Preclinical Therapy

Targeted Chemotherapy for Head and Neck Cancer with a Chimeric Oncolytic Adenovirus Coding for Bifunctional Suicide Protein FCU1

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Abstract

Purpose: Transfer of prodrug activation systems into tumors by using replication-deficient viruses has been suggested to be an effective method for achieving high local and low systemic anticancer drug concentrations. However, most current suicide gene therapy strategies are still hindered by poor efficiency of in vitro gene transfer, inefficient tumor penetration, limited bystander cell killing effect, and need of large prodrug doses. We hypothesized that local amplification provided by a replication competent platform would help overcome these limitations.

Experimental Design: We generated a transcriptionally and transcriptionally targeted oncolytic adenovirus Ad5/3-Δ24FCU1 expressing the fusion suicide gene FCU1. FCU1 encodes a bifunctional fusion protein that efficiently catalyzes the direct conversion of 5-FU, a relatively nontoxic antifungal agent, into the toxic metabolites 5-fluorouracil and 5-fluorouridine monophosphate, bypassing the natural resistance of certain human tumor cells to 5-fluorouracil.

Results: We examined the efficacy of Ad5/3-Δ24FCU1 and the replication-defective control Ad5/3-FCU1 with and without 5-FU. FCU1 expression was confirmed by Western blot, whereas enzymatic conversion levels in vitro and in vivo were determined by high-performance liquid chromatography separation. Significant antitumor effect was observed in vitro and in vivo in a murine model of head and neck squamous cell carcinoma. Although we observed a decrease in viral DNA copy number in vitro and in tumors treated with Ad5/3-Δ24FCU1 and 5-FC, suggesting an effect on virus replication, the highest antitumor effect was observed for this combination.

Conclusions: It seems feasible and efficacious to combine adenovirus replication to the FCU1 prodrug activation system. Clin Cancer Res; 16(9); OF1–10. ©2010 AACR.

Each year, >615,000 new head and neck cancer cases are diagnosed worldwide (1). Approximately 90% of them are squamous-cell carcinomas (HNSCC). HNSCC is an umbrella term that includes cancers at several sites (e.g., oral cavity, pharynx, and larynx) having different etiologies and prognoses while sharing common risk factors and mechanisms of action for 5-FU have been reported in the literature (2). Currently, the standard of care for HNSCC includes surgery, radiotherapy, chemotherapy, monoclonal antibodies, and combinations of these. Despite steady progress in treatment outcomes, the overall 5-year survival rate is below 50%, unchanged for nearly three decades. Surgery can be mutilating and often has significant effects on swallowing and speech. The addition of chemotherapy to radiotherapy has been useful in the context of organ preservation, but has resulted in limited improvement in survival rates (3). Cisplatin and 5-fluorouracil (5-FU) remain the standard chemotherapy agents for HNSCC. Mortality associated with disease and morbidity associated with its treatments have encouraged the pursuit of alternative therapeutic strategies.

5-FU is a pyrimidine analogue that requires cellular uptake and metabolic activation to exert cytotoxicity. As a uracil analogue, it serves as a substrate for the same transport processes and enzymes involved in anabolism and catabolism. As such, 5-FU may be used by several metabolic routes in which it will be converted to its active metabolites (4). Various mechanisms of action for 5-FU have been reported...
including the inhibition of thymidylate synthase by 5-fluoro-2′-deoxyuridine-5′-monophosphate, incorporation of 5-fluorouridine-5′-triphosphate into RNA, and incorporation of 5-fluoro-2′-deoxyuridine-5′-triphosphate into DNA. The reported mechanisms lead to the inhibition of DNA and RNA synthesis and interference with DNA repair (4, 5).

The suicide gene FCU1 encodes a bifunctional chimeric protein that combines the enzymatic activities of Saccharomyces cerevisiae cytosine deaminase (FCY1 or CDase) and uracil phosphoribosyltransferase genes (FUR1 or UPRTase) and efficiently catalyses the direct conversion of 5-fluorocytosine, a relatively nontoxic antifungal drug, and 5-fluorouridine monophosphate (5-FUMP; ref. 6). This bypasses the natural resistance of certain tumor cells to the chemotherapeutic drug 5-FU (6). Furthermore, 5-FU is capable of passive diffusion into and out of cells, resulting in significant bystander effect (7). Importantly, and in contrast to some other prodrug conversion strategies (8), the bystander effect remains and may even increase when the transgene-expressing cell dies.

