CHAPTER 6

Comparison of oncolytic adenoviruses for selective eradication of oral cancer and precancerous lesions

Hester J.T. van Zeeburg
Aafke Huizenga
Arjen Brink
Petra B. van den Doel
Zheng Zhu
Frank McCormick
Ruud H. Brakenhoff
Victor W. van Beusechem

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Abstract

Oncolytic adenoviruses are being investigated as potential anticancer agents. Selective lytic replication in cancer cells is essential for an effective and safe treatment. In this study, we compared 11 oncolytic adenoviruses on relevant cell cultures to assess their utility for treating oral cancer and precancerous lesions. We determined the cytotoxicity of oncolytic adenovirus infection; and calculated selectivity indices for cytotoxicity to cancer cells compared to normal oral keratinocytes and fibroblasts. Keratinocytes were very sensitive to wild type adenovirus serotype 5 (Ad5); 1-to-3-log more than head and neck squamous cell carcinoma (HNSCC) cells. The potencies of oncolytic adenoviruses to kill HNSCC cells within 7 days after infection ranged from approximately 10 times less potent to approximately 10 times more potent than Ad5. The selectivity indices determined on fibroblasts and keratinocytes differed markedly. Two oncolytic adenoviruses were more selective than Ad5 for HNSCC cells compared to fibroblasts and five viruses exhibited selective replication on HNSCC cells compared to keratinocytes. Overall, CRAd-S.RGD with E1A driven by the survivin promoter and an infectivity-enhancing capsid modification exhibited the most favourable cytotoxicity pattern; being very potent in killing HNSCC cells, only slightly less effective than Ad5 in killing preneoplastic keratinocytes and the least toxic to normal keratinocytes.
Introduction

Oncolytic viruses are being developed as new agents to treat cancer\[1,2\]. Oncolytic viruses are considered valuable additions to the arsenal of therapeutic agents to combat cancer. They destroy tumors by preferential lytic replication in cancer cells via mechanisms partly distinct from those of standard cancer therapies such as cytotoxic drugs and irradiation. Moreover, selective production of progeny virus in tumors increases the dose *in situ*, which might yield a higher therapeutic index. Currently, oncolytic viruses derived from adenovirus are the most extensively studied in clinical trials\[1,2\]. Many different oncolytic adenoviruses have been engineered, mainly following two different molecular approaches\[3,4\]. In the first approach, production of early adenovirus proteins essential for viral replication is driven by a tumor-selective promoter, inhibiting replication in non-malignant cells. In the second, adenovirus genes are deleted or mutated, rendering viral replication dependent on complementing alterations in cancer cells. Although each of these manipulations of the adenovirus genome provides a level of cancer cell specificity, none was reported abort replication in non-malignant cells completely. Therefore, oncolytic adenoviruses were made that incorporate multiple modifications, and in general such viruses showed a more strict tumor-selective replication\[5,6,7,8,9\].

Clinical experience with oncolytic adenoviruses has demonstrated their safety, with some evidence of efficacy\[1\]. This has fuelled studies aimed at empowering the cancer cell killing potency of oncolytic adenoviruses, without compromising their selectivity. To this end, adenoviruses were armed with therapeutic transgenes\[10\]. Among these were genes encoding proteins that promote cancer cell lysis and efficient spread of progeny virus, including a dominant-negative mutant of *Iκ-B*, tumor suppressor p53 and fusogenic membrane glycoproteins\[11,12,13,14\]. The impact of the aforementioned selectivity and efficacy enhancing adenovirus genome modifications depends on the biology of the host cancer cell. It can thus be anticipated that different types of cancer each demand a different oncolytic adenovirus offering the highest therapeutic index. In addition, safety of the virus on normal cells may depend on tissue architecture and administration route. Therefore, to identify the most useful oncolytic adenovirus for a particular cancer indication, direct comparisons in relevant model systems need to be made.

