Human adenovirus (HAdV) vectors are intensively investigated for virotherapy of a wide variety of human cancers. Here, we have evaluated the effect of two apoptogenic HAdV5 vectors in an immunocompetent Syrian hamster animal model of head and neck cancer. We established two cell lines of hamster cheek pouch squamous cell carcinomas, induced by treatment with 9,10-dimethyl-1,2-benzanthracene. These cell lines, when infected with HAdV5 mutants lp11w and lp11w/A55 K (which are defective in the expression of either E1B-19 K alone or both E1B-19 K and E1B-55 K proteins) exhibited enhanced apoptotic and cytotoxic responses. The cheek pouch tumor cells transplanted either subcutaneously at the flanks or in the cheek pouches of hamsters readily formed tumors. Intra-tumoral administration of HAdV5-E1B mutants efficiently suppressed the growth of tumors at both sites. Histological examination of orthotopic tumors revealed reduced vascularity and the expression of the viral fiber antigen in virus-administered cheek pouch tumors. These tumors also exhibited increased caspase-3 levels, suggesting that virus-induced apoptosis may contribute to tumor growth suppression. Our results suggest that the apoptogenic HAdV5 vectors may have utility for the treatment of human head and neck cancers.

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In the present study, we chose to investigate the antitumor activity of HAdV5 mutant p11w as well as a second apoptogenic HAdV5 mutant that is defective in coding for both E1B proteins (p11w/Δ55K) in the Syrian hamster animal model. The rationale for the use of these vectors is to exploit their powerful pro-apoptotic activity to kill the tumor cells. Although most of the preclinical studies on the use of HAdV5 vectors employed immunodeficient nude mouse bearing human tumor xenografts, recent studies have exploited the Syrian hamster as an immuno-competent small animal model (which supports HAdV5 replication) to evaluate the oncolytic activity of HAdV5 vectors.20–22 To evaluate the apoptogenic HAdV5 vectors against head and neck tumors, we developed two Syrian hamster cell lines from the cheek pouch squamous cell carcinoma (SCC) induced by the chemical carcinogen DMBA (9,10-dimethyl-1,2-benzanthracene) and determined the oncolytic activity of the HAdV5 vectors in orthotopic and heterotopic tumors induced by these cell lines. Our study appears to be the first in which HAdV5 vectors have been tested in an orthotopic HNSCC tumor model.

MATERIALS AND METHODS

Cells
The human cell lines A549 (alveolar carcinoma), MCF7 (breast carcinoma), HeLa and IMR90 fibroblasts were obtained from the American Type Culture Collection (ATCC, Bethesda, MD, USA). The human embryonic kidney 293 (HEK-293) cells were from Microbiex (Toronto, ON, Canada). The hamster pancreatic carcinoma cell line SHPCC1 was obtained from Dr WS Mould. Normal human bronchial epithelial (NHBE) cells were obtained and grown in a specific medium from Cambrex (Walkersville, MD, USA). Normal human foreskin fibroblasts (HFF) were obtained as a gift from Dr R Ray. A549, 293 and HFF cells were grown in DMEM (Dulbecco's Modified Eagle's Medium; Sigma Life Science, St, Louis, USA) supplemented with 10% fetal bovine serum.

Establishment of Hamster pouch tumor cell lines
Four to six-week-old female Syrian hamsters were anesthetized using 3% isoﬂurane; their cheek pouches were painted with 0.5% DMBA (#D3254, Sigma Aldrich, St, Louis, MO, USA) in paraffin oil (#18512, Sigma Aldrich) using camel hair brush no.4 (#6020-04000, Gordon Brush, Commerce, CA, USA) twice a week for 12 to 14 weeks.24–25 The tumors were aseptically harvested from euthanized animals and transferred to RPMI media without serum. The cells from the tumors were prepared using a commercial kit (Cancer cell isolation kit - #C100XX, Panomics, Affymetrix, Santa Clara, CA, USA) and plated in DMEM supplemented with 20% fetal bovine serum, 10 μg/ml of hydrocortisone, 50 ng/ml of spermine and antibiotics.26 The cells were further cultured in DMEM supplemented with 10% fetal bovine serum with antibiotics. DMBA-induced buccal tumor cells (2 x 106) were transplanted subcutaneously in the flanks. Ten days after injection, the tumors were aseptically removed and the cells were prepared and cloned cell lines (hamster pouch tumor, HPT11 and 12) were established.