Adenoviral gene therapy has been proposed as a novel treatment alternative for advanced cancer (9). Although there is clinical data that has validated the inherent safety and therapeutic potential of the approach, the main conclusion from most cancer trials is that tumor transduction and tumor penetration has been too low for significant therapeutic antitumor effect (10–12). Therefore, oncolytic adenoviruses have been explored for enhanced tumor transduction and amplification of effect (13). These viruses have a cytopathic nature, i.e., the replicative life cycle of the virus results in host cell death. Although oncolytic adenoviruses have been safe and there is some evidence of efficacy, single-agent efficacy has been mostly nonimpressive (14). In contrast, combination with chemotherapy seems quite attractive. Significantly, the only randomized study completed thus far featured treatment of HNSCC with oncolytic adenovirus in combination with 5-FU and cisplatin. Although response rates were improved radically, overall survival was not increased compared with chemotherapy alone (15). Therefore, further advances in the technology are needed.

Infection by most adenovirus serotypes is mediated by the knob region of the fiber binding to the coxsackie adenovirus receptor (CAR). There is a growing body of evidence that a decrease in CAR expression is frequently associated with tumor aggressiveness and that many advanced tumors feature variable and often low expression of CAR (16). Thus, it would be advantageous to transductionally target adenovirus to non-CAR receptors for increased tumor transduction and/or reduced infection of nontarget tissues (17, 18). Various adenoviral capsid modifications have been developed to increase gene delivery to cancer cells (19–22). In this study, we used a 5/3 chimera in which the normal knob fiber of the serotype 5 was replaced by the knob of serotype 3 (19). With regard to HNSCC, we have shown previously that adenoviruses featuring 5/3 serotype chimerism displays significantly enhanced gene delivery and cell-killing effect compared with viruses with wild-type capsids (23).

The oncolytic adenoviruses used in this study feature loss-of-function mutations in the virus genome, which are transcomplemented by features of cancer but not normal cells. They have a 24-bp deletion (Δ24) in the constant region 2 domain of the adenoviral E1A gene (24, 25). Therefore, the E1A protein is unable to bind the retinoblastoma (Rb) tumor suppressor protein for release of E2F and subsequent effective viral replication in noncycling normal cells is impaired. Thus, the virus replicates selectively in cells deficient in the Rb/p16 pathway, including most, if not all, cancer cells (26). Tumor specificity of such adenoviral mutants has been previously shown (13, 17, 18, 24, 25).

In this study, we hypothesized that a transductionally and transcriptionally targeted oncolytic adenovirus, which features a capsid modification and is armed with a prodrug converting suicide transgene, would exhibit enhanced specificity and increased antitumor effect. We constructed the oncolytic adenovirus Ad5/3-Δ24FCU1 and the respective replicative-deficient control Ad5/3-Δ24FCU1 featuring a 5/3-serotype chimeric capsid. Ad5/3-Δ24FCU1 features a 24-bp deletion in E1A and FCU1 in E3. Ad5/3-FCU1 is E1 deleted and FCU1 expression is under the cytomegalovirus (CMV) promotor regulation. Ad5/3-Δ24FCU1 was evaluated in vitro and in vivo for antitumor efficacy in combination with 5-FC.

### Materials and Methods

**Cell culture.** HNSCC low-passage tumor cell cultures UT-SCC8 (supraglottic larynx) and UT-SCC29 (glottic...
larynx; ref. 27) were cultured in DMEM supplemented with 10% FCS (PromoCell GmbH), 1% nonessential amino acids (Life Technologies, Invitrogen) 2 mmol/L glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Sigma). The UT-SCC cells were used in low passage, typically passage 15 to 30.

Human transformed embryonic kidney cell line 293, human lung cancer cell line A549, and Panc-1 pancreatic cancer were obtained from the American Type Culture Collection (ATCC). All cell lines were maintained in the recommended conditions.

