Abstract

Purpose: The purpose of this study was to evaluate a fiber knob replacement strategy to improve infectivity and efficacy of Ad5 fiber chimeric oncolytic viruses for treatment of melanoma and head and neck cancers (HNC).

Experimental Design: Adenoviral receptors and transduction levels were used to determine the level of infectivity of fiber-modified, green fluorescent protein–expressing, replication-deficient viruses in a panel of melanoma and HNC cell lines in vitro. Virus yield and cytotoxicity assays were used to determine the tumor specificity and virus replication-mediated cytotoxicity of the fiber-modified oncolytic viruses in the same panel of melanoma and HNC in vitro. Xenograft tumor models were used to assess the antitumor activity of those fiber-modified chimeric viruses compared with the parental virus.

Results: Marker gene expression following gene transfer of the fiber chimeric vectors in melanoma and HNC cell lines was ~10-fold higher than that obtained with parental Ad5 vector. The fiber chimeric oncolytic variants mediated killing of melanoma and HNC cells that was 2- to 576-fold better than with the parental virus. In addition, fiber chimeric variants produced 2- to 7-fold more progeny virus in tumor cells than the parental virus. Moreover, a high multiplicity of infection was needed for the fiber chimeric viruses to produce cytotoxicity in normal cells. A significantly stronger antitumor response and survival advantage were shown in the tested melanoma and HNC xenograft models following i.t. injections.

Conclusions: In vitro and in vivo studies showed the improved transduction, replication, cytotoxicity, antitumor efficacy, and survival advantage in melanoma and HNC tumor models, suggesting a potential use of these oncolytic agents for the treatment of melanoma and HNCs.

According to the American Cancer Society, in 2005, melanoma and cancers of head and neck (HNC) are expected to represent ~4% and 3%, respectively, of newly diagnosed malignancies in the United States (1). Despite advances in conventional therapies, these two cancer types continue to present a major therapeutic challenge. Surgery is a potentially curable therapy for early-stage HNC and melanoma in majority of patients (2, 3). However, the prognosis is less encouraging for patients with advanced-stage disease, who represent two thirds of all newly diagnosed HNC patients. Patients with recurrent HNC are treated with a combination of surgery, radiation, and/or chemotherapy. In addition to limited efficacy, these treatment modalities for HNC may result in speech abnormalities, swallowing dysfunction, and deformities of the head and neck. Metastasis of melanoma occurs at an early stage of tumor development and is aggressive and refractory to chemotherapy (2). Thus, there is an urgent need for development of effective therapies for melanoma and HNC.

Cancer therapy using adenoviral vectors represents a promising alternative to current conventional treatments. Replication-defective viruses, such as Ad-p53, which express the tumor suppressor protein p53 under the control of cytomegalovirus (CMV) promoter, have been used in local therapy (4, 5), but these replication-defective viruses do not spread to uninfected neighboring cells, which may limit their antitumor activity. To overcome this limitation, replication-competent viruses have been designed, which selectively replicate in tumor tissues, with initially low number of virus copies increasing over time. Such in situ amplification may allow for relatively low, nontoxic doses to be highly effective in elimination of cancer cells and solid tumors. The adenoviral vector Ad dl1520 (ONYX-015), in which the E1b 55-kDa gene is deleted, represents one such class and has been evaluated alone and in combination with cisplatin and 5-fluorouracil in phase II clinical trials for treatment of HNC (6). Limited efficacy was obtained when Ad dl1520 was used alone.

The limited efficacy of Ad dl1520 was attributed mainly to the reduced replication capabilities of the virus as a result of the E1b 55-kDa gene deletion, as well as low and variable
expression levels of the Ad5 receptor coxsackie-adenovirus receptor (CAR) on cancer cells. Furthermore, CAR expression is known to be lost upon progression of certain tumors to malignancy (7). To overcome limitations imposed by low or no expression of CAR on tumor cells, adenoviruses can be engineered to infect cells using alternate receptors that are more abundantly expressed. Because expression of CD46 is relatively high on tumor cells, a retargeted vector that uses CD46 as a cellular receptor may increase the level of tumor cell transduction. To improve gene transfer and mediate efficient oncolysis of these cells, fiber chimeric vectors, in which the fiber knob was replaced with the knob domain of adenovirus serotype 3 or 35, were generated. The infectivity of Ad5/3 fiber chimera used in this study was further enhanced by insertion of the CD8αaCD8b targeting ligand (RGD motif) at the COOH terminus of the protein. Marker gene expression mediated by the fiber chimeric vectors in melanoma and HNC cell lines was severalfold higher than that obtained with parental Ad5 vector. The differences in specific viral receptor levels of the cell lines correlated with the levels of transduction mediated by these vectors. Based on the data, fiber chimeric structures were built into OAVs, in which expression of the E1a gene was placed under the control of the tumor-selective promoter E2F-1.

In the current study, the infectivity, cytotoxicity, replication levels, and antitumor efficacy of the fiber chimeric oncolytic viruses have been evaluated in several melanoma and HNC models.

