Targeting of a conditionally replicative adenovirus agent to human squamous cell carcinomas of the head and neck

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Abstract. Conventional cancer treatments are not adequate for the majority of most patients stricken with squamous cell carcinomas of the head and neck (SCCHN). Conditionally replicating adenoviruses (CRAds) represent a promising new modality for treating of neoplastic diseases, including SCCHN. Specifically, CRAd agents infect tumor cells and selectively replicate within them, thus causing their death while sparing surrounding normal cells in the host. Oncolysis results from the replicative life cycle of the virus, which lyses infected tumor cells and releases viral progeny for propagation of infection and resultant lysis of neighboring cancer cells, sparing normal host cells. However, to date there have been two main limitations to successful clinical application of these CRAd agents: poor infectivity and poor tumor specificity. Here we report the construction of a CRAd agent, CRAd-CXCR4.F5/3, in which the adenovirus E1 gene is driven by a tumor-specific CXCR4 promoter, and the viral infectivity is enhanced by a fiber modification, F5/3, containing an Ad3 knob chimeric fiber protein. As expected, this agent improved both of the viral infectivity and tumor specificity as evaluated in established SCCHN tumor cell lines and in primary tumor tissues from multiple patients. As an added benefit, the activity of the CXCR4 promoter was low in human liver as described previously. Based on these data, the CRAd-CXCR4.F5/3 is a promising novel CRAd agent for SCCHN targeting with low host toxicity.

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Abbreviations: bp, base pairs; CMV, cytomegalovirus; CRAd, conditionally replicating adenovirus; MOI, multiplicity of infection; pfu, plaque-forming units; vp, viral particles; TSP, tumor-specific promoter

Key words: CXCR4 gene, tumor-specific promoter, transcriptional targeting, adenoviral vector

Introduction

Conventional treatment options are not adequate for the majority of SCCHN patients. The failure of these approaches occurs because these tumors are remarkably resistant to chemotherapy and radiation, both of which work in large part by damaging the DNA of rapidly dividing tumor cells (1). The use of replicative viral agents (virotherapy) represents a novel approach for such neoplastic diseases. The most studied of virotherapy is the one originally generated (*dl*1520) by Barker and Berk (1) and used initially by the McCormick group as a selective vector, named ONYX-015 (2). This viral vector originally was believed to only replicate in p53-defective cells (present in ~50% of human tumors), however, this mechanism has subsequently been questioned (3). Based on significant antitumor activity demonstrated using ONYX-015 both *in vitro* and *in vivo* experiments, the preclinical potential of virotherapy led to their rapid translation into human clinical trials, including those targeting recurrent head and neck (4), pancreatic (5), colo-rectal (6), ovarian (7), and hepatobiliary cancer (8). An ideal viral agent would thus possess two characteristics: i) the capacity to selectively infect tumor versus non-tumor cells, which we denote as 'infectivity'; and ii) a relative preference for replication in tumor versus non-tumor cells, which we denote as 'specificity'. However, both viral infectivity and specificity are poor in currently available conditionally replicative viral vectors. Thus, the development of infectivity enhanced conditionally replicative viral vectors with high specificity for SCCHN is the goal.

The limitation of poor infectivity with current non-replicative and replicative Ad systems has been found to result from a relative paucity of the primary receptor for

adenovirus type 5, the Coxsackie-Adenovirus Receptor (CAR), on the surfaces of tumor cells relative to their cell line counterparts (9,10). On this basis, it has been proposed that gene delivery via 'CAR-independent' pathways may be required to circumvent this key aspect of tumor biology (11,12). Many approaches have been described to enhance the viral infectivity by alternative vector tropism. Specifically, Dmitrev *et al* (13) reported that construction of modified adenoviral vectors containing the RGD peptide in the HI loop region, which targets the integrins αvß3 and αvß5 (14) instead of CAR, increased in gene transfer to ovarian cancer cell lines (30-600-fold) and to primary ovarian cancer cells obtained from patients (2-3-fold). Recently, many other approaches have been reported which include targeting Ad to the serotype 3 receptor with a chimeric fiber protein (15,16), targeting Ad to tumor cells with the non-human canine Ad type 1 or 2 knob (17), targeting Ad to a heparin sulfate-containing receptor with an Ad fiber incorporating polylysine (pK7) (18) and targeting Ad to the junction adhesion molecule 1 (JAM1) with an Ad fiber incorporating reovirus sigma 1 fiber (19).