Head and neck squamous cell carcinoma (HNSCC) originates in the mucosal linings of the upper aero-digestive tract, and is diagnosed worldwide approximately 500,000 times each year. Low stage tumors are usually treated by surgery or radiation, while advanced stage tumors are treated by a combination of surgery and postoperative radiotherapy, chemo-radiation or bio-chemo-radiation. Despite advances in therapy, the long-term survival rate of HNSCC patients has increased only moderately over the past decades. An important clinical problem is the frequent development of local
relapse, clinically assigned as local recurrence or second primary tumor in the same or adjacent anatomical area, depending on the distance from the index tumor or the time interval. Many of these relapses are clonally related to the index tumor. Most HNSCCs, particularly those in the oral cavity and oropharynx emerge in preneoplastic fields consisting of genetically altered cells in which multiple mutations accumulate as a result of chronic exposure to carcinogens[15]. These preneoplastic fields can extend up to multiple cm in diameter and are in approximately 80% of cases clinically not macroscopically visible[15]. Because of their dimensions and their invisibility, preneoplastic fields are often left behind in part when the tumor is excised. This puts patients with HNSCC at a high risk of developing local relapse (10-25%) arising from the same preneoplastic field as the first primary tumor[16]. Thus, new treatments aimed at eradication of the precancerous fields are desperately needed to prevent local relapses in treated HNSCC patients. Eradicating preneoplastic fields is also of relevance to prevent malignant transformation of visible leukoplakias and erythroplakias. This is of interest for the sporadic patient population, but in particular for those who are genetically predisposed and at a very high risk for developing squamous cancers, such as patients with Fanconi anemia (FA). FA is an inherited disease characterized by bone marrow failure, congenital abnormalities and cancer predisposition, particularly acute myeloid leukaemia and HNSCC. Since the introduction of bone marrow transplantation for FA patients, allowing treatment of the most life threatening complications, increasing numbers of FA patients survive longer and develop HNSCC. The tumors in these patients are difficult to treat, because FA patients do not sustain radiotherapy and chemotherapy, leaving primary prevention, screening and surgery of the tumors as only options for clinical management. Precancerous fields are often seen in these patients and a secondary prevention strategy of these lesions is urgently awaited. Oncolytic adenoviruses appear very useful to treat HNSCC. Several clinical trials with the oncolytic adenovirus ONYX-015 were performed in HNSCC patients[17,18,19] and the very similar virus H101 is already registered as an HNSCC medicine in China[20]. In addition, oncolytic adenoviruses could potentially also be used to eradicate preneoplastic lesions, because preneoplastic cells carry some of the genetic aberrations found in cancer cells that are exploited to design oncolytic adenoviruses. Moreover, the confinement of HNSCC and its preneoplastic fields to the oral mucosa, allowing exposure to virus via topical administration, makes this a particularly attractive indication for treatment with oncolytic adenovirus. Indeed, the feasibility of administering ONYX-015 to preneoplastic oral lesions by rinsing the oral cavity with a virus suspension was already demonstrated in a clinical trial[21], but the reported efficacy leaves much to be desired.

In this study, we set out to evaluate a panel of oncolytic adenoviruses on cell cultures relevant for treating oral and oropharyngeal cancer and their precancerous fields. We included five human HNSCC cell lines, two of which were derived from FA patients;

104
Oncolytic adenoviruses for oral cancer treatment

A preneoplastic cell culture derived from the resection margin of an excised HNSCC specimen and the two main cellular constituents of the healthy oral mucosa, that is primary oral keratinocytes that form the squamous stratified epithelium and fibroblasts from the underlying connective tissue. The squamous stratified epithelium consists of different layers. Keratinocytes in the basal and suprabasal layers divide, move upwards to more superficial layers, differentiate and lose replication potential. Hence, topical application of adenovirus by mouthwash brings in particular differentiated superficial keratinocytes in contact with virus. To culture keratinocytes resembling this differentiated cell type, we induced primary oral keratinocytes to differentiate by addition of calcium to the culture medium. Keratinocytes cultured for 7 days in the presence of calcium show morphological changes (Supplementary Figure 1A) and show down-regulation of K984 antigen expression and up-regulation of K928 antigen expression (Supplementary Figure 1C, Supplementary Table 1) typical for differentiated keratinocytes.
First, we investigated inherent susceptibility of the different normal and malignant cell types to human adenovirus serotype 5 (Ad5) infection and lytic replication. Infection efficiency was studied using AdCMV-Luc\[^{[23]}\], a replication-defective Ad5 vector expressing firefly luciferase. Cells were subjected to AdCMV-Luc at various multiplicities of infection (MOI) and luciferase activity was measured the next day. All cell cultures showed a linear relation between MOI and luciferase activity in the range of 10-1,000 IU/cell. Figure 1A shows the comparison at 100 IU/cell. AdCMV-Luc did not show preference for infection of malignant or non-malignant cells derived from the oral epithelium. The five HNSCC cell lines showed highly variable susceptibility to infection, with UM-SCC-22A being 70-fold more resistant than VU1365. In addition, cells were analysed by FACS for expression of primary and secondary Ad5 receptors CAR and αvß3 and αvß5 integrins (Supplementary Table 1). This showed that CAR was virtually absent on fibroblasts and expressed at variable levels on the other cell types, while integrins were expressed on all cell types. Neither CAR expression, nor integrin expression levels correlated with infection efficiency (Spearman rank correlation test: CAR, r=0.21, p= 0.66; integrins, r=0.11, p=0.84).