Materials and Methods

Construction of E1-deficient, green fluorescent protein–expressing fiber chimeric Ad5 vectors. To generate Ad5 vector–based fiber chimeras, a shuttle plasmid, pAd5LtRt-Smal, was constructed as described below. The left end (1-1,009 bp) of Ad5 DNA was amplified by PCR using primers 1 (5′-GAACTTCTAGGATACAGGCTAACATCATCATAATATACTT-3′) and 2 (5′-CCCCGGGGGTGGTCCACATACATAATT-3′) and the right terminal 580-bp sequences of Ad5 DNA was amplified using a second set of primers designated primer 3 (5′-AACGTTTCTAGGATACAGGCTAACATCATAATATATACTT-3′) and primer 4 (5′-CCCCGGGGGTGGTCCACATACATAATT-3′). The right terminal 580-bp sequences of Ad5 DNA was amplified using a second set of primers designated primer 3 (5′-AACGTTTCTAGGATACAGGCTAACATCATAATATATACTT-3′) and primer 4 (5′-CCCCGGGGGTGGTCCACATACATAATT-3′). The recognition sequence of I-SceI incorporated into primers 1 and 3 are shown in italics. The first PCR product was digested with EcoRI and Smal, and the second PCR product was digested with HindIII and Smal. The resulting fragments were gel purified and cloned into EcoRI and HindIII sites of pBlueScript (Stratagene, La Jolla, CA) by three-way ligation to generate pAd5-LtRtSmal. The plasmid pFLAd5.CMV5-GFP, which contains the Ad5GFP vector genome bordered by I-SceI sites, was generated by combining the Smal-linearized pAd5LtRt-Smal and the genomic DNA of Ad5.CMV5-GFP (QiBiogene, Carlsbad, CA) in Escherichia coli.

The fiber chimeric vectors were generated in several steps. The plasmid pFLAd5.CMV5-GFP was digested with Smal, and the fragment containing the left and the right terminal fragments (1-3047 and 32652-33231 nucleotides, respectively) of Ad5.CMV5-GFP was gel purified and self-ligated to generate pAd5LtRtSmaI-CMV5-GFP. The plasmids pFLAd5.CMV5-GFP-ST3H and pFLAd5.CMV5-GFP-ST3H-RGD, which contain the full-length Ad5 DNA with the green fluorescent protein (GFP) coding region replacing the E1 coding region and containing chimeric fiber coding regions, were generated by combining Smal-linearized pAd5LtRtSmaI-CMV5-GFP and the genomic DNA of Av9ΔβgΔST3H and Av9 lacZ-RGD, respectively. The gene encoding shaft portion of the fiber protein in Av9 lacZ-RGD was derived from Ad5, whereas the knob-coding region was obtained from Ad3. The chimeric fiber protein of Av9 lacZ (8) was further modified by incorporation of the CGGCGGGGGGGCGCCDRCRGDCF ligand at the COOH terminus of the protein to generate Av9 lacZ-RGD. Previously, the COOH terminus of Ad3 fiber knob was shown to be optimal for insertion of targeting ligands (9, 10). To generate fiber chimeric vectors, the full-length plasmids were digested with I-SceI and transfected into PER.C6 cells (11).

Construction of fiber chimeric Ad5 oncolytic viruses. In the first step in generating these viruses, a full-length plasmid, pFLAd5, was constructed by combining the Smal-linearized pAd5LtRtSmaI and genomic DNA of Ad5 in E. coli. Next, pFLAd5 digested with XhoI and the DNA fragment containing the left and right terminal fragments of Ad5 was gel purified and self-ligated to generate pAd5-LtRtXhoI. Using PCR, the entire fiber-coding region from pAd5-LtRtXhoI was deleted, and a recognition sequence for SwaI was inserted to generate pAd5-LtRtXhoI-Smal. Combining XhoI-linearized pAd5LtRtXhoI-Smal and the genomic DNA of modified Ar20pAE2hGM (12) generated the plasmid pFLAr20pAE2hGMdelfiber containing the full-length Ar20pAE2hGM DNA minus the fiber-encoding region. The modified Ar20pAE2hGM was generated from Ar20pAE2hGM by deleting an extra ATG upstream of the E1a start codon and hereafter known as OV1193. A recombinant plasmid, pFBSE5T3H (13), containing the gene encoding Ad5 fiber shaft and Ad35 fiber knob was digested with XhoI and EcoRV, and the fragment containing the gene for chimeric fiber region was gel purified. The plasmid pFLAr20pAE2hGMdelfiber containing the gel-purified fragments in E. coli. A fiber chimeric oncolytic adenovirus, OV1194, was generated by digesting pFLAr20pAE2hGM-ST3H with I-SceI and transfected into PER.C6 cells.

To generate OV1195, the oncolytic virus containing Ad5 shaft and Ad3 knob and RGD targeting motif, a 3.16-kb EagI and KpnI restriction enzyme fragment containing the gene-encoding chimeric fiber protein was obtained from genomic DNA of Av9 lacZ-RGD cloned into pBlueScript. The gel-purified fragment was combined with SwaI-linearized pFLAr20pAE2hGMdelfiber in E. coli to generate pFLAr20pAE2hGMdelfiber containing the RGD targeting motif. The resulting plasmid was digested with I-SceI and transfected into PER.C6 cells to generate OV1195.

Other adenoviruses used in the study include wild-type Ad5, Add3/132, and Add1/1520. Add3/132 is a replication-defective vector with a deletion in the E1a gene used as a negative control vector. Add1/1520 and Ad-p53 are used as in-class standards because these vectors are currently being tested in clinical trials.

Tumor cell lines and normal cells. All tumor cell lines used in the study were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Human head neck cancer cell lines used in the study include A253 [American Type Culture Collection (ATCC), Manassas, VA, HTB-41], A431 (ATCC), FaDu (ATCC, HTB-43), SCC-9 (ATCC, CRL-1629), Detroit 562 (ATCC, CCL-138), Cal 27 (ATCC, CRL-2095), RPMI 2650 (ATCC, CCL-30), and HLAC-79. Human skin malignant melanoma cell lines used in the study include A375-luc (ATCC, CRL-1619 modified to express luciferase), A2058 (ATCC, CRL-11147), C32 (ATCC, CRL-1585), SK-Mel-28 (ATCC, HTB-72), WM-266-4 (ATCC, CRL-1676), and G-361 (ATCC, CRL-1424). The primary human diploid fibroblast line WI-38 (ATCC, CCL-75) and lung fibroblast line MRC-5 (ATCC, CCL-171) were used as normal cells, and these were grown in EEM4 supplemented with 10% fetal bovine serum.