The improvement of poor tumor specificity with current replicative Ad systems has also recently been exploited by using a tumor specific promoter (TSP) to drive the E1 expression resulting in the viral replication being restricted in normal host cells, but not tumor cells, thereby avoiding the toxicity of the CRAd agent. An ideal tumor-specific promoter (TSP) for transcriptional targeting exhibits selective high activity in tumor cells (termed a 'tumor on' phenotype) and exhibits low activity in the endogenous sink, i.e. the liver (termed a 'liver off' phonotype). Many TSPs have been explored for specific cancers, such as prostate-specific antigen for prostate cancer, and α-fetoprotein promoter for hepatocarcinoma (20,21). However, there is no strong evidence of a tumor specific promoter (tsp) for targeting SCCHN. In our laboratory, we have exploited several tumor-specific promoters, including the Cox-2, Mk, VEGF, SLPI, TERSTS, CXCR4 and survivin promoters. All these promoters target different types of tumors, but they have not yet been screened in SCCHN. In this study, we tested all 7 promoters in four SCCHN cell lines and found that CXCR4 is the most active compared to the rest of the promoters in SCCHN cells. Müller *et al* have demonstrated that CXCR4 expression was undetectable in normal epithelial cells but markedly up-regulated in cancer cells (22). Others have described the chemokine, SDF-1, and its receptor, CXCR4, to have critical roles in determining the metastastic destination of tumor cells (23-26). In our previous work (27), CXCR4 promoter activities were analyzed in pancreatic, breast, and ovarian cancer cell lines with melanoma, breast cancer and lung cancer primary cells derived from patient material. The CXCR4 promoter had a 'tumor on' and 'liver off' phenotype in both *in vitro* and *in vivo* experiments when a recombinant adenoviral vector (reAd5-CXCR4.Luc), in which a luciferase reporter gene is driven by the CXCR4 promoter, was employed. Recently, we verified that a CXCR4-based CRAd agent, CRAd-CXCR4.RGD, targets human lung cancer (28).

In this study, we constructed a conditionally replicative adenoviral vector, in which the Ad E1 gene was regulated by using the CXCR4 promoter as a TSP and viral infectivity was enhanced with the capsid modification, F5/3, by which the adenoviral vector was targeted to tumor cells via a CAR-independent pathway. We verified that infectivity was enhanced and the selected vector replicated in both the SCCHN cancer cell lines and primary cells obtained from three patients. From our published data (28), the activity of the CXCR4 promoter was seen to be low in human liver compared to three other tumor promoters which previously had been used for targeting to other cancers. From these data, the CRAd-CXCR4.F5/3 is a promising novel agent for targeting human SCCHN with resultant low host toxicity.

Materials and methods

Cells and tissues. Human SCCHN tumor cell lines, SCC1, SCC22A and SCC27 were kind gifts from Dr Thomas Carey (The University of Michigan School of Medicine, Ann Arbor, MI) and the FaDu cell line was transferred by Michael Mathis from LSU Shreveport to UAB. The 911 cells were a kind gift from Dr Van Der Eb (Leiden University, The Netherlands) and non-transformed human skin fibroblasts were a kind gift from Dr Suresh Boppana (Childrens Hospital of Birmingham). Cells were maintained in Dulbecco's modified Eagle's medium. Each medium was also supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cells were incubated at 37˚C in a 5% CO_2 environment under humidified conditions.

Specimens of SCCHN, not needed for diagnostic purposes, were collected by two of us (ELR and MJM), following IRB approval. To generate tissue slices, tissue was cut in consecutive 0.5-mm-thick slices using the Krumdieck tissue slicer (Alabama Research Development, Munford, AL). Sequential slices were then cultured in 24-well-plates in RPMI medium supplemented with 10% bovine fetal serum, 100U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml insulin. Cultures were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Three tissue slices were examined per group.

Recombinant adenoviruses. All recombinant adenoviruses including Ad5-CXCR4.Luc (Ad5-C.Luc) (27), Ad5-Cox-2.Luc (29), Ad5-SLPI.Luc (30), Ad5-MK.Luc (31), Ad5-VEGF.Luc (32), Ad5-TERSTS.Luc (33), Ad5-Survivin.Luc (Ad5-S.Luc) (34), Ad5-CMV.Luc, Ad5-CMV.Luc.RGD, Ad5-CMV.Luc.F5/3, Ad5-CMV.Luc.FCN1 (17) and Ad5-CMV.Luc.FCN2 (35) were generated in this laboratory at UAB. The CRAd genomes were constructed via homologous recombination in *Escherichia coli* as previously described (28). The CRAd-CXCR4.RGD (CRAD-C.RGD) and CRAd-CXCR4.F5/3 (CRAd-C.F5/3) vectors have the following characteristics: i) The CRAd agent contains the human CXCR4 promoter nucleotide -191/+88 (36) which replaced Ad5 nt 346-521 (NCBI nucleotide database accession no. BK000408), which is the region of the Ad5 native E1 promoter. The CXCR4-controlled E1 expression cassette was placed in the original E1 region of the Ad5 gene; ii) A RGD-4C capsid modification was inserted into the Ad fiber knob region or a chimeric F5/3 contained a chimeric fiber protein possessing the Ad3 knob for enhancement of Ad infectivity (13); iii) The E3 gene was retained in the Ad genome for elevating the

Table I. The characteristics of adenoviral agents used in this study.