Next, we compared the inherent susceptibility of the cells to Ad5-induced cell kill. To this end, we infected cells with an Ad5 dilution titration and measured cell viability 7 days later. Replication-defective AdCMV-Luc was taken along as control for replication-independent adenovirus particle toxicity. The inverse of the virus dose required to decrease cell viability by 50% (1/LD\(_{50}\)) was used as a measure for cytotoxic activity. Figure 1B shows that of all cell types tested, keratinocytes were the most sensitive to Ad5-induced cell kill and fibroblasts were the most resistant (i.e., more than 3-log difference). The five HNSCC cell lines differed considerably in their susceptibility to Ad5-induced cell kill, with most sensitive VU1365 cells being >250-fold more susceptible than most resistant UM-SSC-14C cells. Infection and cell killing efficiencies correlated weakly (Spearman rank correlation test: r=0.71, p=0.09), suggesting that Ad5 cytotoxicity on the tested cells could be partly dependent on infection efficiency, but that other intrinsic cellular properties seem to be important as well. Notably, none of the HNSCC cell lines were as sensitive to Ad5 as keratinocytes. This underscores the importance of introducing tumor-selective modifications in the adenovirus genome, in particular to reduce lytic replication in keratinocytes. In the context of treating oral cancer or preneoplastic lesions by mouthwash, protecting fibroblasts against the virus seems less important, as these cells are likely to be less accessible and appear relatively resistant to Ad5 cytotoxicity. Notably, we do not know whether this also holds true for fibroblasts in submucosal fibrosis that is frequently observed in subjects with the habit of chewing betel nuts. These fibroblasts do not behave normally and might
be more susceptible for the effects of adenovirus infection.

To identify a most useful oncolytic adenovirus for treating oral cancer or dysplasia, we measured the HNSCC cell killing potency of 11 different oncolytic adenoviruses comprising six different modifications providing tumor-selectivity and three modifications described to affect killing potency. The characteristics of the oncolytic adenoviruses used are shown in Table 1. Modifications conferring tumor-selective replication included deletion of the p300/CBP-binding motif in the N-terminal region of E1A[7,24],...
deletion of a pRB-binding motif in the E1A-CR2 region\textsuperscript{[25,26]}, deletion of the entire \textit{E1B55K} gene\textsuperscript{[27]} or abrogation of the p53 binding site in \textit{E1B55K} by single amino acid R240A substitution\textsuperscript{[28]}, and regulation of E1A expression by two different tumor-selective promoters, that is the survivin promoter\textsuperscript{[29]} and the CXCR4 promoter\textsuperscript{[30]}. Modifications that affect oncolytic potency include presence or absence of the adenovirus E3 region\textsuperscript{[31,32]}, expression of tumor suppressor p53\textsuperscript{[13,14]} and infectivity-enhancement by inserting a cyclic RGD motif in the fibre capsid protein\textsuperscript{[33]}. Three independent experiments to determine the LD\textsubscript{50} were performed in triplicate for each virus on each cell type. Figure 2a shows, by way of example, results of Ad5 and three oncolytic viruses in one of the experiments on VU1131 cells. Because different cell lines showed highly variable inherent sensitivity to adenovirus-induced cell death (see Figure 1b), we normalized data by the LD\textsubscript{50} of Ad5. This normalization also corrects for inter-experimental variation. Supplementary Figure 2 shows the normalized oncolytic potencies of all test viruses on the five HNSCC cell lines; Figure 2B shows the mean results of each virus on the complete set of HNSCC cell lines. The AdΔ24-type viruses were quite consistent in their cytotoxicity against HNSCC cells. In contrast, viruses with multiple E1 modifications or with tumor-selective promoters were much more variable in their potency against individual HNSCC cell lines. Mean oncolytic potencies differed from approximately 10-times less potent than Ad5 to approximately 10-times more potent. Of note, these differences that became evident after only 7 days of culture are expected to amplify upon extended virus propagation. Consistent with findings by others\textsuperscript{[8,26,34,35]}, ONYX-015 was attenuated compared to Ad5, whereas AdΔ24 was similarly effective as Ad5. In our experiments, the latter was not influenced by the presence or absence of the E3 region, whereas insertion of an RGD motif or a p53 expression cassette yielded a small potency enhancement. Previous studies reported more profound effects in other cancer cell types\textsuperscript{[14,31,33,36]}. Not unexpectedly, introduction of second or third modifications in the E1 region attenuated the killing potency of the virus. In contrast, CRAd-S.RGD and CRAd-CXCR4.RGD were on average more potent against HNSCC cells than Ad5, but with considerable differences between cell lines. These viruses showed a particularly strong cytotoxicity on VU1131 cells. The presence of an RGD motif or E3 region seems essential for this effect, as AdSurE1 which lacks E3 and RGD but is otherwise identical to CRAd-S.RGD, was rather ineffective against VU1131 cells. This was surprising, because in the context of AdΔ24-type viruses the effect of RGD and E3 was minimal (compare Ad5-Δ24.RGD to AdΔ24). Figure 2c shows the selectivity indices of the viruses, calculated from their mean relative cytotoxicities on HNSCC cells versus normal cells. Keratinocyte proliferation was reduced, but not entirely blocked, by addition of calcium; fibroblast proliferation was not inhibited. Most oncolytic adenoviruses did not show appreciable selectivity for HNSCC cells compared to fibroblasts. Their potency attenuation or enhancement compared to Ad5 was similar on HNSCC cells and fibroblasts, yielding selectivity indices around 1. The exceptions were AdΔ24-p53 and CRAd-S.RGD, which were approxi-
Oncolytic adenoviruses for oral cancer treatment