Receptor density and transduction efficiency determination. Single-cell suspensions of tumor cells were incubated with mouse antibody directed against CAR (Rmcb, Upstate Biotechnology, Charlottesville, VA; 1:500 final dilution), CD46 (clone E4.3, BD Biosciences Pharmingen, San Diego, CA; 1:200 final dilution), α3β1 (Chemicon International, Temecula, CA; 1:500 final dilution), or α4β1 (Chemicon International; 1:500 dilution) for 30 minutes at 4°C. Subsequently, cells were washed twice with PBS containing 4% fetal bovine serum and incubated with secondary FITC-conjugated anti-mouse IgG (BD Biosciences Pharmingen; 1:200 final dilution) for 30 minutes at 4°C. After two washings with PBS containing 4% fetal bovine serum, the cells were suspended in PBS.
and analyzed by flow cytometry. Transduction efficiency mediated by Ad5GFP and fiber chimeric vectors was determined by infecting a selected panel of melanoma and HNC cell lines at 50 viral particles per cell. Infected cells were incubated at 37°C for 24 hours, and transduction efficiencies were determined by flow cytometry.

Cytotoxicity assays. Assays were done on a panel of tumor and normal cell lines using the Promega CellTiter aqueous nonradioactive cell proliferation kit (Promega, Inc., Madison, WI). The data were analyzed using GraphPad Prism 4 (San Diego, CA) analysis software.

Virus production assays. Cells were seeded in a six-well dishes 1 day before adenoviral infection. On the next day, cells were infected with oncolytic viruses at 50 viral particles per cell. After 3 hours of incubation, infection medium was removed, and cells were rinsed twice with serum-free medium and replaced with 3 mL of growth medium and incubated at 37°C for 72 hours. The cell lysate was serially diluted on 293 cells and incubated for 5 days and scored for CPE by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent. The level of virus replication in excised tumors following i.t. administration of oncolytic viruses was determined at 3 and 7 days following final virus injection by the same method. Tumor extracts were prepared and used as described previously (14) to determine the levels of virus replication in the xenografts.

In vivo efficacy studies. Female NCR (nu/nu) mice (4-6 weeks of age; body weight of 18-20 g) were purchased from Simonsen Laboratories (Gilroy, CA). Mice were injected s.c. in the right flank with 5e6 RPMI 2650 cells in Matrigel or 2e6 A375-luc cells (injection volume of 100 to 150 Lmm3 [volume = W x (L/2)2 / 2, where W = width, L = length, in mm3], animals (n = 16) were randomly distributed into several treatment groups and received a total of four i.t. injections, given once every other day, of 1 x 1010 virus particles or PBS in a 50-L mm3 dose volume. The size of tumors was measured twice weekly in two dimensions. When tumor volumes reached 2,000 mm3, or when the tumors became necrotic, animals were euthanized. Tolerability of the vector treatment was monitored by weekly body weight measurements. Three animals from each group were selected at random on study days 3 and 7 following the final treatment, bled, and euthanized, and tumors were collected. Tumors were cut into halves, with one half used to detect virus replication, and the other half used to determine viral spread within xenografts by immunohistochromy. For histologic examination, 5-mm paraffin sections of the tumor tissue were stained for hexon protein using the DAKO ARK kit (Carpinteria, CA) following the protocol given by the manufacturer. The remaining half of the tumor was used to isolate the virus, and virus titer was determined by infecting AE1-2a cells.

Results

Receptor density and transduction efficiency determination. Ad5 entry into cells depends on the interaction of the viral fiber knob with CAR and the interaction of penton base protein with αvβ3 and αvβ5 integrins. Melanoma and HNC cells are known to be relatively more resistant to Ad5 infection (15–17). It has also been shown that Ad5 tropism can be expanded by replacing the Ad5 fiber knob with a fiber knob derived from other adenoviral serotypes (8, 18, 19). In this study, the infection efficiencies mediated by Ad5GFP, Ad5GFP-5T3H, and Ad5GFP-5T3H-RGD were analyzed. These vectors have the fiber knobs from Ad5, Ad3, and Ad3 respectively, with a RGD motif insertion in the 3 knob. A GFP reporter gene incorporated into these adenoviral vectors allowed for direct measurement of infection efficiency by flow cytometry. Figure 1B and C shows the percentage of GFP-transduced cells. Among the HNC cell lines tested, Ad5GFP-mediated gene transfer was variable and ranged from 2% to 48% (Fig. 1B). The HNC cell lines FaDu, Detroit 562 (2%), A253, and A431 (4%) cells were extremely resistant, whereas SCC-9 (10%) and Cal27 (12%) cells were moderately resistant, and RPMI 2650 (32%) and HLaC 79 (48%) were more susceptible to Ad5GFP infection. In contrast, the two fiber chimeric vectors mediated higher (21-59%) transduction than did Ad5GFP. Infection mediated by fiber chimeric vectors in three HNC squamous cell carcinoma cells line (FaDu, SCC-9, and HLaC 79) was 1.5- to 2-fold higher than that in two epidermoid cell lines (A-253 and A431) and a pharyngeal carcinoma cell line (Detroit 562). Melanoma cell lines were 5- to 50-fold less susceptible to Ad5GFP infection compared with control 293 and AE1-2a cells (Fig. 1C). Of the six tested melanoma cell lines, WM-266-4 (2%), C32 (2.5%), and G-361 (4%) were extremely resistant; SK-MEL-28 (10%) was moderately resistant, whereas A2058 (17%) and A375-luc (18%) cells were more susceptible to Ad5 infection. The two fiber chimeric vectors mediated significantly higher (27-92%) rates of infection in the melanoma cell lines (Fig. 1C). In two normal human fibroblast cells, MRC-5 and WI-38, Ad5GFP mediated very low (5-7%) infection. In contrast, the two fiber chimeric vectors mediated higher levels (39-80%) of infection in these two cell types.