Virus name	Promoter	Reporter	E1	E3	Modification	Replication
Ad5-CMV.Luc	CMV	Luciferase	No	No	No	No
Ad5-Cox-2.Luc	Cox-2	Luciferase	No	No	No	No
Ad5-CXCR4.Luc	CXCR4 (C)	Luciferase	No	No	No	No
Ad5-Mk.Luc	Midkine	Luciferase	No	No	No	No
Ad5-TERT.Luc	TERT	Luciferase	No	No	No	No
Ad5-VEGF.Luc	VEGF	Luciferase	No	No	No	No
Ad5-SLPI.Luc	SLPI	Luciferase	No	No	No	No
Ad5-Survivin.Luc	Survivin (S)	Luciferase	No	No	No	No
Ad5-CMV.Luc.RGD	CMV	Luciferase	No	No	RGD4C	No
Ad5-CMV.Luc.F5/3	CMV	Luciferase	No	No	F5/3	No
Ad5-CMV.Luc.FCN1	CMV	Luciferase	No	No	FCN1 (canine virus type 1 knob)	No
Ad5-CMV.Luc.FCN2	CMV	Luciferase	No	No	FCN2 (canine virus type 2 knob)	No
Adwt	Native	No	Yes	Yes	No	Yes
CRAd-CXCR4.RGD	CXCR4 (C)	No	Yes	Yes	RGD4C	Yes
CRAd-CXCR4.F5/3	CXCR4 (C)	No	Yes	Yes	F5/3	Yes

oncolytic effect of the CRAd agents (37); and iv) A poly-A signal was inserted between the inverted terminal repeat (ITR) and the CXCR4 promoter to stop any non-specific transcriptional activity of the ITR and to retain the tumor specificity of the CXCR4 promoter.

Briefly, DNA fragments containing nucleotides -191/+88 were cut with *Bam*HI and *Hin*dIII restriction endonucleases from the clone pBSKCAT/CXCR4 3B/4-1[5' Δ3] (38), and subcloned into the plasmid pBSSK (Stratagene, La Jolla, CA) by use of the same restriction sites. A SV40 poly-A (PA) fragment was then cut with *Xba*I/*Bam*HI from a pGL3B vector (Invitrogen, Carlsbad, CA) and inserted into the pBSSK by use of the same restriction sites. A generated clone named pBSSK/PA/CXCR4 was used to create shuttle vectors. DNA fragments containing both an SV40 PA and the CXCR4 promoter were cut with *Not*I/*Xho*I, and subcloned into the same sites of the pScsE1 plasmid (from Dr D.M. Nettelbeck, Erlangen, Germany) which contained a DNA fragment from nucleotide number 522-3924 (GenBank sequence AD5001). This fragment covered both the Ad5 E1A and E1B coding region and was amplified from the genomic vector pXC-1 (39). Thus the plasmid, pScsE1/PA/CXCR4 was generated.

The Ad vectors, pVK503c and pVK500F5/3 (40), were kind gifts from Dr V. Krasnykh (M.D. Anderson Cancer Center, Houston, TX), and contained either fiber modification of RGD4C or F5/3, respectively. After cleavage with *Pme*I, the shuttle vector, pScsE1/PA/CXCR4, was recombined with *Cla*I linerized pVK503c to generate a CRAd genome with a RGD4C-modified fiber (CRAd-CXCR4.RGD) and with pVK500F5/3 to generate a CRAd genome with a F5/3-modified fiber (CRAd-CXCR4.F5/3). The resultant plasmids encoding the CXCR4 promoter in the CRAds were linearized with *Pac*I and transfected into 911 cells using Lipofectamine (Qiagen, Valencia, CA). Generated viruses were propagated in A549 cells, a lung tumor cell line, in which the CXCR4 gene is positively expressed (36) and the promoter is active, and purified by double CsCl density gradient centrifugation, followed by dialysis against phosphate-buffered saline (PBS) containing 10% glycerol. The viruses were titrated by plaque assay in 293 cells, and vp number was determined spectrophotometrically based on absorbance at a wavelength of 260 nm. The viruses were stored at -80˚C until use.

Wild-type Ad5 (Adwt) and Ad5-CXCR4.Luc were used as replication positive and negative controls, respectively, in the CRAd agent analysis. The characteristics of all adenovirus vectors used in this study are shown in Table I.

Transcriptional and transductional evaluations in vitro. SCCHN cell lines ($5x10^4$ cells/well), including FaDu, SCC-1, SCC-22A and SCC-27, were plated on 24-well plates in 1 ml of medium. The next day, cells were infected with recombinant Ads, Ad5-CMV.Luc, Ad5-Cox-2.Luc, Ad5-CXCR4.Luc (Ad4-C.Luc), Ad5-VEGF.Luc, Ad5-TERT.Luc, Ad5-SLPI.Luc, Ad5-MK.Luc, or Ad5-Survivin.Luc (Fig. 1) for transcriptional evaluation, or Ad5-CMV.Luc.RGD, Ad5-CMV.Luc.F5/3, Ad5-CMV.Luc.FCN1 or Ad5-CMV.Luc.FCN2 (Fig. 2) for transductional evaluation at 100 vp/cell for 2 h in 200 μl of the medium containing 2% of FCS. Cells were then washed once with 1 ml of PBS and 1 ml of the medium containing 10% of FCS was added to each well. After 48-h incubation, cells were washed with PBS, luciferase activity was determined using the Reporter Lysis Buffer and Luciferase Assay System of Promega (Madison, WI) following the manufacturer's protocol. Experiments were performed in triplicate and luciferase activities were standardized to the relative light unit (RLU) values of the CMV promoter (the CMV promoter activity is set as 100%). The transcriptional and transductional levels of the Ads in SCCHN cells were thus evaluated by expression activity of the luciferase reporter gene. Detected