Subcutaneous tumor assay in nude mice
Hamster cheek pouch SCC tumor (HPT12) cells (4 x 103) were injected subcutaneously in 100 μl volume into 5–6-week-old female athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN, USA) into the hind flanks to establish subcutaneous tumors. Tumor-bearing animals were randomized before virus injection. When the tumor volumes reached about 100–200 μl, the animals were intratumorally injected with 100 μl vehicle (PBS + /–) alone or with 1 x 1010 PFU of p11w or p11w/Δ55K for 6 consecutive days (total 6 x 1010 PFU per animal). Tumor measurements were performed twice weekly.

Western blot
Cells were grown in 60 mm tissue culture dishes and washed twice with PBS. Cells were lysed in NuPAGE sample buffer and heated in boiling water for 5 min. The extracts were fractionated by SDS-PAGE (NuPAGE 4–12% gel, NOVEX, Life Technologies, Grand Island, NY, USA) and subjected to western blotting. The following antibodies were used: anti-cytokeratin 19 (sc-25724, Santa Cruz Biotechnology, Dallas, TX, USA), cytokeratin 8 (17514-1-AP, Proteintech, Chicago, IL, USA), cytokeratin 14 (22221-1-AP, ProteinTech), anti-EGFR (sc-03, Santa Cruz Biotechnology), anti-actin (sc-1615, Santa Cruz Biotechnology), caspase-3 (9662, Cell Signaling Technology, Danvers, MA, USA), PARP (poly ADP-ribose polymerase; 556362, BD Bioscience, San Jose, CA, USA), E1A (05–595, EMD Millipore, Billerica, MA, USA), E1B-19K (gift from Dr M Green) and E1B-55K (gift from Dr Peter Yaciuk) Abs.

Viral growth assay
A549, NHBE and HFF cells were plated in 35 mm dishes and at 60–70% confluency, were infected with HAdV5 mutants. A549 and NHBE cells were infected at 10 PFU per cell whereas HFF cells were infected at 100 PFU per cell. One hour after infection, the cells were washed three times with a medium containing no serum and fed with 2.5 ml of DMEM containing 5% fetal calf serum. After 40 h, the cells and the medium were harvested and the viruses were released by freeze, thaw and sonication and the virus yield was estimated by plaque assay on 293 cells.

Determination of virus titer in hamster serum
At the end of the experiments, hamsters were killed, blood was collected in sterile tubes without anticoagulant from each animal and allowed to stand for 1 h (Leica–BioFlex 20–24 min). The sera were centrifuged at 1500 x g for 10 min and the serum was removed and frozen at −80 °C. The serum samples were titered by plaque assay on 293 cells.

Neutralizing antibody titration
All serum samples were heat inactivated at 56 °C for 30 min before titration. They were diluted two fold across 96-well plates in four replicates. One column without serum sample and the other column without virus were run as controls. All wells except virus control were incubated with 100 PFU per well of HAdV5 wt. After 1 h, 5 x 103 A549 cells in growth medium were added to each well and incubated for 8–10 days at 37 °C. At the end of the incubation period neutral red (30-fold dilution of neutral red in PBS) was added to the cells and incubated for 1 h at 37 °C. The plates were gently rinsed with PBS two times followed by the addition of 100 μl of 5% acetic acid in water. After 10 min at room temperature, the plates were read at 550 nm using a plate reader and the neutralizing antibody titers were calculated.

Histology
Solid tumors were fixed in formalin and 2–3 mm thick slices were excised with a sharp scalpel through the middle of each tumor. The slices were fixed for another 24 h in formalin, processed into paraffin in a Miles Scientific Tissue-Tek VIP autosectioner, embedded in cassettes in a Leica EG1150H/11000 cryostat (Biosystems, Buffalo Grove, IL, USA) embedding station, and sectioned at 5 μm with a Leica RM2255 microtome using disposable steel blades. After collection on Super Frost glass slides (Thermo Fischer Scientific, Waltham, MA, USA), the sections were stained with hematoxylin and eosin in a Leica Autostainer XL and after coveringslipping with Leica Surgipath MM24 mounting medium were photographed with an Olympus BX41 microscope/DP72 digital camera system (Olympus, Center Valley, PA, USA). Contrast and brightness were adjusted using Microsoft Power Point.