To elucidate the biological basis of infection or resistance to infection, cellular levels of adenoviral receptors in melanoma and HNC cells were determined by the degree of binding of monoclonal antibodies that bind to CAR, CD46, and αvβ3 and αvβ5. Figure 1B depicts the percentage of HNC cell types that are receptor positive. CAR was expressed at varying levels in all eight human HNC cell lines. Virtually no CAR expression was seen in FaDu and Detroit 562 cells. Overall, the percent CAR-positive cells ranged from 2% to 92% among the HNC cell lines. In contrast, all eight tested HNC cell lines expressed very high levels of CD46, ranging from 64% to 95%, but extremely low levels of αvβ3 and αvβ5 integrins.

In the melanoma cell lines, expression levels of CAR were low and variable, ranging from 0.3% to 39%, with the highest level found in A2058 cells (Fig. 1C). The expression levels of CD46 in these cell lines were variable and ranged from 4% to 69%. With the exception of A375-luc (28%), all tested melanoma cells expressed very low levels of αvβ3 integrins (1-3%). In contrast, the expression levels of αvβ3 in a majority of melanoma cell lines were relatively high (32-62%). As expected, nearly all 293 and AE1-2a cells expressed high levels of CAR and CD46 (Fig. 1C). Extremely low level expression of CAR and αvβ3 integrins but high levels of CD46 and αvβ5 integrins were seen in MRC-5 and WI-38 cells.

Construction of oncolytic adenoviruses. The oncolytic adenoviruses used in this study are shown in Fig. 1A. These viruses differ from the wild-type Ad5 genome in three different regions. First, the tumor-specific promoter (EZF-1) replaced the native E1a promoter, thus selectively regulating E1a expression and limiting viral replication to tumor cells with Rb pathway defects. Second, the SV40 polyadenylation signal site was inserted downstream of the packaging signal. Third, the cDNA encoding human granulocyte-macrophage colony stimulating (hGM-CSF) factor replaced the open reading frame—encoding 19-kDa glycoprotein (gp19k) of the E3 region under the control of the endogenous viral E3 promoter. However, the presence of the hGM-CSF gene would not alter the relative potency of the vectors in the studies reported here, which were done either in vitro or in immune-deficient mice that lack the
capability to respond to hGM-CSF stimulation of the immune system. The presence of the hGM-CSF gene is not further discussed. Finally, the fiber knob region of Ad5 was replaced with the corresponding region of Ad35 (OV1194) or Ad3 (OV1195) in fiber chimeric viruses (Fig. 1A), and the OV1195 was further modified with the RGD targeting motif.

Cytotoxicity assays. The cytolytic potential of the oncolytic adenoviral variants was assessed in eight HNC and five melanoma cell lines by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays. Two other agents, Ad5DL1520 and Ad-p53, were used for comparison. In addition to tumor cell lines, MRC-5 and WI-38 cells were included as primary cells to assess the selectivity of the viruses. The cytotoxicity data are summarized in Tables 1 and 2 Replication-defective, Ad5DL1520-negative control virus was not significantly cytotoxic in any tested tumor cell lines, and the EC50 value could not be determined at the vector particle doses used. The Ad5 wild-type virus was the most cytotoxic in the majority of the tested HNC cell lines, with EC50 values ranging from 1 to 38 viral particles per cell. The Ad5 OAV and both fiber chimeric OAVs were more cytotoxic than Ad-p53, with lower EC50 values [i.e., more cytotoxic recorded for the Ad5 OV1193 (1- to 12-fold), the Ad5-35 chimera OV1194 (8- to 237-fold), and the Ad5-3-RGD chimera OV1195 (16- to 576-fold); Table 1]. OV1193 (1- to 8-fold) and both fiber chimeric viruses OV1194 (5- to 130-fold) and OV1195 (18- to 270-fold) were also more cytotoxic than AdDL1520. Most interestingly, the fiber chimeric viruses OV1194 was 3- to 35-fold more cytotoxic, and OV1195 was 4- to 47-fold more cytotoxic than OV1193 (Ad5 fiber knob) in these HNC cells (Table 1).

Melanoma cell lines were relatively more resistant to Ad5 wild-type mediated cytotoxicity, with higher EC50 values ranging from 6 to 1,141 viral particles per cell (Table 2). Compared with parental virus OV1193, the fiber chimeric viruses OV1194 and...
OV1195 were 2- to 113-fold and 8- to 188-fold more cytotoxic, respectively, in melanoma cell lines. In two of five tested melanoma cell lines, the OV1194 and OV1195 were more cytotoxic than Ad5 wild type. In these cells, Ad-p53 was the least potent with very high EC90 values, and the Ad5-3-RGD chimera OV1195 was the most potent. In three of five tested melanoma cell lines, Ad/l520 mediated better killing than the Ad5 OAV1193. In contrast, the cytotoxicity of the fiber chimeric viruses was severalfold greater than that of Ad/l520. In MRC-5 and WI-38, Ad5-wild type was the most cytotoxic (Table 2). The cytotoxicity of OV1194 and OV1195 was 4- to 9-fold and 4- to 20 fold more than that of OV1193 in these cell types respectively, but both fiber chimerics were less than Ad5 wild type.