Figure 2. Relative cytotoxicity of oncolytic adenoviruses on cultured primary oral keratinocytes and fibroblasts and on HNSCC cell lines. Cytotoxic activity of Ad5 and 11 different oncolytic adenoviruses was determined as described in the legend to Figure 1b. (A) Log MOI - cell survival dose-response curves for Ad5 and three oncolytic adenoviruses from a typical experiment on VU1131 cells. The box below the graph shows the calculated LD_{50} values. (B) Average cytotoxicity of oncolytic adenoviruses on five HNSCC cell lines relative to Ad5, calculated from the median normalized LD_{50} values (i.e., LD_{50} Ad5 / LD_{50} oncolytic adenovirus) on each cell line determined in three independent experiments. (C) Average normalized cytotoxicity indices of oncolytic adenoviruses on five HNSCC cell lines, relative to keratinocytes (black bars) or fibroblasts (striped bars). Data shown are the values from (B) divided by the median normalized LD_{50} values determined on keratinocytes or fibroblasts, respectively.


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mately 10-times more selective than Ad5 on HNSCC cells versus fibroblasts. In contrast, large differences over 4 orders of magnitude were observed in the selectivity of oncolytic viruses for HNSCC cells compared to keratinocytes. AdΔ24-type viruses, except Ad5-Δ24.RGD, were at least as toxic to keratinocytes as to HNSCC cells. Surprisingly, several of these viruses were even more toxic to keratinocytes than to HNSCC cells. AdΔΝΔ24.55K(R240A) that incorporates three E1 modifications intended to provide oncolytic selectivity yielded a selectivity index similar to that of AdΔ24 and 50-fold lower than that of Ad5. Adding more E1 modifications to the Δ24-deletion did thus not improve oncolytic adenovirus selectivity. In contrast, Ad5-Δ24.RGD, ONYX-015 and the three promoter-driven viruses showed selectivity indices ranging from 4 for Ad5-Δ24.RGD to 171 for CRAd-S.RGD. Hence, RGD-modification, E1B55K gene deletion and tumor-selective E1A expression contributed to selective cytotoxicity on HNSCC cells compared to keratinocytes.