**Construction of adenoviruses.** To create the oncolytic Ad5/3-Δ24FCU1, we used a plasmid pTHSN-FCU1. Briefly, the plasmid was constructed by digesting pTHSN, a plasmid containing the E3 region of the adenoviral genome, with Muni/BsiWI, and inserting FCU1 into the resulting 965 bp 6.7K/gp19K deletion of E3A. The FCU1 gene was extracted from the plasmid pCl-neoFCU1 (28) using the restriction enzymes XhoI/SmaI. pAdEasy-1.5/3-Δ24FCU1 was generated by homologous recombination in *Escherichia coli* BJ5183 cells (Qbiogene, Inc.) between FspI-linearized pTHSN-FCU1 and SrfI-linearized pAdEasy-1.5/3-Δ24 (19), a rescue plasmid containing the serotype 3 knob and a 24-bp deletion in E1A. The genome of Ad5/3-Δ24FCU1 was released by *PacI* digestion and subsequent transfection to 911 cells. The virus was propagated on A549 cells and purified on cesium chloride gradients. The viral particle concentration was determined at 260 nm and standard TCID50 on 293 cells was done to determine the infectious particle titer.

To construct the E1-deleted control virus Ad5/3-FCU1, the FCU1 gene was extracted from the plasmid pCl-neoFCU1 (28) using the restriction enzymes XhoI/SmaI. pShuttle-CMV was digested with EcoRV and the FCU1 fragment was ligated into the multiple cloning site under the control of the CMV immediate early promoter to generate pShuttle-CMV-FCU1. Homologous recombination was done between pAdEasy-1.5/3 and Pmel-linearized pShuttle-CMV-FCU1 to construct pAdEasy-1.5/3-FCU1. The genome of Ad5/3-FCU1 was released by *PacI* and transfected into 293 cells. The virus was propagated on 293 cells and purified on cesium chloride gradients. The viral particle concentration was determined at 260 nm and standard plaque assay on 293 cells was done to determine the infectious particles.

Viruses were propagated as reported (19) and their main features of the other viruses, Ad5Luc1 (19), Ad5/3-Δ24 (18), and AdTG14800 (28), are described in Fig. 1. Viruses were propagated in low passage, typically passage 15 to 30.

**Western blot analysis.** UT-SCC8, UT-SCC29 or 293 tumors were infected with Ad5/3-Δ24FCU1 and Ad5/3-Δ24FCU1 at 10 virus particles (VP) per cell and were incubated for 24 h. Cell lystate proteins (30 mg) or bacterially expressed and purified FCU1 protein (150 mg ref. 29) were run on a 7.5% SDS-PAGE gel under reducing conditions and were transferred onto a nitrocellulose membrane. The membrane was incubated with rat anti-FCU1 serum (Transgene SA) at 1 mg/mL, washed, and incubated with secondary antibody coupled to horseradish peroxidase (Chemicon). Signal detection was done by enhanced chemiluminescence (GE Healthcare).

**Enzymatic assays.** In vitro CDase and UPRTase activities in PANC-1 cells were determined using 5-FC (Toronto Research Chemicals, Inc.) and 5-FU (Sigma) as substrates. PANC-1 cells (2 × 10^6 cells) were infected with each Ad vector at a VP per cell of 25. Twenty-four-hours later, enzymatic assays were determined using high-performance liquid chromatography (HPLC) separation as described in Erbs et al. (30).

The CDase activity in tumors and plasma of nude mice bearing s.c. HNSCC tumors infected with the indicated vectors at a dose of 3 × 10^8 VP/d (days 0, 2, and 4) was determined using HPLC separation. 5-FC was given i.p. at 250 mg/kg/d and PBS injections were used as negative controls. Plasma was separated by centrifugation from blood collected through the tail vein in heparinized tubes on day 4, 1 h post–5-FC i.p. injection. Animals were killed 1 h post–5-FC i.p. injection on day 57 and tumors were harvested. The tumors were homogenized, and CDase and UPRTase activities were determined using 5-FC and 5-FU as substrates, respectively.