**Virus production assays.** Virus production is another important variable for measuring the potency of oncolytic adenovirus. Selected panels of HNC cell lines were infected with Ad5 wild type, Ad/l520, OV1193, OV1194, or OV1195 at 50 viral particles per cell. Cell lysates were titrated on 293 cells by limiting dilution to determine the titers in TCID50/mL. 293 cells have higher levels of CAR expression (~90%) compared with the CD46 levels (~69%). Thus, the titers expressed in 293 cellshave higher levelsof CARexpression due to the unequal expression of the receptors. The results of these assays are shown in Fig. 2A and B. In general, the cell lines varied widely in their ability to support infection and produce progeny virus, with SCC-9 being the most permissive among the tested cell lines, and FaDu being the least permissive among the tested cell lines. Of the tested viruses, Ad5 wild type produced the highest amount of infectious virus particles, and Ad/l520 produced the lowest amount of infectious virus particles (Fig. 2A). The two fiber chimeric oncolytic viruses, OV1194 and OV1195, produced 2- to 7-fold more virus than Ad5 OV1193 and 2- to 29-fold more virus than Ad/l520. Tumor cells were also infected with the replication-defective virus Ad/l312 as a negative control, and no significant virus replication was observed (data not shown). In majority of the melanoma cell lines, Ad wild type virus generated the most infectious viral particles followed by OV1194 and OV1195 (Fig. 2B). The infectious titers of Ad/l520 and OV1193 were similar, and OV1194 replicated slightly better than OV1195 in melanoma cell lines. The titers of chimeric vectors were 4.4- to 8.4-fold less than Ad5wt titers in MRC-5 cells, and it was about 3- to 10-fold less than the Ad5wt titers in WI-38 cells.

**In vivo efficacy studies.** S.c. xenograft models of RPMI 2650 and A375-luc in nude mice were used to evaluate the antitumor efficacy of the oncolytic viruses. A sham-treated group (PBS) was included to account for any effects caused by direct injection into tumors. Mice in vector treatment groups (Ad/l312, OV1193, OV1194, and OV1195) received 1 × 1010 virus particles per injection on study days 1, 3, 5, and 7, for a total of four injections. None of the virus-injected animals exhibited any clinical symptoms of toxicity or loss of body weight (data not shown). Three weeks after treatment, the relative tumor volumes in the RPMI 2650 xenograft study remained close to the starting volume for the group receiving OV1194 (Fig. 3A). A slight increase in tumor volume was noted in mice injected with OV1193 and OV1195. In contrast, during the same period, the tumor volumes of mice injected with PBS or Ad/l312 increased to 257% and 179% of starting volumes, respectively. From study day 25 onwards, the mean tumor volumes of mice treated with OV1193 and OV1195 increased continually, and by study day 60, the tumor volumes increased to 953% and 605% of starting tumor volumes respectively. On the other hand, the tumor volumes of mice treated with OV1194 increased only to 145% in comparison.

All the mice in the PBS and Ad/l312 control groups died or were euthanized with large tumors by study day 74, with a median survival time (MST) of 51 days. By study day 74, all but one mouse had to be euthanized from the group treated with OV1193 (Ad5 fiber knob), and MST was 63 days. In contrast, 6 of 10 mice survived for the duration of the study in the OV1194 group, and MST was not reached during the study period. In mice treated with OV1195, 4 of 10 mice survived, with a MST of 70 days (Fig. 3B).

In the melanoma A375-luc xenograft model, significant inhibition of tumor growth was observed following i.t. injection of all the oncolytic viruses (Fig. 4A). By study day 25, tumors in the PBS- and Ad/l312-treated groups had increased by 9- to 10-fold in volume from study day 1. During the same period, tumor volumes of mice treated with OV1193, OV1194, or OV1195 increased by 5.5-, 3-, and 1.9-fold, respectively. By study day 70, complete tumor regression was noted in 3 of 10 mice treated with OV1193 and OV1194 and in 4 of 10 mice treated with OV1195. MST was 53 and 72 days, respectively, in the OV1193 and OV1194 treatment groups, and 90 days in the OV1195 treatment group. MST for the PBS- and Ad/l312-treated mice was 39 and 46 days, respectively (Fig. 4B).

To verify the therapeutic efficacy was due to viral replication, tumors were harvested at days 3 and 7 following treatment. Virus production assays were performed to confirm replication in the tumors. Virus production of all the oncolytic viruses (Fig. 4B).