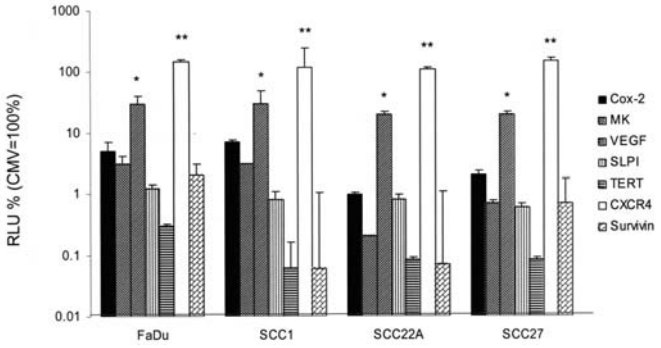

Figure 1. Comparison of promoter activities in SCCHN cells. Four SCCHN cell lines, FaDu, SCC1, SCC22A and SCC27, were used in these experiments. SCCHN cells ($5x10^4$) were plated on 24-well plates and infected at a MOI of 100 vp/cell with Ad5-CMV.Luc, Ad5-Cox-2.Luc, Ad5-Mk.Luc, Ad5-VEGF.Luc, Ad5-SLPI.Luc, Ad5-TERT.Luc, Ad5-CXCR4.Luc, or Ad5-Survivin.Luc, respectively. Luciferase activities were analyzed 48 h later. Results are shown as relative light units (RLU) of luciferase activity. The % of luciferase activity = (RLU induced by TSP)/(RLU induced by the CMV promoter) x 100. The mean value ± SE of triplicate samples is shown.

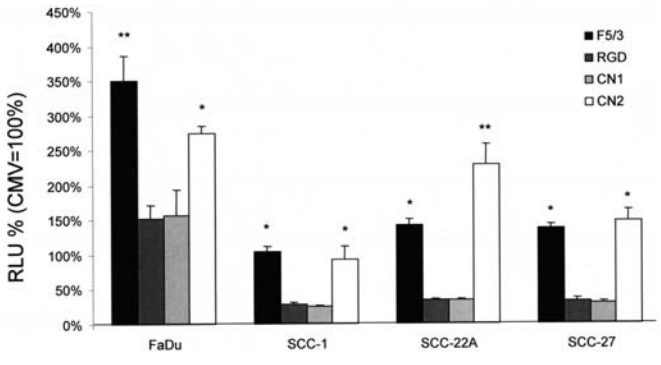

Figure 2. Comparison of transductional activity in SCCHN cells with different capsid modified adenovirus vectors. SCCHN cells ($5x10^4$ cells) were plated on 24-well plates and infected at a MOI of 100 vp/cell withAd5-CMV.Luc.RGD, Ad5-CMV.Luc.F5/3, Ad5-CMV.Luc.FCN1 and Ad5-CMV.Luc.FCN2, respectively. Luciferase activities were analyzed 48 h later. Results are shown as relative light units (RLU) of luciferase activity. The % of luciferase activity = (RLU induced by TSP)/(RLU induced by the CMV promoter) x 100%. The mean value ± SE of triplicate samples is shown.

activities of the luciferase reporter gene in Ad vectors were normalized by the expression activity of Ad5-CMV.Luc for both transductional and transcriptional level evaluation.

Binding of the CRAd agent to the surfaces of tumor cells. SCCHN cell lines ($5x10^4$ cells/well), including FaDu, SCC-1, SCC-22A and SCC-27, were plated on 24-well plates in 1 ml of medium. Cells were infected with 1, 10 or 100 vp/cell of Ad5-C.Luc, CRAd-C.RGD, CRAd-C.F5/3 or Adwt in infection medium containing 2% FBS. After a 3-h incubation at 37˚C, DNA was isolated from cells with the DNeasy Tissue Kit (Qiagen). The Ad5 E4 gene was detected in DNA samples by using an oligo-pair (forward primer, 5'-GGAGTGCGCCG AGACAAC-3', and reverse primer, 5'-ACTACGTCCGGCG TTCCAT-3') and a probe (5'-6-FAM-TGGCATGACTACGA CCAACACGATCT-TAMRA-3'). Real-time PCR was performed as described elsewhere (28). Ad E4 gene copy numbers were detected and normalized against human ß-actin.