Finally, we tested ONYX-015, AdΔ24-p53 and the two Survivin promoter-driven viruses in comparison to wild type Ad5 on M3 cells derived from the resection margin of an excised head and neck tumor. M3 cells exhibit continuous growth in calcium-
free keratinocyte growth medium and show loss of heterozygosity at chromosomal locations 3p and 9p, consistent with a preneoplastic state (Chapter 4). Figure 3 shows that Ad5 killed M3 cells growing in calcium-free medium with a 1/LD$_{50}$ of 0.34 and M3 cells with reduced growth in calcium-containing medium with a 1/LD$_{50}$ of 0.08. The sensitivity of M3 to Ad5 was within the range observed for HNSCC cell lines; and well below the sensitivity of normal keratinocytes (see Figure 1A). Of the four oncolytic adenoviruses tested, only AdΔ24-p53 was at least as potent as Ad5 in killing growing M3 cells. The other viruses were 3- to 7-fold attenuated. Arresting M3 growth reduced the killing potency of most viruses including Ad5. This is consistent with the general requirement of S-phase progression for effective adenovirus replication. AdΔ24-p53 displayed the strongest attenuation by cell cycle suppression. The exception was ONYX-015, which killed M3 cells with similar efficiency in the presence or absence of calcium. This was remarkable, because it has been reported that replication of E1B55K mutant adenovirus is restricted by the cell cycle[37]. Our observation suggests that at least this restriction does not substantially affect induction of cell death in M3 cells.

In summary, we have tested a large panel of oncolytic adenoviruses for efficacy against HNSCC cells and preneoplastic cells versus toxicity on differentiated keratinocytes and fibroblasts. To enable extrapolation of our findings to clinical data we included ONYX-015 that has been tested for HNSCC tumor treatment and for eradication of preneoplastic fields by oral rinse procedure. Normal keratinocytes appeared much more sensitive to adenovirus infection than HNSCC cells, preneoplastic oral keratinocytes and primary fibroblasts. This extends a previous observation made using in vitro engineered oral epithelia. In that study, epithelia prepared using normal keratinocytes were more sensitive to exposure to Ad5 than epithelia prepared using p53 mutant, p16 deficient cells derived from a severe dysplasia[38]. Surprisingly, most AdΔ24-type viruses were more toxic to keratinocytes than Ad5, whereas they were similarly or less toxic to fibroblasts. Our observation that selectivity indices determined on fibro-
Oncolytic adenoviruses for oral cancer treatment

blasts and keratinocytes differed markedly underscores the importance of evaluating the safety of oncolytic viruses on normal cells relevant for the intended clinical application. For topical application to the oral mucosa we consider testing the safety on keratinocytes most important. In this context, five viruses showed a better efficacy/safety profile than wild type Ad5. Of these, CRAd-S.RGD appears a particularly promising lead for development of an oncolytic adenovirus oral rinse treatment, being eight-fold more potent against HNSCC cells, only three-fold less potent against preneoplastic keratinocytes cultured from the oral mucosa and approximately 2-log more selective towards oral keratinocytes than Ad5. However, as a consequence of the very high intrinsic susceptibility of differentiated oral keratinocytes to adenovirus replication, even CRAd-S.RGD with 2-log improved selectivity would be expected to cause considerable toxicity to the normal mucosa. Therefore, it seems remarkable that topical application of ONYX-015 to the oral mucosa has shown a beneficial effect on dysplasias without apparent toxicity[21]. Perhaps the explanation could be sought in the specific tissue architecture of the normal oral mucosa. A previous study reported that CAR expression is absent on superficial cells of the oral mucosa[39]. We found reduced CAR expression and undetectable integrin expression on superficial mucosa cells (Supplementary Figure 3). Decreased receptor expression could thus render the differentiating oral mucosa relatively resistant to topical adenovirus infection; perhaps even to infectivity-enhanced viruses with the RGD-modification. The in vitro experimental model applied in our study does not address this tissue architecture. Our findings suggest that an undamaged mucosal layer, preventing uptake in normal keratinocytes, might be critical to prevent toxicity problems when applying current generation oncolytic adenoviruses using an oral rinse.
Supplementary Figure 3. Immunohistochemical staining of adenovirus receptor expression on human oral mucosa. Sections (5 µm) were made from fresh frozen tissue of an uvulopalatopharyngoplasty, air-dried and fixed using acetone. Sections were blocked using 2% (v/v) normal rabbit serum (DAKO) and incubated overnight at 4°C with primary antibodies against αvβ3 (biotin-labelled; 10 µg/ml; Ebioscience, Hatfield, UK), αvβ5 (P1F6; 10 µg/ml; Abcam, Cambridge, UK), Ly6-D (E48 hybridoma supernatant) and CAR (RmcB hybridoma supernatant). Next, sections were washed and incubated with 1:500 diluted RαM F(ab')2 Biotin (DAKO) followed by incubation with streptavidin-biotin complex labelled with horseradish peroxidase (sABC-HRP; DAKO). Antibody binding was visualized using DAB with H2O2. Sections were counterstained with haematoxylin and cover slipped with Kaiser’s glycerin. Monoclonal antibody E48 served as a positive control, since its target antigen Ly6-D is known to be expressed in all cell layers of the oral mucosa at very high level. CAR expression was clear on the surface of cells in the lower cell layers of the mucosa and decreased upon keratinocyte differentiation. Expression of αvβ3 and αvβ5 integrins was almost absent and confined to the basal layers of the oral mucosa and decreased upon keratinocyte differentiation. Expression of αvβ3 and αvβ5 integrins was almost absent and confined to the basal layers of the oral mucosa. Note that positive integrin staining was observed on endothelial cells of blood vessels in the underlying connective tissue indicating that the antibody and staining worked well.