**Table 1. Cytotoxicity of oncolytic viruses and control viruses in HNC cell lines**

<table>
<thead>
<tr>
<th></th>
<th>A253</th>
<th>SCC-9</th>
<th>FaDu</th>
<th>A431</th>
<th>Detroit 562</th>
<th>CAL 27</th>
<th>HLAC 79</th>
<th>RPMI 2650</th>
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<tr>
<td>Ad5</td>
<td>8 ± 2</td>
<td>4 ± 1</td>
<td>38 ± 3</td>
<td>1 ± 0.3</td>
<td>10 ± 2</td>
<td>4 ± 1</td>
<td>0.23 ± 0.05</td>
<td>2 ± 1</td>
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<td>Ad/l520</td>
<td>690 ± 16</td>
<td>53 ± 10</td>
<td>633 ± 14</td>
<td>781 ± 18</td>
<td>1893 ± 22</td>
<td>155 ± 12</td>
<td>33.6 ± 7</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>Ad-p53</td>
<td>1,027 ± 15</td>
<td>379 ± 12</td>
<td>284 ± 8</td>
<td>1,636 ± 18</td>
<td>1,249 ± 14</td>
<td>1,153 ± 16</td>
<td>20.3 ± 2</td>
<td>98 ± 8</td>
</tr>
<tr>
<td>OV1193</td>
<td>276 ± 11</td>
<td>86 ± 6</td>
<td>171 ± 7</td>
<td>209 ± 12</td>
<td>240 ± 8</td>
<td>94 ± 6</td>
<td>5.3 ± 1</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>OV1194</td>
<td>22 ± 3</td>
<td>12 ± 3</td>
<td>8 ± 2</td>
<td>6 ± 1</td>
<td>19 ± 3</td>
<td>14 ± 3</td>
<td>5.1 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
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<td>3 ± 1</td>
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<td>6 ± 2</td>
<td>7 ± 2</td>
<td>2 ± 1</td>
<td>4.3 ± 1</td>
<td>4 ± 1</td>
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</table>

NOTE: Cyclin D is overexpressed in A253, SCC 9, FaDu, A431, Detroit 562, and CAL 27 cell lines, which leads to Rb pathway defects (30). Rb pathway status is unknown for HLAC 79 and RPMI 2650 cell lines. 3-((4,5-Dimethylthiazol-2-yl)-5-((carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays were done as described in Materials and Methods. Average EC50 value ± SD of the three replicates.
virus injection, and the amount of virus and hexon-positive cells were determined. On study day 3, RPMI 2650 and A375-luc tumors that had been treated with oncolytic viruses had ~1,000-fold more virus than tumors treated with replication-defective Ad\textsubscript{dl}1520. RPMI 2650 xenografts treated with OV1193 and OV1195 had 2- to 3-fold more virus than those treated with OV1193 (Fig. 5A). The RPMI 2650 tumors treated with any of the oncolytic viruses had 1- to 2-fold more virus on study day 7 than on study day 3. A375-luc xenografts treated with OV1194 and OV1195 had 1.2- to 4.3-fold more virus than those treated with OV1193 on study day 3 and 6.5- to 12.5-fold more virus on study day 10 (Fig. 5B). In contrast to the observations in the RPMI 2650 tumor model, A375-luc tumors that were treated with the oncolytic viruses had 1.1- to 6-fold more virus on study day 3 than on study day 7 (Fig. 5B). Tumors that were treated with oncolytic viruses also displayed hexon-positive staining cells (Fig. 5C and D). None of the mice bearing RPMI 2650 or A375-luc

Table 2. Cytotoxicity of oncolytic viruses and control viruses in melanoma cell lines and normal cells

<table>
<thead>
<tr>
<th></th>
<th>A375-luc</th>
<th>C32</th>
<th>G-361</th>
<th>A2058</th>
<th>WM-266-4</th>
<th>MRC-5</th>
<th>WI-38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad5</td>
<td>32 ± 3</td>
<td>1,141 ± 21</td>
<td>27 ± 3</td>
<td>6 ± 2</td>
<td>1,038 ± 15</td>
<td>16 ± 2</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>Ad5/1520</td>
<td>526 ± 12</td>
<td>1,400 ± 20</td>
<td>90 ± 7</td>
<td>56 ± 4</td>
<td>1,710 ± 17</td>
<td>NA</td>
<td>218 ± 5</td>
</tr>
<tr>
<td>Ad-p53</td>
<td>1,193 ± 16</td>
<td>7,500 ± 27</td>
<td>1,268 ± 24</td>
<td>80 ± 6</td>
<td>1,962 ± 22</td>
<td>NA</td>
<td>143 ± 3</td>
</tr>
<tr>
<td>OV1193</td>
<td>343 ± 12</td>
<td>1,887 ± 26</td>
<td>390 ± 12</td>
<td>35 ± 3</td>
<td>2,259 ± 21</td>
<td>544 ± 7</td>
<td>1,389 ± 12</td>
</tr>
<tr>
<td>OV1194</td>
<td>40 ± 4</td>
<td>64 ± 4</td>
<td>183 ± 6</td>
<td>21 ± 2</td>
<td>20 ± 2</td>
<td>59 ± 3</td>
<td>333 ± 6</td>
</tr>
<tr>
<td>OV1195</td>
<td>16 ± 3</td>
<td>27 ± 3</td>
<td>49 ± 5</td>
<td>14 ± 2</td>
<td>12 ± 2</td>
<td>26 ± 2</td>
<td>338 ± 13</td>
</tr>
</tbody>
</table>

Note: Cyclin D is overexpressed in C32 and WM-266-4 cell lines (31). p16 is negative in A375-luc and G361 cell lines (32), and Rb protein is absent in A2058 cell line (33), all of which leads to the defective Rb pathway. Rb pathway is not defective in MRC-5 and WI-38 cell lines. Average of the three replicates. Average EC\textsubscript{50} value ± SD of the replicates.