Immunohistochemistry
Slides of 5 μm thick formalin-fixed paraffin sections were de-paraffinized and subjected to antigen retrieval using DIVA Decloaker solution (Biocare Medical, Concord, CA, USA). Following quenching of endogenous peroxidase with 3% hydrogen peroxide in PBS for 30 min, the sections were washed in PBS and blocked (1% bovine serum albumin, 5% normal goat serum, 0.05% TX100 in PBS) for 30 min at room temperature in a
humidified sealed container. Sections were then incubated overnight at 4°C in antifiber adenovirus Ab4 monoclonal antibody (4D2; MS-1027-P, NeoMarkers, Freemont, CA, USA) or anti-caspase-3 antibody (9662, Cell Signaling) diluted to 1:200 in 1/10th blocking buffer in a sealed humidified container. Negative controls were incubated in nonimmune isotype-specific mouse IgG (Sigma I5381) at the same IgG protein concentration as for the antifiber primary antibody. The sections were then washed in PBS and incubated for 1 h at room temperature in goat anti-mouse IgG conjugated to peroxidase (Sigma A4416) diluted to 1:400 in 1/10th blocking buffer. After washing in PBS, the sections were incubated in peroxidase substrate (Sigma D4168) for 5 min and the reaction was stopped by rinsing in water. The sections were counter-stained with hematoxylin, dehydrated, coverslipped with Leica Surgipath MM24 mounting medium and photographed as described above. Histology and immunohistochemistry staining were performed at the Research Microscopy and Histology Core, Saint Louis University School of Medicine.

RESULTS

HAdV5 vectors

We have previously reported that the replication-competent apoptogenic mutant of HAdV5, lp11w exhibited enhanced cytolytic activity in human HNSCC cells in vitro.27 The apoptogenic activity of lp11w is conferred by a mutation that obliterates the ORF for the viral anti-apoptotic protein E1B-19K.27 In the present study, we also used an additional vector that is defective in coding both E1B-19K and E1B-55K proteins (Figure 1a). We decided to include the mutation in E1B-55K, because HAdV5 mutants have been reported to exhibit restricted replication in certain normal human cells.28 We rationalized that this vector, designated here as lp11w/Δ55K, would incorporate both the enhanced apoptotic activity in cancer cells and reduced toxicity to normal cells. As expected, the newly engineered mutant (lp11w/Δ55K) was deficient in the expression of both E1B proteins whereas lp11w was deficient in the expression of only E1B-19K (Figure 1b). In addition, lp11w/Δ55K also expressed reduced levels of E1A proteins. We assessed the replication of these vectors in comparison with HAdV5 wt in NHBE cells and in normal HFF (Figure 1c). In NHBE, replication of both lp11w and lp11w/Δ55K was significantly reduced compared with HAdV5 wt. In contrast, the replication of lp11w was not impaired in HFF whereas replication of lp11w/Δ55K was severely impaired. These results suggest that the lack of E1B-19K in these vectors may reduce toxicity to normal epithelial cells during oncolytic applications to head and neck cancers. The combination of both E1B-19K and 55K mutations may retard replication in both NHBE and HFF.

Figure 1. HAdV5 mutants and replication in normal cells. (a) Diagrammatic illustration of HAdV5 mutants. The top panel shows the proteins expressed from early regions E1B and E3. Mutants lp11w and lp11w/Δ55K express wt E1A and E3 regions while the control mutant d312 contain deletions in these regions. The mutations within the E1B region are indicated. (b) Expression of E1A and E1B proteins by HAdV5 mutants. (c) Replication of HAdV5 mutants in normal human bronchial epithelial (NHBE) and human foreskin fibroblast (HFF) cells. The virus yield from infected NHBE and HFF cells were determined by plaque assay on 293 cells. HAdV, human adenovirus.