**Fig. 1.** Construction of a double-targeted and FCU1-armed oncolytic adenovirus. Schematic illustration of Ad5/3-Δ24FCU1 and replication-deficient Ad5/3-FCU1 as well as the unarmed oncolytic adenovirus Ad5/3-Δ24 and the replication-deficient viruses Ad5/3Luc1 and AdTG14800. Ad5/3-Δ24FCU1 and Ad5/3-Δ24 contain a 24-bp deletion in the constant region 2 of the adenoviral E1A gene. This deletion reduces replication in quiescent normal cells but allows effective oncolysis of tumor cells with a defective Rb/p16 pathway. All viruses except AdTG14800 contain a chimeric fiber with the knob domain of Ad3 in the Ad5 capsid. FCU1 is a fusion enzyme of CDase and UPRTase activities and its expression is under the control of the native E3 promoter in Ad5/3-Δ24FCU1 and under the CMV promoter in Ad5/3-FCU1 and AdTG14800. Ad5/3Luc1 encodes for luciferase. Enzymatic activation of 5-FC by FCU1 allows bystander cell killing of neighboring cells, which potentiates the antitumor effect associated with virus replication.
collected. Tumors were homogenized using a Polytron homogenator. Tumor or plasma samples were quenched with 1 mL of ethyl acetate/2-propanol/0.5 mM acetic acid solution (84:15:1). The organic supernatant was constituted in 50 μL of water and analyzed by HPLC as described above using 50 mMol/L phosphoric acid adjusted to pH 2.1 as mobile phase.

**In vitro cell killing.** HNSCC low-passage tumor cell cultures were seeded at 1.5 × 10^4 cells per well on 96-well plates. On the next day, cells were infected with the indicated viruses at given multiplicities of infection. Viruses were diluted in growth media with 2% FCS and cells were infected for 1 h at 37°C. After infection, cells were washed and incubated in 5% FCS. At 48 h after infection, cells were exposed to various concentrations of 5-FC for 5 to 7 d, before cell viability was determined according to the manufacturer's protocol (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Promega).

**Antitumor activity of Ad5/3-Δ24FCU1 in subcutaneous tumor model.** All animal experiments were approved by the Experimental Animal Committee of the University of Helsinki and the Provincial Government of Southern Finland. Human HNSCC explant xenografts were established by injecting 3 × 10^6 UT-SCC8 cells mixed with Matrigel (BD Pharmingen) into the flanks of 5- to 6-wk-old female NMRI/nude mice (Taconic). These cells were selected because they grow reliably in mice (23). After 7 d, tumors (n = 8/group, 4-6 mm in diameter) were injected with a volume of 50 μL thrice every other day with the indicated vectors at dose of 3 × 10^8 VP (days 0, 2, and 4) and control tumors were injected with DMEM only. 5-FC was given i.p. at 250 mg/kg/d thrice (from day 1-11, from day 28-38, and from day 48-56) and PBS injections were used as negative controls. The formula (length × width^2 × 0.5) was used to calculate tumor volume.

**Quantitative real-time PCR.** Total DNA was extracted from tumors using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's protocol and DNA concentration was measured by spectrophotometry. PCR amplification was based on primers and probe targeting the E4 gene (31). Human β-actin primers and probe were used as internal control to normalize the number of viral DNA copies for the amount of genomic DNA (32).

The real-time PCR conditions for each 20-μL reaction were as follows: 2× LightCycler480 Probes Master Mix (Roche), 500 nmol/L each of forward and reverse primer, 150 nmol/L of each probe, and 5 μL extracted DNA. PCRs were carried out in a LightCycler (Roche) under the following cycling conditions: 10 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 60°C, and 10 min at 40°C. All samples were tested in duplicate of three samples. A regression standard curve was established using DNA extracted from serial dilutions of pAd5easy plasmid (from 10^6 to 10^1 copies). Known amounts of human genomic DNA (500, 50, 5, 0.5, and 0.05 ng) were used to generate a standard curve for the β-actin gene.