Fig. 2. A, vector production in HNC cell lines. The cells were infected at 50 virus particles per cell for 3 days, and the cell lysates were titered on 293 cells to determine TCID\textsubscript{50}/mL produced during the initial infection as described above. The data were plotted for each vector produced in each of the selected tumor cell line. B, vector production in melanoma cell lines. The cells were infected at 50 virus particles per cell for 3 days, and the cell lysates were titered on 293 cells to determine TCID\textsubscript{50}/mL produced during the initial infection as described in Materials and Methods. The data were plotted for each vector produced in each of the selected tumor cell line. Columns, average; bars, SD.
xenografts that were treated with oncolytic viruses had elevated liver enzymes in serum on study days 3 and 7 (data not shown), suggesting that there may have been no or minimal spread of oncolytic virus to the liver following i.t. injections.

**Discussion**

Ad5-based oncolytic viruses may not efficiently transduce some types of human tumor cells, and strategies to overcome this limitation are currently being evaluated. To this end, we have selected melanoma and head and neck tumor cells to explore the basis of their relative resistance to adenoviral infection and evaluated the fiber knob replacement strategy as a means to improve the infectivity of adenoviruses.

Using specific antibodies to adenoviral receptor proteins, CAR and integrins, a wide variation in receptor densities among melanoma and HNC cell lines was evident. Melanoma and HNC cell lines were infected with adenoviral vectors expressing GFP to determine the influence of receptor density on gene transfer. GFP expression was variable among tested cell lines and correlated with the level of primary adenovirus receptors, with low CAR density resulting in low levels of Ad5-mediated gene transfer. As a means to increase adenoviral infection of the refractory tumor cells, a fiber knob replacement strategy was evaluated. The fiber knobs derived from Ad3 and Ad35, which have been found to recognize abundantly expressed cellular receptors, were used to construct the fiber chimeric viruses. In addition, the Ad5/Ad3 fiber chimera was further modified by the insertion of the RGD targeting ligand at the COOH terminus of the fiber knob. Earlier studies have shown that the insertion of similar ligands had no affect on fiber trimerization and generally resulted in improved infectivity of Ad5/Ad3 fiber chimeric vectors (9, 10). The cellular receptor CD46 recognized by Ad35 (20) is up-regulated in many types of cancer (21). The receptor of Ad3 is currently not known, with one group suggesting that CD80 and CD86 function as coreceptors (22), and another group suggesting that CD46 is the Ad3 receptor (23). A majority of melanoma and HNC cell lines tested in the study expressed high levels of CD46. The high density of CD46 correlated

**Fig. 3.** A, antitumor efficacy in the RPMI 2650 human head and neck tumor xenograft tumor model. Nude mice bearing s.c. RPMI 2650 tumors were injected i.t. with PBS, Add/312, or oncolytic adeno viruses with four doses of $1 \times 10^{10}$ viral particles per mouse per injection, once every other day. Tumor sizes were measured externally with calipers. Points, mean volumes of 10 treated mice; bars, SE. All the OAV-treated groups were statistically different from the PBS-treated group: OV1193 versus PBS ($P < 0.05$), OV1194 versus PBS ($P < 0.0001$), OV1195 versus PBS ($P < 0.0001$). Both chimeric virus–treated groups were statistically different from OV1193–treated groups: OV1194 versus OV1193 ($P < 0.0001$), OV1195 versus OV1193 ($P < 0.005$). B, survival curves for RPMI 2650 tumor-bearing animals. Survival of tumor-bearing animals ($n = 10$) after treatment with PBS, Add/312, OV1193, OV1194, or OV1195 were monitored until study day 84 and plotted using GraphPad Prism. The chimeric virus–treated groups showed statistical survival advantage over the other groups: OV1193 versus PBS ($P = 0.06$), OV1194 versus PBS ($P = 0.0006$), OV1195 versus PBS ($P = 0.0013$), OV1194 versus OV1193 ($P = 0.01$), OV1195 versus OV1193 ($P = 0.05$).

**Fig. 4.** A, antitumor efficacy in A375–luc human melanoma xenograft tumor model. Nude mice bearing s.c. A375–luc tumors were injected every other day i.t. with PBS, Add/312, or oncolytic adenoviruses with four doses of $1 \times 10^{10}$ viral particles per mouse per injection. Tumor size was measured externally with calipers. Points, mean tumor volumes of 10 treated mice; bars, SE. All the OAV–treated groups were statistically different from the PBS–treated group: OV1193 versus PBS ($P < 0.05$), OV1194 versus PBS ($P < 0.0001$), OV1195 versus PBS ($P < 0.0001$). Both chimeric vector–treated groups were statistically different from OV1193–treated group: OV1194 and OV1195 versus OV1193 ($P < 0.05$). B, survival curves for A375–luc tumor-bearing animals. Survival of tumor-bearing animals ($n = 10$) after treatment with PBS, Add/312, OV1193, OV1194, or OV1195 were monitored until study day 98 and plotted using GraphPad Prism. The chimeric virus–treated groups showed statistical survival advantage over PBS–treated group: OV1193 versus PBS ($P = 0.12$), OV1194 versus PBS ($P = 0.05$), OV1195 versus PBS ($P = 0.04$).
with efficient Ad5-5T35H-GFP and Ad5-5T3H-RGD-GFP mediated gene transfer.

The level of the secondary adenoviral receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, was very low in HNC cells. Lower levels of transduction mediated by Ad5GFP correlated well with low expression levels of CAR and integrins. On the other hand, the higher transduction efficiency of fiber chimeric viruses correlated with levels of CD46 but not with expression levels of $\alpha_v\beta_3$ and $\alpha_v\beta_5$. 