Analysis of replication of CRAd agents in tumor cell lines. SCCHN cell lines ($5x10^4$ cells/well), including FaDu, SCC-1, SCC-22A and SCC-27, were plated on 24-well plates in 1 ml of medium. Cells were infected with 100 vp/cell of Ad5-C.Luc, CRAd-C.RGD, CRAd-C.F5/3 or Adwt in infection medium containing 2% FBS, and incubated at 37˚C in a 5% CO_2 environment. After a 3-h incubation, infection medium was taken off, the cells were washed three times to remove uninternalized viruses, and the cells then placed in fresh culture medium with 10% FBS. Media from triplicate wells were collected 1 (24 h after viral infection), 3, and 9 days later. DNA was extracted from 200 μl of media with the DNeasy Tissue Kit (Qiagen). The Ad5 E4 gene was detected in DNA samples by real-time PCR as described above. Ad E4 gene copy numbers were detected and normalized against human ß-actin.

In vitro analysis of cytocidal effects. The *in vitro* cytocidal effect of the CRAd-C.RGD was analyzed by determining the viability of the test cells with crystal violet staining after infection. Briefly, 25,000 cells (FaDu, SCC-1, SCC-22A, SCC-27 and non-transformed human skin fibroblasts)/well were plated on 12-well plates. Cells were infected at 625, 125, 25, 5, 1, or 0 vp/cell with Ad5-C.Luc, CRAd-C.RGD, CRAd-C.F5/3 or Adwt in infection medium. Three hours later, the infection medium was replaced with the appropriate complete medium. After 10 days of cultivation, the cells were fixed with 10% buffered formalin for 10 min and stained with 1% crystal violet in 70% ethanol for 20 min, followed by washing 3 times with tap water and air-drying. Trypan-blue exclusion experiments were also performed as described previously (41).

Replication of CRAd-CXCR4 in human SCCHN tumor slices. Excess tissue from three human SCCHN tumor specimens not needed for diagnostic purposes were obtained from E.L. Rosenthal and M. Mathis who are co-authors in this study. To generate SCCHN tissue slices, tissue was cut using the Krumdieck tissue slicer under the tissue culture conditions as described above. The tissue slices were infected with 500 vp/cell of Ad5-C.Luc, CRAd-C.RGD, CRAd-C.F5/3 or Adwt in fresh infection medium as described above. Three tissue slices were included per group. After 24 and 72-h incubation times, respectively, total DNA was extracted from the human tumor slices via the DNeasy Tissue Kit (Qiagen). DNA samples were treated with DNase free RNase to remove possible RNA contamination and stored at -80˚C until use. Ad E4 gene copy numbers were quantified as described and normalized with human ß-actin. We reported viral copy number as E4 copies/ng DNA. The amount of DNA in each sample was determined by using a human ß-actin probe, oligo-pair and a series of diluted genomic DNA samples (50, 5, 0.5 and 0.05 ng DNA).

Statistical analysis. The Student's t-test was employed for statistical analysis where $P<0.05$ was considered to be statistically significant.

Results

Evaluation of tumor-specific promoters in vitro. To screen for a SCCHN tumor-specific promoter, the activities of 7 tumor-specific promoters (TSPs), i.e. the Cox-2, Mk, VEGF, SLPI, TERT, CXCR4, and survivin promoters, were evaluated in four human SCCHN cell lines: FaDu, SCC-1, SCC-22A and SCC27. The backbone structures of the Ad vectors were identical for all constructs which additionally contained a luciferase reporter gene derived from a pGL3 plasmid. Thus the only difference in these Ad vectors was the promoter driving luciferase reporter gene expression. As there is a direct correlation between luciferase expression and promoter activity, a higher activity of the promoter results in higher luciferase gene expression. Therefore, the levels of the Ad transfected luciferase in tumor cells were normalized to that of an Ad5-CMV.Luc vector which had an identical backbone but utilized the CMV promoter to regulate the expression of luciferase gene. As shown in Fig. 1, two out of 7 TSPs (the VEGF and CXCR4 promoters) exhibited higher promoter activity than the other TSPs in the SCCHN cell lines. The mean activities in the four cell lines were 25% ($P<0.05$) for the VEGF promoter and 132% ($P<0.01$) for the CXCR4 promoter compared to that of the CMV promoter. The mean activities of the remaining promoters were <10% in these same cell lines. These data provide the evidence that the two promoters have a 'tumor on' phenotype.

