. injections in left and right flanks with $1 \times 10^7$ JHU006 or JHU012 cells suspended in 100 µl of Hank’s buffered saline solution. Six to 8 tumors were propagated in each group.

Ten days after injection, animals were reanesthetized with tribromoethanol. Skin flaps were raised and tumors exposed. Measurements were made in three dimensions using calipers. Tumors were then injected using a 100-µl Hamilton syringe and 26 gauge needle with 8.5 × $10^7$ JHU006 or JHU012 cells suspended in 100 µl of Hank’s buffered saline solution. Six to 8 tumors were propagated in each group.

Cells were then incubated at 37°C for 5 min) were prepared. TS primer from the appropriate buffer were added to individual samples, incubated, and put through according to the manufacturer’s instructions for cell culture samples with $^{32}$P used for the detection of telomerase activity. Briefly, cells were lysed in 100 µl of 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid lysis buffer containing RNase inhibitor (100 units/ml), and the protein concentration according to the manufacturer's instructions for cell culture samples with $^{32}$P used for the detection of telomerase activity.

Telomerase Assay

Cells were plated at a density of $3 \times 10^5$ log phase cells/well in 6-well tissue culture plates and allowed to adhere overnight. Media were removed and cells incubated with either Ad-RB94 or DL312 at an MOI of 10 or mock-treated with PBS in 500 µl media for 4 h, after which 3.5 ml of media were added. Cells were then incubated at 37°C and evaluated daily for 0–5 days. For the determination of telomerase activity, cells were trypsinized, neutralized with media, and pelleted by centrifugation at 3000 rpm for 5 min and supernatant aspirated. Cells were then resuspended in PBS and repelleted, and the cell pellets were stored at ~80°C. Protein concentrations were determined by ELISA following the standard protocol for the Bio-Rad Protein Assay and BSA standard (Bio-Rad, Hercules, CA). Telomerase activity was detected using the TRAPEZE Telomerase Detection Kit (Intergen, Purchase, NY) according to the manufacturer’s instructions for cell culture samples with $^{32}$P used for the detection of telomerase activity. Briefly, cells were lysed in 100 µl of 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid lysis buffer containing RNase inhibitor (100 units/ml), and the protein concentration was determined. Samples were diluted to 100 ng/ml, and both native and heat-inactivated samples (85°C for 5 min) were prepared. TS primer from the kit was labeled using T4 polynucleotide kinase and $^{32}$P, and then Taq polymerase deoxynucleotide triphosphates, a labeled primer, mixed with the appropriate buffer were added to individual samples, incubated, and put through a two-step PCR-based amplification process to generate a ladder of products with 6-base increments when subjected to electrophoresis. Samples were subjected to electrophoresis on a 12% polyacrylamide gel. Gels were dried, and reaction products visualized through autoradiography and then quantified by Phosphomager analysis. Total product generated was determined as described in the Intergen protocol.

Fig. 1. Detection of RB94 and endogenous wild-type RB proteins in JHU006 and JHU012 cell lines by Western blot after Ad-RB94 gene transfer. Each lane represents 1 × 10^7 JHU006 or JHU012 cells after transfection with Ad-RB94, DL312, or no-treatment control, then run on an 8% SDS-PAGE gel. All lanes show baseline 110-kDa wild-type endogenous RB expression reactive to the anti-RB antibody. Cell lines transfected with Ad-RB94 show an additional 94-kDa immunoreactive band. Cell lines transfected with control adenovirus DL312 or no-treatment control (JHU022) do not exhibit the 94-kDa band.