Statistical analysis

The data from multiple samples were compared by Student’s t-tests analysis, two-way ANOVA by Bonferroni multiple comparisons. Significant values were defined as those with $P$-values $< 0.05$. 

$\text{Oncolytic activity of HAdV5 vectors in hamsters}$

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HPT cell lines

The HPT model is a well-accepted orthotopic head and neck cancer model. As HAdV5 replicates in the Syrian hamster, it has been shown to be a valuable immune-competent model to investigate the oncolytic activity of HAdV5 vectors (reviewed in Wold and Toth). To evaluate the HAdV5-E1B vectors for head and neck cancers, we first explored the utility of the HPTs induced by painting with the chemical carcinogen DMBA. However, we encountered difficulty in quantifying the effect of intratumoral administration of the viral vectors in this system due to the heterogeneity of large number of tumors induced by DMBA on the painted area. Therefore, we decided to use syngeneic pouch tumor cell lines that could be transplanted in the pouch for more quantitative evaluation of HAdV5-E1B vectors. We established two clonal cell lines derived from pouch SCCs (HPT) induced by treatment with DMBA. We examined the expression of the SCC markers, cytokeratins 8, 14 and 19 in these cell lines (Figure 2a). Both cell lines expressed readily detectable levels of all three cytokeratins. To determine whether the viral vectors induce cytolysis of these cell lines, the cells were infected with HAdV5 wt or different mutants and cell lysis was quantified by the LDH (lactate dehydrogenase) release assay (Tollefson, 1996) (Figure 2b). In both HPT cell lines, mutant lp11w induced substantially increased release of LDH compared with HAdV5 wt. The mutant lp11w/Δ55K induced intermediate levels of LDH release. These results suggest that both E1B mutants may be more cytotoxic to the HPT cell lines than HAdV5 wt. Between the two E1B mutants, lp11w may be a more potent cytolytic agent than lp11w/Δ55K.

Apoptotic activity of E1B mutants in hamster cells

It is well known that HAdV mutants deficient in the expression of the viral anti-apoptosis protein E1B-19K induce apoptosis in cultured animal cells. We examined the apoptotic activity of the E1B mutants in the hamster tumor cell lines by analyzing the activation of caspase-3 as well as proteolytic processing of PARP and EGFR. Both E1B mutants induced efficient caspase-3 activation (loss of pro-caspase-3 and formation of 19 and 17 kD subunits) and proteolytic processing of PARP and EGFR (caspase target proteins), suggesting efficient apoptosis induction by lp11w and lp11w/Δ55K in both HPT11 and 12 cell lines (Figure 3a). We also measured the levels of apoptosis by FACS analysis of cells stained with annexin V-APC and the nonvital stain PI. In both cell lines infected with either E1B mutants, there was increased apoptosis as indicated by annexin V-staining and total cell death (PI-staining) (Figure 3b and c). The cell population in the transition phase (apoptosis to dead, positive for both Annexin V and PI-staining) also showed comparable patterns. These results show that both E1B mutants are apoptogenic in the HPT cell lines.

Tumor growth suppression by E1B mutants

We first determined the effect of the viral vectors lp11w and lp11w/Δ55K in the mouse xenograft model (Figure 4a). Athymic mice were transplanted with HPT12 cells subcutaneously and the tumors were then treated by intratumoral injection with different HAdV5 mutants. In this tumor model, both lp11w and lp11w/Δ55K suppressed tumor growth efficiently compared with d312 (replication-defective control). Although HAdV5 wt inhibited tumor growth, the effect was less compared with the E1B mutants. We then determined the effect of lp11w and lp11w/Δ55K in subcutaneous hamster flank tumors induced by the transplanta- tion of the two hamster tumor cell lines. The hamsters bearing HPT12-induced tumors were treated with different HAdV5 mutants by intratumoral injection and the changes in tumor volumes were monitored for 3 weeks. In tumors injected with mutant d312, there was substantial increase in volumes (Figure 4b). In contrast, there was no significant increase in tumor volumes in animals injected with lp11w or lp11w/Δ55K during the period of observation compared with animals injected with d312. The oncolytic activities of the E1B mutants were also assessed against tumor-bearing hamsters that were subcutaneously transplanted in the flanks with the tumor cell line HPT11. In these tumors, both E1B mutants efficiently inhibited tumor growth compared with the administration of vehicle alone (Figure 4c). These results suggested that the apoptogenic E1B mutants efficiently inhibited tumor growth both in the xenograft and syngeneic models transplanted with the HPT cell lines.