Evaluation of capsid modification in vitro. To screen the fiber modification for viral infectivity enhancement, four capsid modifications, RGD (13), F5/3 (40), CN1 (canine adenovirus type 1 knob) (17) and CN2 (canine adenovirus type 2 knob) (34), were generated, via a CAR-independent pathway as described previously. All the Ad vectors again had an identical backbone, the difference being the incorporation of these alternative modifications in the Ad fiber region. The luciferase levels of the modified Ad vectors in the SCCHN tumor cells were normalized to that of the Ad5-CMV.Luc vector which had the same backbone as the native Ad5 fiber. The Ad vector with the F5/3 modification, Ad5-CMV.Luc.F5/3, exhibited the highest reporter activity among the 4 SCCHN cell lines tested. They were 350%, 105%, 141% and 136% (mean 183%, $P<0.05$) in FaDu, SCC-1, SCC-22A and SCC27 cell lines, respectively, when compared to that of the Ad5-CMV.Luc with native fiber. The Ad vector carrying the CN2 fiber, Ad5CMV.Luc.FCN2, also exhibited high reporter activity in the 4 cell lines. They were 274%, 91%, 227%, and 147% (mean 184%, $P<0.05$) in FaDu, SCC-1, SCC-22A and SCC27 cell lines, respectively, compared to that of the Ad5-CMV.Luc with native fiber. The mean RLU percent using other two vectors, Ad5CMVLuc. RGD and Ad5CMV.Luc. FCN1, when examined in the four cell lines were less than 100%. These data strongly argue that the F5/3 and CN2 fiber modifications should be excellent candidates for viral infectivity enhancement in SCCHN directed CRAd agents.

Transductional activities of capsid modified CRAds in SCCHN cells lines. Most human tumors contain only low levels of the coxsackie-adenovirus receptor (CAR), the natural endogenous receptor for human adenovirus serotypes 2 and 5. As described in Materials and methods, we constructed two CRAd agents, CRAd-C.RGD and CRAd-C.F5/3, in which the Ad E1 gene was under the control of the same CXCR4 promoter in both CRAd agents, but each with a different fiber modification, RGD (low transductional level in SCCHN cell lines) and F5/3 (high transductional level in SCCHN cell lines), respectively. We tested the transductional activities of these two CRAd agents in the four SCCHN cell lines. To avoid viral replication, we infected tumor cells for only 3 h and immediately isolated DNA from them. We had previously developed evidence that 18-24 h was required to detect Ad DNA in the medium, which corresponded to the life cycle of Ad from its entry into the tumor cells to its release. The adenoviral copy number was determined by real-time PCR and normalized to the housekeeping gene, ß-actin. As shown in Fig. 3, we compared the transductional levels (including viral binding and internalization) of two CRAd agents carrying different capsid modification, RGD and F5/3, in tumor cell lines. The results indicate that the CRAd-S.F5/3 had the highest binding activity to the tumor cells tested. The F5/3 fiber modification enhanced the transduction efficacy 9.3-, 9.0-, 1.3- and 1.1-fold for CRAd-C.F5/3 in FaDu, SCC-1, SCC22A and SCC-27 cell lines, respectively, compared to Adwt and 8.5, 7.9, 4.4 and 2.2-fold to CRAd-C.RGD in same cell lines, respectively, in the same conditions at a MOI of 1000 vp/cell. The transductional levels of CRAd-C.RGD were slightly higher than that of Ad-C.Luc and slightly lower than that of Adwt both of which carried a native fiber at all the MOI doses tested. From the transductional data, the F5/3 modification in CRAd agents exhibited the higher activity for binding to SCCHN cell surfaces compared to the RGD modification.

The evidence for CRAd agent replication in tumor cell lines. To further assay CRAd replication, SCCHN cancer cells (10^5) were plated in a 24-well plate and cells were infected with 100 vp/cell of Ad5-C.Luc, CRAd-C.RGD, CRAd-C.F5/3 or Adwt. After a 3-h infection, the cells were washed three times with PBS to remove free non-internalized viruses and provided with fresh medium. The presence of the E4 gene was determined by using real-time PCR from DNA samples extracted from the medium after 1, 3, and 9 days post-infection. The data shown in Fig. 4 demonstrate that the replication rate of Ad-C.Luc as a non-replicative control is low. The E4 copy number was less than one log increased both at days 3 and 9 when compared to that at day 1 in all four cell lines. The replication rate of replicative vectors, including CRAd-C.RGD, CRAd-C.F5/3 and Adwt, increased two to three logs when comparing days 3 and 9 to that of day 1. The E4 copy number of CRAd-C.F5/3 and Adwt increased mainly between days 1 and 3, then reached a plateau indicating rapid viral replication by day 3. After that, the viral replication rate slowed. E4 copy number of CRAd-C.F5/3 was higher than that of CRAd-C.RGD at all time-points examined in each cell line, although the E4 copy number of CRAd-C.F5/3 was only modestly higher than that of Adwt at all time-points tested in the cell lines.

CXCR4 based CRAds induce cytotoxicity in SCCHN cell lines. To evaluate cytotoxicity of CRAd agents in SCCHN cell lines, we used the CXCR4 based CRAd agents, CRAd-