Fig. 2. Real-time quantification RT-PCR analysis of RB expression levels. Human HNSCC xenograft tumors (JHU006) were treated with Ad-RB94 or DL312 or given no treatment as a control. A, expression levels of wild-type RB mRNA in tumors using the primer pair designed to span a region missing in RB94. The expression levels in each of the treated groups were identical, and there were no statistical differences (P = 0.51–0.82). B, expression levels of total RB and RB94 mRNA in tumors using the primer pair designed to span a segment common to both wild-type RB and RB94. A highly significant increase in the expression level of total RB and RB94 mRNA was detected in the group transfected with Ad-BR94 when compared with the groups treated with DL312 and no-treatment control. The differences were statistically significant at P = 0.004 (RB94 versus DL312 and RB94 versus no-treatment). There was no statistical difference between DL312 and no-treatment groups (P = 0.82).
Apoptosis Studies

The effect of Ad-RB94-induced apoptosis was evaluated on tumors generated from JHU006 and JHU012 cell lines implanted in nude mice as described above. The ApopTag Peroxidase in Situ Apoptosis Detection Kit and protocols (Intergen) were used to detect early DNA fragmentation associated with apoptosis. In brief, tumor samples were fixed in formalin, embedded in paraffin using standard procedures, and tissue samples were cut in 7-μm sections and placed on superfrost glass slides. Sections were deparaffinized with xylene, then rinsed in absolute ethanol, and washed in 95% ethanol then 70% ethanol. Protein digesting enzyme (20 mg/ml) was applied to the specimens for 15 min at room temperature, then washed with dH2O, and rinsed in 3% H2O2 to quench endogenous peroxidase. Specimens were dried, and equilibration buffer was applied directly to the sample for 10 s at room temperature. Buffer was removed, and Working Strength Terminal Deoxynucleotidyltransferase Enzyme was applied for 1 h at 37°C. Working Strength Stop/Wash Buffer was then applied and incubation continued for 10 min. Specimens were washed with PBS, and antidigoxigenin peroxidase conjugate was applied for 30 min at room temperature. Specimens were then washed in PBS, and peroxidase substrate was applied for 3–6 min. Specimens were washed in dH2O, and methyl green counterstain was applied. Slides were mounted and viewed via microscopy. Representative sections of tumors were identified, and cells stained positive for apoptosis were counted using IPLab software (Scanalytics, Fairfax, VA).

Statistical Analysis

Statistical analysis was performed using StatMost software to determine statistical significance.

RESULTS

Expression of RB94 in Vitro after Ad-RB94 Gene Transfer

Western blot analysis of RB protein expression following Ad-RB94 gene transfer revealed two bands reactive to the anti-RB antibody at both 110 kDa (wild-type RB) and 94 kDa (RB94) in JHU006 and JHU012 cell lines (Fig. 1). A 94-kDa band was not seen in either cell line treated with DL312 or buffer control.

Expression of RB94 in Vivo after Ad-RB94 Gene Transfer

Real Time Quantification RT-PCR. To confirm whether the tumors transfected with Ad-RB94 indeed expressed RB94, we used LightCycler RT-PCR analysis using mRNA extracted from these tumors. LightCycler system is a real-time quantitative analysis of expression of a gene of interest versus the housekeeping gene cytochrome. Tumors derived from the JHU006 cell line were used for this
Ad-RB94 suppresses tumor growth in vivo. Ad-RB94 showed strong tumor suppression in the nude mouse xenograft tumor model. Xenografts were established and treated as described in “Materials and Methods.” Animals treated with Ad-RB94 had a significant reduction in tumor size when compared with DL312 group ($P = 0.02$, both cell lines) and to the no-treatment group ($P = 0.01$, both cell lines; Fig. 5, A and B).

Ad-RB94 gene transfer induces G2/M cell cycle arrest. To investigate the mechanism behind the antitumor activity, cell cycle studies were performed to determine the fate of cells after Ad-RB94 gene transfer. JHU006 and JHU012 were transfected with Ad-RB94 or DL312 at an MOI of 1:10 or given no treatment. Flow cytometry was performed along with cell cycle analysis (Figs. 6, A–C). In repeated experiments, flow cytometry showed that Ad-RB94-transfected cells arrested in the G2-M phase of the cell cycle, whereas cells transfected with DL312 maintained a cell cycle distribution with no G2-M arrest that was identical to the no-treatment group.