**Apoptosis immunostaining.** Cryosections of 4- to 5-μm thickness of frozen tumors embedded in Tissue Tek OCT (Sakura) were prepared and fixed in aceticone for 10 min at −20°C. As primary antibody, we used a rabbit monoclonal antibody against active caspase-3 at dilution 1:200 for 1 h at room temperature (BD Pharmingen, TM,}

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**Fig. 2. Evaluation of FCU1 protein expression and specific CDase and UPRTase activities.** A, UT-SCC8, UT-SCC 29, or 293 cell lysates were analyzed by Western blot for specific detection of the FCU1 protein (M, 42,000). Lane 1 (left to right), cells infected with Ad5/3-Δ24FCU1; lane 2, cells infected with Ad5/3-FCU1; lane 3, bacterially expressed and purified FCU1 protein. Cellular lysates or purified FCU1 protein were loaded at 30 mg or 150 ng per lane, respectively. Virus infections were done at 10 VP per cell. B, CDase and (C) UPRTase activities in PANC-1 cells were determined using 5-FC and 5-FU as substrates. Twenty-five VP per cell were used, and 24 h later, HPLC separation was done. Columns, mean of three independent experiments; bars, SD.
Further, sections were incubated according to manufacturer instructions with the LSAB2 System-horseradish peroxidase kit (K0673, DakoCytomation). Bound antibodies were visualized using 3,3′-diaminobenzidine (Sigma). Lastly, sections were counterstained with hematoxyline and dehydrated in ethanol, clarified in xylene, and sealed with Canada balsam. Representative pictures were captured at ×20 magnification using a Leica DM LB microscope equipped with an Olympus DP50 color camera.

The immunohistochemistry images of tumor cryosections stained for apoptosis were analyzed using Matlab from MathWorks. The color space of each image was simplified to the 10 most representative different colors by using K-means algorithm (33). The simplified colors were labeled as being red, blue, or background. The resulting areas were filtered based on shape features to increase accuracy (34).

Statistical analysis. Statistical analyses were done using a two-tailed Student’s t test and the nonparametric Mann-Whitney U test (SPSS 17.0, SPSS, Inc.). For all analyses, a P value of <0.05 was deemed statistically significant.

Results

FCU1-expressing oncolytic and replication-deficient adenoviruses retain their efficacy of infecting cells in vitro and express high levels of functional FCU1. To investigate whether a FCU1-expressing oncolytic adenovirus would retain its oncolytic activity and add to it the activity of the prodrug conversion activity of FCU1, we generated two different 5/3 chimera adenoviruses bearing the FCU1 gene. The constructed viruses are shown in Fig. 1. The coding sequence of FCU1 was introduced into the 6.7K/gp19K deletion of adenoviral E3A (replication-competent Ad5/3-Δ24FCU1) or into the deleted E1 under the CMV promoter (replication-deficient Ad5/3-FCU1). Virus structures were confirmed by PCR and sequencing (data not shown).

Cellular expression of the FCU1 fusion protein after adenoviral infection was confirmed by Western blot on UT-SCC8, UT-SCC29, and 293 using a rat antibody directed against FCU1 (Fig. 2A). Ad5/3-Δ24FCU1 and Ad5/3-FCU1 expressed the expected 42-kDa FCU1 protein (Fig. 2A) at 24 h after infection.

Confirmation of expression of functional FCU1 by Ad5/3-Δ24FCU1 and Ad5/3-FCU1 was examined by measuring the enzymatic activities of FCU1 as previously described (30). The CDase and UPRTase activities were determined by the analysis of the enzymatic conversions of 5-FC to 5-FU and 5-FU to 5-FUMP, respectively. This analysis indicates that increased CDase and UPRTase activities were found in cells infected with Ad5/3-Δ24FCU1, Ad5/3-FCU1, and AdTG14800, whereas no CDase and UPRTase activities were detectable in mock-infected cells or in Ad5/3-Δ24 or Ad5/3Luc1-infected cells (Fig. 2B and C). The difference observed in CDase and UPRTase activities between Ad5/3-FCU1 and AdTG14800 may be due to the differences
in functional versus physical titers (VP/PFU titers were 89 and 34, respectively). The highest CDase and UPRTase activities were observed in cells infected with Ad5/3-Δ24FCU1 (Fig. 2B and C). Taken together, our data indicate that infection with Ad5/3-Δ24FCU1 leads to high expression of functional FCU1 in cancer cells.