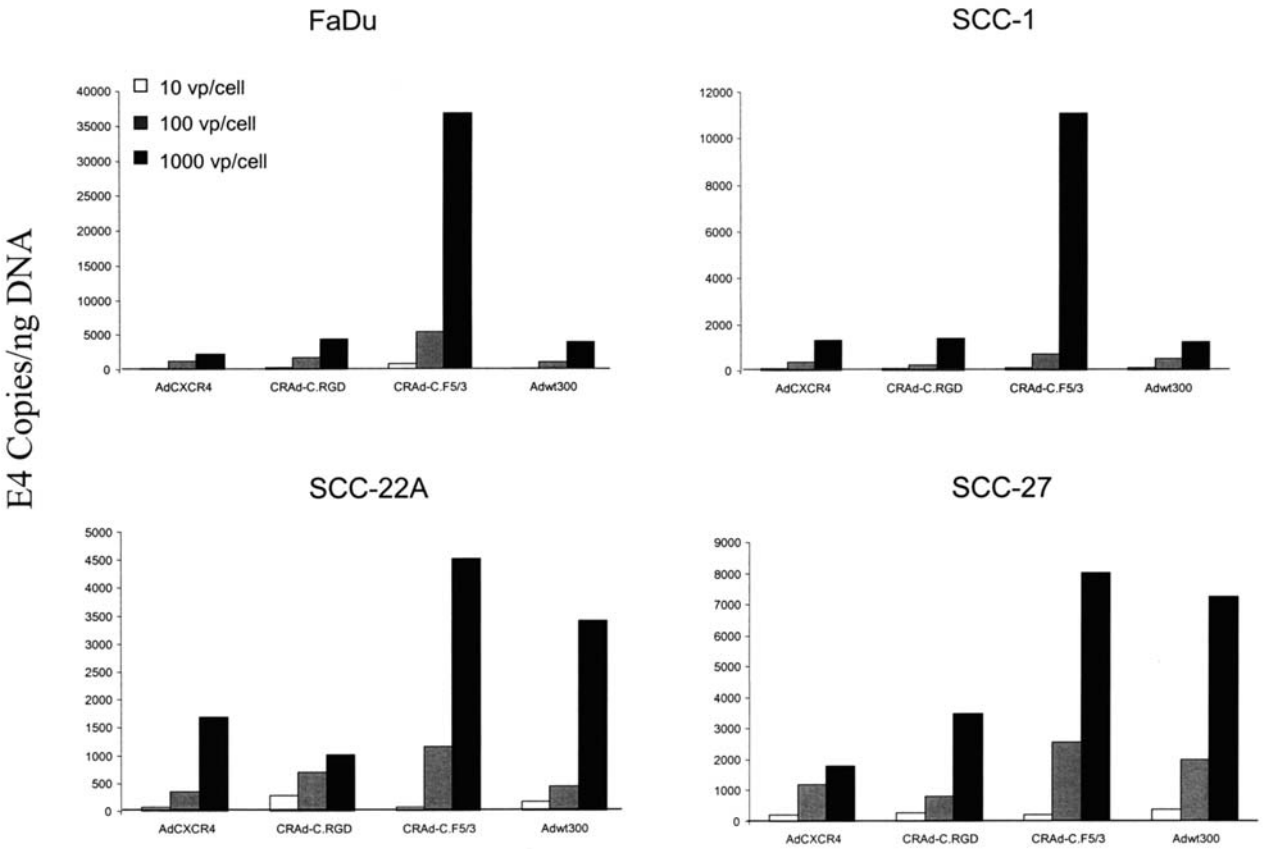

Figure 3. Transductional activities of modified CRAd agents in SCCHN cells. The SCCHN cells ($5x10^4$ cells) were plated on 24-well plates and infected at a MOI of 1,000, 100 and 10 vp/cell of Ad5-C, CRAd-C.RGD, CRAd-C.F5/3 or Adwt, respectively. After a 3-h infection, the cells were washed 3 times with PBS to remove un-infected free adenoviral vectors. The DNAs were isolated from these cells and the Ad E4 gene was determined by using RT-PCR. An internal standard, the GAPDH gene, was used for normalizing the DNA amount and the E4 copy number. The ordinate is shown as E4 copies/ng DNA.