Since the E1B mutants exhibited significant antitumor activity against subcutaneous tumors in the flanks of athymic mice and...
hamsters, we then determined the effect of these mutants against orthotopic tumors in hamster pouches. The HPT cell lines were transplanted submucosally in the Syrian hamster cheek pouches. Both cell lines formed tumors in a more accelerated fashion in the pouches than in the flanks. As there was no significant difference in tumor growth in the pouch of animals treated with the vehicle or dl312 (results not shown), the effects of E1B mutants were compared with the effects of vehicle administration in these tumor assays. Between the two cell lines, HPT12 formed faster growing tumors than HPT11. Intratumoral administration of lp11w and lp11w/Δ55K inhibited the growth of tumors induced by both cell lines (Figure 5). We note that in some instances, viral administration induced transient inflammation in the tumors that skewed the measurements at certain time points (for example, Figure 5a, 7 days).

Tumor histology

Histological examination of the pouch tumors collected after sacrifice at 17 days from vehicle-treated animals revealed clear submucosal localization (Figure 6a, top panel) and increased angiogenesis and vascular ectasia with thrombosis, whereas tumors administered with the viruses exhibited reduced vascularity (Figure 6a, lower panel). The virus-treated tumors also showed large areas of confluent necrosis (Figure 6b, top panels, indicated by arrow heads) and focal areas of dystrophic calcification (Figure 6b, lower panels, indicated by arrows) compared with vehicle-administered tumors. Further, the tissues surrounding the virus-administered tumors also contained more cells that resembled aggregates of the plasma cells (Figure 6c, top panels) and infiltration of mast cells (Figure 6c, lower panels, indicated by arrows) than the tissues surrounding the vehicle-administered
tumors. The presence of features such as chromatin condensation associated with apoptosis was also prevalent in virus-treated tumors (Figure 6d).

We then analyzed the pouch tumor samples and the plasma from virus-treated animals at the end of the study for infectious virus by plaque and infectious center assays on sensitive human (293) cells. However, these assays did not reveal the presence of infectious virus at detectable levels (results not shown). We also analyzed the tumor sections by immunocytochemistry to detect the viral fiber antigen (Figure 7a) and caspase-3 (Figure 7b). In tumors treated with lp11w, focal positive staining patterns for fiber at discrete locations were observed (Figure 7a-3); whereas in tumors treated with lp11w/Δ55K (Figure 7a-4), more extensive staining was noted. These results suggest that the virus-treated tumors may support at least limited late viral gene expression. The virus-treated tumors were also significantly positive for caspase-3 staining, suggesting virus-induced apoptosis.

The sera from tumor-bearing hamsters collected at the end of the tumor assays were analyzed for antiviral antibodies (Table 1).
These results revealed the presence of neutralizing antibodies against HAdV5. These results suggest that the treatment of hamsters with the cytolytic viral vectors induced significant antibody response against viral antigens.

**DISCUSSION**

We have investigated the potential utility of two apoptotically proficient HAdV5 vectors against two head and neck model hamster cell lines in the immunocompetent Syrian hamster model.