The oncolytic adenovirus expressing FCU1 showed increased cell killing activity in combination with 5-FC prodrug. We evaluated the combined oncolytic efficiency of Ad5/3-Δ24FCU1 with 5-FC on different HNSCC low-passage tumor cell cultures (Fig. 3) and 293 cells (data not shown). The oncolytic effect of Ad5/3-Δ24FCU1 in the absence of prodrug resulted in 32.5% and 98.1% maximum cell killing, on UT-SCC8 and UT-SCC29, respectively (Fig. 3A and B). On UT-SCC8, the maximum cell killing was further increased by 27% when 10 μmol/L 5-FC was added (Fig. 3A). As expected, Ad5/3-Δ24FCU1 and its counterpart with no transgene in E3 (Ad5/3-Δ24) showed no statistical difference in cytotoxicity in the absence of 5-FC. The addition of 5-FC did not statistically increase cytotoxicity in tumor cells infected with Ad5/3-Δ24, which does not express FCU1, except at higher doses, when some unspecific toxicity was seen (Supplementary Fig. S1B).

With nonreplicating Ad5/3-FCU1 or AdTG14800, no cytotoxicity was observed in the absence of 5-FC (data not shown). In the presence of 10 μmol/L 5-FC, the highest cell killing rates were 4.5% to 18.7% for Ad5/3-FCU1 and 9.6% to 7.8% for AdTG14800 on UT-SCC8 and UT-SCC29, respectively. In UT-SCC8, the addition of 5-FC to Ad5/3-Δ24FCU1-infected tumor cells statistically increased cytotoxicity in a prodrug dose-independent manner (Fig. 3C). In UT-SCC29, cell killing was increased with some 5-FC concentrations in a nondose-dependent manner (Supplementary Fig. S1A). Thus, enhanced cell killing was seen with the combination of Ad5/3-Δ24FCU1 + 5-FC and even small prodrug concentrations yielded benefit. The Ad5/3-Δ24FCU1 + 5-FC combination therapy was more effective than either treatment alone.

Decreased virus replication in HNSCC low-passage tumor cell cultures treated with 5-FC. Because it has been reported that 5-FC might hinder vaccinia virus replication by interfering with its DNA replication machinery (29), we sought to investigate whether that was true also in the context of oncolytic adenovirus. UT-SCC8 HNSCC low-passage tumor cell cultures treated with the combination of
Ad5/3-Δ24FCU1 and 0.1 μmol/L 5-FC revealed a 9.2-fold (P < 0.01) decrease in DNA copy number compared with Ad5/3-Δ24FCU1 only-treated tumors (Fig. 3D).

Coadministration of Ad5/3-Δ24FCU1 with 5-FC showed significantly enhanced antitumor activity in a subcutaneous tumor xenograft model. To investigate whether our observation in vitro were translatable into an in vivo model of HNSCC cancer, UT-SCC8 cells were inoculated in nude mice. Mice were randomized into six groups and treated intratumorally with 1 × 10⁶ VP or growth medium only on days 0, 2, and 4. 5-FC (250 mg/kg/d) was given i.p. (from day 1-11, from day 28-38, and from day 48-56). Ad5/3-Δ24FCU1 with 5-FC showed a significant antitumor effect compared with mock or 5-FC alone (P < 0.01 and P < 0.05, respectively; Fig. 4). Further, the antitumor efficacy of Ad5/3-Δ24FCU1 + 5-FC was statistically increased when compared to Ad5/3-Δ24FCU1 alone, Ad5/3-Δ24 alone, or to Ad5/3-Δ24 + 5-FC (P < 0.05, P < 0.01, and P < 0.001, respectively). No statistical difference was observed between Ad5/3-Δ24FCU1 alone and Ad5/3-Δ24 alone (P = 0.86), confirming the in vitro data that the FCU1 transgene expression does not affect the antitumor potency of the virus.