Fig. 5. A, viral replication in RPMI2650 xenografts following i.t. administration of oncolytic viruses. At days 3 and 7 following final virus injection, extracts from 1 mg of tumor tissue were prepared and titrated on AE1-2a cells as described in Materials and Methods and used to determine the amount of virus. Columns, average TCID$_{50}$/mL ($n = 3$); bars, SD. B, viral replication in A375-luc xenografts following i.t. administration of oncolytic viruses. At days 3 and 7 following final vector injection, extracts from 1 mg of tumor tissue were prepared and titrated on AE1-2a cells as described in Materials and Methods and used to determine the amount of virus. Columns, average TCID$_{50}$/mL ($n = 3$); bars, SD. C, immunohistochemical detection of hexon protein in RPMI2650 xenografts injected with oncolytic viruses. Seven days following final virus injection, tumors were harvested ($n = 3$) and fixed in buffered formalin. Sections of paraffin-embedded tumor tissue were stained for hexon protein using the DAKO ARK kit. D, immunohistochemical detection of hexon protein in A375-luc melanoma xenograft injected with oncolytic viruses. Seven days following final virus injection, tumors were harvested ($n = 3$) and fixed in buffered formalin. Sections of paraffin-embedded tumor tissue were stained for hexon protein using the DAKO ARK kit. Magnification $\times 20$. 

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the integrins. These findings in HNC cell lines suggest that the primary receptor but not αvβ3 and αvβ5 integrins plays a role in mediating Ad5GFP-5T3H and Ad5GFP-5T3H-RGD infection. Alternatively, other RGD-binding integrins may act as coreceptors and facilitate internalization of adenoviruses in HNC cells. In fact, RGD-binding integrins αvβ3 and αvβ5 have been shown to be up-regulated and play a role in internalization of adenoviruses in HNC cell lines (15).

To evaluate the potential of fiber chimeric viruses for treatment of melanoma and HNCs, replication-selective vectors in which the native E1a promoter was replaced by the tumor-selective promoter E2F-1 were generated. In these viruses, tumor-selective replication was achieved by transcriptional targeting. Tumor selectivity of the E2F-1 promoter in the context of oncolytic adenoviral viruses has been shown previously (12, 24–26). The E2F-1 promoter selectivity is based on the derepression of the promoter in Rb pathway–defective tumor cells. In nondividing quiescent cells, E2F binds to the tumor suppressor protein pRB and thus represses the transcriptional activity from promoters with E2F-binding sites, including the E2F-1 promoter itself (27). By this type of transcriptional targeting, the lytic properties of the virus will progress selectively in Rb pathway–defective cancer cells, sparing normal, with potentially improved safety profiles (24). Rb pathway defects are found in a majority of tumor types, including HNC and melanoma (28, 29). Tumor selectivity of the E2F-1 promoter was further enhanced by an insertion of the SV40 polyadenylation signal sequence between the E2F-1 promoter was further enhanced by an insertion of the SV40 polyadenylation signal sequence between the E2F-1 promoter and the E2F-1 promoter activity (24). Although these oncolytic viruses also encoded the cDNA for hGM-CSF, the activity of this gene in eliciting antitumor response in the studies described here is irrelevant, as hGM-CSF is not biologically active in mice. In addition, the studies described here were carried out in immunodeficient animals.

The results of the cytotoxicity and virus yield assays presented here correlated well with the CAR and CD46 density on these cell lines. Cytotoxicity mediated by fiber chimeric viruses was greatly enhanced compared with OV1193 (Ad5 fiber knob). These results suggest that loss of potency resulting from replacement of E1A promoter with E2F-1 promoter in OV1193 may be compensated in fiber chimeric vectors by improved viral cell entry.

To be useful as therapeutic agents, oncolytic viruses must also have restricted growth in normal cells. In this study, the cytotoxicity and replication of fiber chimeric viruses were attenuated in MRC-5 and WI-38 cells compared with wild-type Ad5. Although the chimeras transduced normal cells better and were more cytotoxic in normal cells than an Ad5-based oncolytic virus, improved selectivity for tumor cells compared with Ad5-based oncolytic viruses was still seen; that is, the increase in cytotoxicity of the chimeric vectors in tumor cells was greater than that in normal primary cells. This improved tumor selectivity of the fiber chimeric viruses, despite their increased transduction of normal cells, is likely due to the selective transcriptional targeting conferred by the E2F-1 promoter.

Fiber chimeric viruses were more efficacious than OV1193 in reducing tumor volumes and prolonging survival of mice in tumor xenograft models. The increased antitumor efficacy may be attributed to the high expression levels of specific virus receptors on tumor cells, leading to efficient uptake and production of infective progeny virus, which is then available for spread into neighboring tumor cells.

Although extensive virus replication was noted in the tumor, there were no signs of vector-associated toxicities in the animals. It should be noted, however, that the safety of the chimeric viruses cannot be fully assessed in normal mice because the mouse homologue of CD46 is expressed only in the testes, limiting the potential infective spread of the virus to normal tissues. A complete assessment of the safety of the chimeric viruses will have to be conducted in CD46-expressing transgenic mouse strains or in other species that express CD46.

In conclusion, these studies have shown that the density of primary receptors on melanoma and head and neck tumor cells is a key determinant of adenoviral entry and infection. Using tumor-selective fiber chimeric oncolytic adenoviral variants, we have shown that enhanced entry results in improved cytotoxicity and viral yields and significantly improved antitumor efficacy and survival in xenograft tumor models. Thus, oncolytic adenoviral variants incorporating both transcriptional and transductional targeting may be attractive candidates for clinical application in treatment of melanoma and HNCs.

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References
Enhanced Gene Transfer and Oncolysis of Head and Neck Cancer and Melanoma Cells by Fiber Chimeric Oncolytic Adenoviruses

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