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**Figure 6.** Histology of hamster cheek pouch tumors treated with HAdV5 vectors. Tumor sections were stained with hematoxylin-eosin and photographed. The size bars indicate 100 μm (top three panels), 50 μm (fourth panel), 100 μm (fifth panel) and 25 μm (sixth panel). (a) Vascularity of treated tumors. (b) Necrosis and calcification in treated tumors. (c) Presence of cells like plasma cells. (d) Presence of apoptotic features. HAdV, human adenovirus.
Most preclinical studies with oncolytic HAdV vectors have employed xenografts of human cancer cell lines in athymic mice. Recently, the Syrian hamster has been exploited as an immuno-competent model that is permissive for HAdV5 replication (reviewed in Wold and Toth30). A limitation for the use of the hamster system has been the availability of suitable syngeneic tumor cell lines. Here, we established two cell lines from the Syrian hamster cheek pouch SCCs that readily form orthotopic tumors upon submucosal transplantation in the cheek pouches as well as heterotopic tumors at the flanks upon subcutaneous transplantation. Between the two vectors, lpl11w exhibited enhanced cytolytic activity in the two hamster cell lines than lpl11w/Δ55K in vitro (Figure 2b). However, both vectors suppressed the growth of orthotopic and heterotopic tumors at comparable levels. Thus, it is possible that the virus-induced apoptotic response might contribute to their oncolytic activity. Extensive analysis of the tumor samples and the plasma from virus-treated hamsters at the end of the study did not provide evidence for the presence of infectious virus (results not shown). However, immunocytochemistry analysis of the tumor samples revealed the presence of different levels of the viral fiber antigen (Figure 7a). Thus, it is possible that there might be low levels of abortive/productive viral

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**Figure 7.** Immunocytochemistry of virus-treated pouch tumors. Immunocytochemistry analysis was performed with tumor sections using antifiber antibody (a) or caspase-3 antibody (b). (1) Representative vehicle-treated tumor section. (2) Section of tumor treated with lpl11w/Δ55K and stained with the isotype (mouse IgG) antibody control. (3) Section of tumor treated with lpl11w and stained with the antifiber or caspase-3 antibody. (4) Section of tumor treated with lpl11w/Δ55K and stained with the antifiber or caspase-3 antibody. The size bars indicate 200 μm (A1 and 2) and 50 μm (A3 and 4 and B1–4).
replication within the tumors. These tumors also contained hallmarks of apoptosis such as chromatin condensation (Figure 6d) and activation of caspase-3 (Figure 7b). Thus, the apoptotic activity of the two vectors may restrict the level of viral replication in the tumors as well as contribute to reduced tumor growth. Considering the lack of detectable levels of infectious virus within the tumors, it is possible that the apoptotic activity of the two vectors might be the driver behind their strong oncolytic activity. It should be noted that other investigators who studied other oncolytic HAdVs vectors that do not exhibit enhanced apoptosis also failed to detect significant viral replication in virus-treated hamsters at late times after infection.36,37 The cytolytic activity of these vectors may also facilitate the release of complete or incomplete virus particles from infected tumor cells contributing to the antiviral immune response (Table 1) and tumor growth inhibition.

One of the characteristic features of HNSCC is overexpression of EGFR18,39 which form the basis for the treatment of these cancers with EGFR antagonists. We have previously shown that in HNSCC cells infected with lp11w, there was a dramatic downregulation of EGFR as a result of caspase-mediated proteolytic processing of EGFR as well as through viral E3-RID protein-mediated receptor cleavage.30,41 In the hamster tumor cell lines infected with lp11w and lp11w/ΔSSK, we have observed efficient proteolytic processing of EGFR (Figure 3). As both viruses possess intact early genes, the cell lines that we have developed should be useful in studying various chemotherapeutic agents in combination therapy with the apoptotic vectors studied here. As the hamster pouches are immune privileged sites, it may also be possible to directly investigate the tumor growth inhibitory activities of the viral vectors against HNSCC under different immunomodulatory conditions.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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**Table 1.** Anti-HAdVs antibody in sera from tumor-bearing hamsters treated with adenoviral vectors

| Serum (HPT11) | Serum (HPT12) |
|--------------|--------------|
| **Subcutaneous tumors** | | |
| Vehicle | — | Vehicle | 40 |
| lp11w | 720 | lp11w | 180 |
| lp11w/ΔSSK | 960 | lp11w/ΔSSK | 240 |
| **Pouch tumors** | | |
| Vehicle | — | Vehicle | — |
| lp11w | 480 | lp11w | 1440 |
| lp11w/ΔSSK | 640 | lp11w/ΔSSK | 640 |

Abbreviations: HAdV, human adenovirus; HPT, hamster pouch tumor. Neutralizing antibody titers are expressed as the highest reciprocal serum dilutions. Average antibody titers from two animals from each group are presented. Hyperimmunized serum raised against HAdV5 wild-type virus from hamster was used for positive control.
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