In vivo cytosine deaminase activity. On day 4, mice treated with Ad5/3-Δ24FCU1 and 5-FC in the xenograft study (Fig. 4A) showed a significantly higher 2.4-fold decrease in the plasma levels of 5-FU– compared with 5-FC only–treated mice and a 1.6-fold decrease compared with Ad5/3-FCU1– and 5-FC–treated mice (Fig. 4B). This suggested the FCU1-mediated metabolism of 5-FC. No levels of 5-FU were detected by HPLC separation in the plasma of any of the groups (Fig. 4C), but 5-FU could be detected in tumors treated with Ad5/3-Δ24FCU1 and 5-FC (Fig. 5A), confirming that the activation of 5-FC into their active components is localized to the tumor site.

On day 57, tumors from the xenograft study (Fig. 4A) were collected and 5-FU could be detected only in tumors treated with Ad5/3-Δ24FCU1 and 5-FC (Fig. 5C). Similar to the 5-FU levels in plasma, a statistically significant 2.6-fold decrease in tumor 5-FU levels was seen compared with 5-FC only–treated mice. In Ad5/3-FCU1– and 5-FC–treated mice, the difference was 1.9-fold (Fig. 5B).

Decreased virus replication in vivo in mice treated with 5-FC. Similarly to in vitro results, HNSCC tumors treated with the combination of Ad5/3-Δ24FCU1 and 5-FC revealed a 9-fold (P = not significant) decrease in DNA copy number compared with Ad5/3-Δ24FCU1 only–treated tumors (Fig. 6A).

The combination of Ad5/3-Δ24FCU1 and 5-FC enhances apoptosis in xenograft model of HNSCC. The combination of Ad5/3-Δ24FCU1 with 5-FC resulted in an increased activity of CDase (Fig. 5) as well as a statistically significant reduction of tumor size compared with the treatment of virus alone (Fig. 4). Because it has been shown that 5-FU and its derivatives promote apoptosis (4), we asked whether the observed tumor reduction was associated with increased apoptosis. As expected, 2.7- and 3-fold increased apoptosis was observed in tumors treated with the combination of Ad5/3-Δ24FCU1 and 5-FC compared with Ad5/3-Δ24FCU1 alone or 5-FC alone (Fig. 6).

Discussion

The chemotherapeutic 5-FU has been used for the treatment of several cancer types for over 50 years (4). Despite the efficacy of 5-FU and other therapies currently used for...
treatment of recurrent HNSCC, the median survival is still <1 year. The efficacy of 5-FU and other chemotherapeutic agents is restricted not only by the natural resistance of certain tumors but also by severe side effects associated with the treatments.

The CDase/5-FC system delivered by a replication-deficient adenovirus has been used to reduce the side effects of 5-FU by promoting a localized activation of 5-FC to 5-FU (12). The clinical trial revealed positive safety results. However, tumor transduction and penetration were too low for significant therapeutic antitumor effect (12). Oncolytic adenoviruses have been explored for enhanced tumor transduction and amplification of effect (19). In addition, we have previously shown synergy between oncolytic adenoviruses and 5-FU (23). Therefore, we armed an oncolytic adenovirus with FCU1 gene. In this approach, tumor cells are killed due to virus replication and by 5-FU and 5-FUMP, and additional benefit may result from the synergy of the approaches (23) and the direct bystander effect of passive diffusion of 5-FU that can kill untransduced neighboring tumor cells (7). In addition, side effects of the treatments are nonoverlapping, which might facilitate increased efficacy without increasing toxicity.

Erbs et al. (28) reported a replication-deficient adenovirus serotype 5 (Ad5) expressing FCU1. They show an inhibition of tumor growth in a subcutaneous model of human colorectal tumor in mice. We found that the efficacy of this system can be augmented by using a replication-competent adenovirus. One reason is the higher transgene level achieved because of the replication of the virus genome and transgene therein (Fig. 2A; ref. 35). With regard to in vivo efficacy, we saw a 5-fold reduction of tumor growth compared with the nontreated group.

In this respect, interesting results have also been published by Foloppe and colleagues (29), who showed a 10-fold reduction on tumor growth in a model of colorectal cancer by vaccinia virus expressing FCU1. In contrast, oncolytic adenoviruses have not been previously used.
for targeting FCU1/5-FC suicide system. Although other viruses have their merits, adenoviruses and oncolytic adenovirus in particular are appealing for HNSCC because of the promising clinical data already available from randomized and nonrandomized trials (10, 12, 14, 15). The growing body of clinical data available on adenovirus in HNSCC treatment facilitates clinical translation of the approach described here.