Ad-RB94 gene transfer inhibits telomerase activity in vitro. A recent paper had suggested a link between telomerase activity, senescence, and wild-type RB expression (6). To further elucidate the process by which RB94 inhibits tumor growth, we assessed the level of telomerase activity in JHU006 and JHU012 cell lines after transfection with Ad-RB94, DL312, or after no treatment using a PCR-based TRAP assay. Cells were collected on days 0–6 after transfection, lysed, and protein concentrations were determined. TRAP assays were then performed on equal amounts of cellular extracts and subjected to PAGE. The gels were dried and autoradiographed. A decrease in telomerase activity as quantified by PhosphorImager analysis was observed on day 5 and beyond in both JHU006 and JHU012 cell
lines transfected with Ad-RB94, which was confirmed in multiple assays. No definitive reduction was observed on days 0–4 (data not shown). After Ad-RB94 transfection, there was an average 58% decrease in telomerase activity in the JHU006 (Fig. 7A) and a 41% reduction in the JHU012 cell line (Fig. 7B) when compared with the no-treatment group, and these differences from the control were statistically significant ($P < 0.05$). For both cell lines, there was no significant statistical difference between the DL312 and no-treatment groups.

Ad-RB94 Induces Apoptotic Cell Markers in Vivo. Having witnessed robust tumor growth suppression with Ad-RB94 in vitro and in vivo, an examination of apoptosis-related molecular markers was performed on tumor sections. An apoptosis detection kit was used to detect early DNA fragmentation associated with apoptosis (Fig. 8A). The apoptotic index for the Ad-RB94-treated tumors was found to average 33% (Fig. 8B), significantly higher than that obtained for the DL312 and no-treatment groups.

DISCUSSION

We have demonstrated that the 94-kDa protein encoded by Ad-RB94 gene transfer significantly suppresses HNSCC tumor growth both in vitro and in vivo. Furthermore, we have shown for the first time that the expression of RB94 protein correlates with a significant inhibition of telomerase activity, cell cycle arrest in the G2-M phase, and induction of apoptosis in vivo.

The nuclear phosphoprotein RB tumor suppressor gene product regulates cell growth by repressing DNA transcription and preventing cell division (2, 3, 5). Activity of the RB phosphoprotein is negatively regulated by cell cycle, cyclin-dependent kinases through increased
phosphorylation of the protein. When expressed in tumor cells, RB has been shown to cause growth suppression both in vitro and in vivo, however, the effect was limited because of the rapid inactivation of the wild-type RB protein (3, 5). RB94 is a truncated form of RB, missing the NH$_2$-terminal 112 amino acids but still possessing many of the NH$_2$-terminal and COOH-terminal phosphorylation sites and the pocket domain (3, 11). RB94 has been shown to have an even greater efficacy in tumor suppression than RB probably because of its longer half-life and the tendency to remain in the active, hypophosphorylated state (3, 5). Our study confirms that an adenoviral vector efficiently delivers the RB94 gene into targeted cells and suppresses tumor cell growth both in vivo and in vitro.

Previous studies on wild-type RB expression in tumor cell lines reported cell cycle arrest at G$_0$-G$_1$ (6, 11). One possible explanation for the G$_2$-M cell cycle arrest seen in HNSCC cells could be that the endogenous wild-type RB interacts or interferes with RB94. To control for this, we infected RB$^{-/-}$ SAOS-2 cells (American Type Culture Collection, Manassas, VA) with Ad-RB94. Contrary to previously published papers showing G$_0$-G$_1$ arrest after wild-type RB transfection, (6, 11) Ad-RB94 transfection in SAOS-2 cells induced the same shift to G$_2$-M arrest after that we observed in HNSCC cells (data not shown). Alternatively, the NH$_2$ terminus truncation and subsequent hypophosphorylated RB94 protein may result in differential binding of other factors involved in the S to G$_2$-M transition. The literature supports an evolving theory that differential phosphorylation of the 16 consensus sites in the RB protein may alter S-phase completion to cause cell cycle arrest at G$_2$-M (4, 11–14).