Supplementary Figure 1. Effect of calcium addition on differentiation of oral keratinocytes in vitro. (A.) Normal oral keratinocytes were cultured in KGM as described in the legend to Figure 1, with or without addition of calcium at 1.8 mM. After 7 days, phase contrast light microscopy pictures were taken at 10-times original magnification. Under the influence of calcium, cells undergo a clear morphology change. The expression of keratinocyte antigens K928 and K984 on cultured keratinocytes is given in supplementary Table 1. (B.) Uniform expression of K928 antigen and differential expression of K984 antigen on keratinocytes in basal and superficial layers of the normal oral mucosa. 5 µm frozen tissue sections of a uvulopalatopharyngoplasty specimen were air-dried and fixed using 2% paraformaldehyde. Sections were blocked using normal rabbit serum (1:50; DAKO, Everhee, Belgium), incubated with supernatant of hybridoma cell lines producing K928 or K984 antibodies, washed, incubated with 1:100 RαM-HRP (DAKO) and developed with diaminobenzidine (DAB) and H2O2. Sections were counterstained with haematoxylin and cover slipped with Kaiser’s glycerin. K928 is expressed in all cell layers of the squamous stratified epithelium, including the most differentiated keratinocytes. In contrast, K984 expression is confined to the lower 2-3 cell layers of the oral mucosa, containing the more primitive keratinocytes.
Oncolytic adenoviruses for oral cancer treatment

FACS analysis of HNSCC cell lines, fibroblasts and keratinocytes (cultured with or without 1.8mM calcium for 7 days) for CAR, αvβ3 integrin, αvβ5 integrin, K928 and K984. Cells were harvested using trypsin and washed once with PBS, 0.5% BSA and 0.02% azide (PBA). Cells were incubated with primary or directly labelled antibodies for 1 hour on ice. Antibodies used were 10 µg/ml biotin-labelled anti-αvβ3 (Ebioscience); 25µg/ml anti-αvβ5 (P1F6; Abcam); undiluted anti-CAR (RmcB) hybridoma supernatant; undiluted P5D4 hybridoma supernatant (negative control); 1:100 diluted K928-dyelight-488; 1:100 diluted K984-dyelight-633; and 1:100 diluted human IgG labelled with dyelight-488 or 633 (negative controls). Direct fluorescent labelling was performed using the Dyelight microscale labelling kit (Thermo Scientific Pierce, Etten-Leur, The Netherlands) according to the manufacturer’s protocol. Cells incubated with unlabeled antibodies were washed twice using PBA and incubated for 1 hour on ice with secondary antibody RαM-PE (1:100; DAKO) or with Streptavidin-APC (1:300; Ebioscience). Cells were washed three times and fixed using 1% paraformaldehyde. Cells were analysed using a Becton-Dickinson FACS ARIA. Values given are relative mean fluorescence intensities compared to the corresponding negative control staining.

Supplementary Table 1.

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Supplementary Figure 2. Normalized cytotoxicity of oncolytic adenoviruses on five HNSCC cell lines. Cytotoxic activity of Ad5 and 11 different oncolytic adenoviruses was determined as described in the legend to Figure 1b. Data shown are the median ratios LD_{50} Ad5/LD_{50} oncolytic adenovirus, calculated from three independent experiments.
References