Based on our in vitro results, we selected an in vivo dose of 250 mg/kg/d of 5-FC, resulting in a total cumulative dose of 7000 mg of 5-FC per mouse, whereas in a previous study featuring replication-incompetent adenoviral delivery of FCU1-1, a concentration of 1000 mg/kg/d was used with a total cumulative dose of 20,000 mg/mouse (28). In addition, in another study featuring replication-incompetent adenoviral delivery of FCU1-1, only 1000 mg/kg/d was significant in tumor growth suppression, whereas 500 or 200 mg/kg/d were not able to suppress tumors (30). 5-FC is a relatively well-tolerated substance widely used for the treatment of fungal infections in humans (36). In mouse studies, the maximum tolerated dose has been reported as 1800 mg/kg/d (37). Thus, local amplification provided by a replication-competent platform seems to overcome the requirement for large prodrug doses.

We showed that oncolytic adenoviruses can effectively express the fusion enzyme FCU1 as a transgene in cancer cell lines and HNSCC explants. In addition, we found that it was possible to combine oncolytic adenovirus replication with 5-FC conversion by FCU1 and obtain increased cell killing (Fig. 3). This has been of concern previously because 5-FU and 5-FUMP block DNA and RNA replication and inhibit virus replication (38, 39). Viral oncolysis together with 5-FC enzymatic conversion resulted in better antitumor activity in vivo than either treatment alone. The increased efficacy suggests that the additional cell killing and bystander effect provided by 5-FU and 5-FUMP, together with the putative synergy with oncolytic adenovirus (23), are more important than the relative reduction in virus replication.

Importantly, no levels of 5-FU were detected in the plasma of mice treated with Ad5/3-Δ24FCU1 and 5-FC. In contrast, we found 5-FU in the tumors even 52 days after the last virus injection, suggesting selective and localized conversion of 5-FC in the tumor, with minimal systemic exposure. Because Ad5/3-Δ24FCU1 is a tumor-selective virus in mouse models with human xenograft tumors, replication and subsequent FCU1 expression is only expected in tumor cells (13, 17, 18, 24). Similar to what has been reported in vitro for vaccinia virus (29), we observed in vitro and in vivo a decrease in virus replication when 5-FC was added to Ad5/3-Δ24FCU1. This might explain the unexpected outcome of Ad5/3-Δ24FCU1 with 1 μmol/L 5-FC (Fig. 3C). Nevertheless, tumor growth inhibition in mice was not compromised. Instead, efficacy was better with the combination, which might increase the therapeutic window of the approach because enhanced efficacy together with less virus replication might mean less replication-associated side effects. Lastly, we observed an increase in apoptosis in tumors treated with Ad5/3-Δ24FCU1 + 5-FC comparing to Ad5/3-Δ24FCU1 or 5-FC alone, confirming the mechanism of action of 5-FU and 5-FUMP (40). Because the data on apoptosis and oncolytic adenovirus replication are somewhat discordant (41–44), perhaps in part due to the antiapoptotic activities of E1B and E4 (44, 45), we assume that the increase in apoptosis seen here may be chiefly due to the 5-FU-mediated bystander effect.

In summary, Ad5/3-Δ24FCU1 with 5-FC seemed to result in improvements in antitumor activity. There were no side effects seen in these experiments, but this requires further studies. The efficacy of the approach can eventually be further improved in a multimodal approach with HNSCC conventional therapies e.g., radiotherapy or cetuximab (23). Our data provide a rationale for the clinical translation of 5/3-Δ24FCU1 as an oncolytic viral vector for treating HNSCC. Future studies are needed to optimize these results, especially vector and produg administration modalities and timing. Because the p16-Rb pathway is defective in many, if not all, solid tumors (26), Ad5/3-Δ24FCU1 + 5-FC is an attractive system for improving the treatment of many other types of cancer refractory to available treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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