We show that RB94 expression in HNSCC tumor cells leads to apoptotic tumor cell death in a xenograft nude mouse model and apoptotic-like cytological changes in multiple in vitro studies. The number of cells expressing RB94 in our study was slightly higher than the number observed undergoing apoptosis, strengthening a cause and effect relationship. Similar studies with wild-type RB expressed in several tumor cell lines failed to detect the induction of apoptosis and showed instead the development of a senescence-related marker and a senescent phenotype with large, flattened cells with low nucleus to cytoplasm ratios (6). The missing NH$_2$ terminus in the truncated RB94

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**Fig. 7.** Ad-RB94 gene transfer inhibits telomerase activity. PCR-based TRAP assays were used to assess the level of telomerase activity in JHU006 and JHU012 cell lines after transfection with Ad-RB94, DL312, or after no-treatment control. Reaction products were subjected to electrophoresis on a 12% polyacrylamide gel then quantified by PhosphoImager analysis. Total product generated was determined as described by the manufacturer’s protocol. After transfection with RB94, both cell lines JHU006 (A) and JHU012 (B) had a significant decrease in telomerase activity when compared with controls.

**Fig. 8.** Ad-RB94 gene transfer induces apoptotic cell markers in vivo. The ApopTag Peroxidase in Situ Apoptosis Detection Kit and protocols (Intergen) were used to detect early DNA fragmentation associated with apoptosis in JHU006 cell line after transfection with Ad-RB94, DL312, or no-treatment control. A, a representative tumor section originating from JHU006 cell line demonstrates 33% positive apoptotic staining cells after Ad-RB94 gene transfer, whereas only a few positive cells were observed in the controls. Multiple sections of tumor were analyzed, and cells staining positive for apoptosis were counted using the IPLab software, and data were converted into a graph. B, JHU006 exhibited a distinct increase in early apoptotic markers after transfection with RB94.
protein may provide a switch between these two mutually exclusive events. This hypothesis is supported by studies reporting that a hypophosphorylated form of RB that undergoes proteolytic cleavage has been associated with apoptosis and other studies linking wild-type RB to the regulation of apoptosis (2, 15, 16).

Telomeres are repetitive DNA repeats found on the ends of chromosomes that are necessary for their stability. During cellular division, telomeres are typically shortened by ~2–3 kbp, and a critical threshold for telomeric length is believed to exist beyond which cells will not divide and will instead become senescent. The ability to maintain telomeres is a critical step toward achieving cellular immortality (7, 8, 17, 18). Ninety percent of HNSCC cancers achieve this goal by activating telomerase, a ribonucleoprotein which extends telomeres through an RNA reverse transcriptase mechanism (7, 8, 17, 18). Wild-type RB has been shown to inhibit telomerase in some cancer cells, (6) and we have found that RB94 gene expression can also inhibit telomerase activity in HNSCC cell lines. This knowledge that RB94 can suppress telomerase activity may be useful in adjuvant therapy with radiation as tumors with high telomerase expression have been found to be less radiosensitive than those with reduced telomerase expression (20). As reported recently, telomerase activity is also associated with resistance to apoptosis in tumor cells. This study demonstrates that the inhibition of telomerase activity by Ad-RB94 gene transfer is accompanied with the induction of apoptosis in HNSCC cell lines. RB94 gene therapy, by reducing telomerase expression and arresting tumor cells in the most radiosensitive G2-M stage of the cell cycle, can be an innovative modality to sensitize tumors to radiation.

Additional studies are under way to elucidate the molecular specifics of RB94 activity and to test our hypothesis for its efficacy in a combination therapeutic treatment modality for head and neck cancers.

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