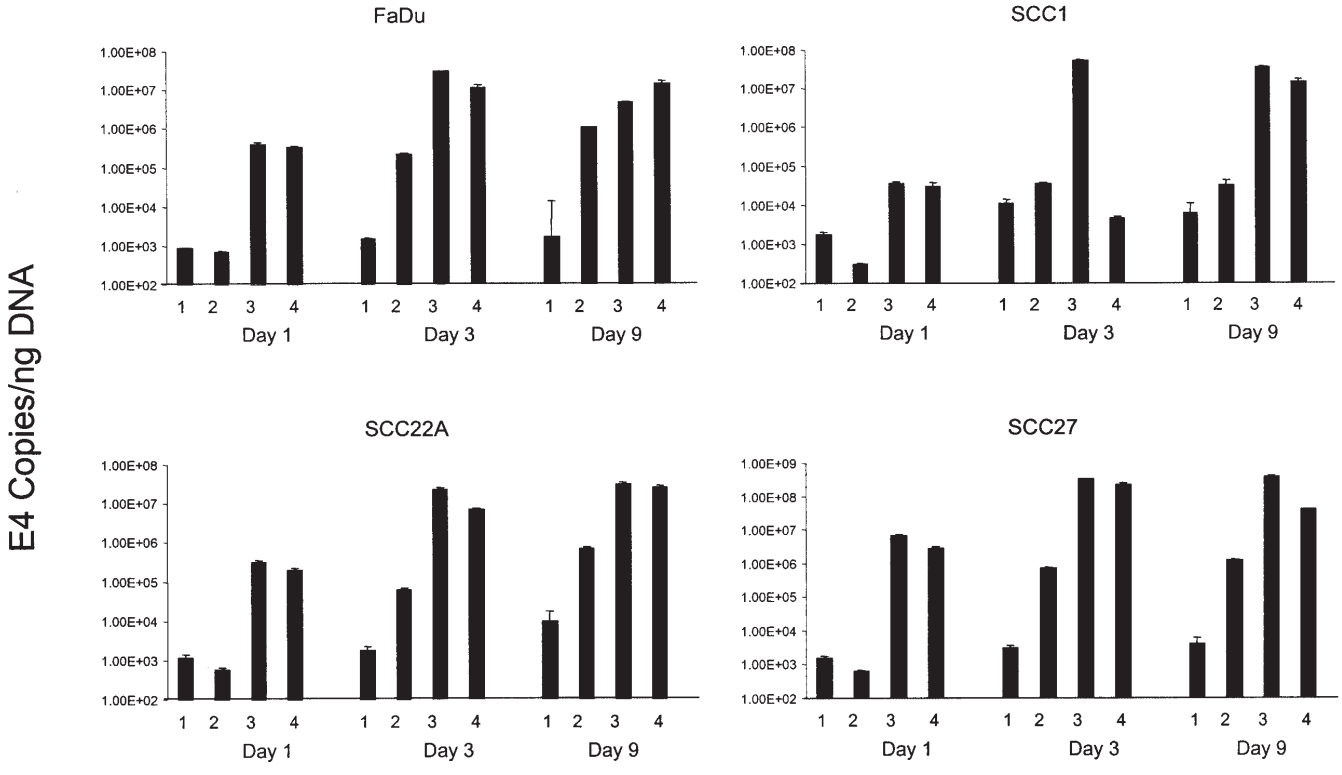

Figure 4. Replication rates of modified CRAd agents in SCCHN cells. The SCCHN cells ($5x10^4$ cells) were plated on 24-well plates and infected at a MOI of 100 vp/cell of Ad5-C, CRAd-C.RGD, CRAd-C.F5/3, or Adwt, respectively. Days 1, 3 and 9 post-infection, 200 μl of medium was collected and spun to remove the cell debris. The DNAs were isolated from the media and the Ad E4 gene was determined by using RT-PCR. 1, Ad5-CXCR4. 2, CRAd-C.RGD. 3, CRAd-C.F5/3. 4, Adwt.

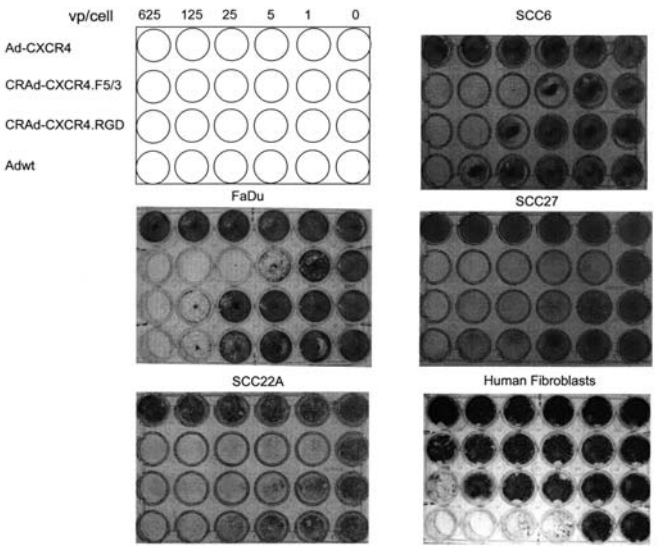

Figure 5. Cytotoxic efficiency of CXCR4-based CRAd agents on SCCHN cell lines. Cells ($5x10^4$) from each cell line of SCCHN or human skin fibroblasts were plated onto 24-well plates, and infected with Ad5-C, CRAd-C.RGD, Ad5-C.F5/3 and Adwt at the indicated MOIs or mock-infected. Cells were stained with crystal violet after a 10-day incubation as described in Materials and methods.

C.RGD and CRAd-C.F5/3, as oncolytic anti-tumor agents which were evaluated for their cell-killing effect in variety of SCCHN cell lines. Cytotoxicity was evaluated after 10 days of incubation via crystal violet staining (Fig. 5). While the replication-incompetent AdCXCR4Luc vector had no cytotoxic effect even at 625 vp/cell, the CXCR4-based CRAds induced strong cytotoxicity in all SCCHN cell lines tested, including FaDu, SCC-1, SCC-22A and SCC-27. Nearly 100% of cells were killed even at the minimal dose, 1 vp/cell, in the two cell lines, SCC-22A and SCC-27, 5 vp/cell in FaDu cells and 25 vp/cell in SCC-1 cells. A similar oncolytic effect can be seen in the four cell lines by using 5-10-fold higher doses of CRAd-C.RGD and 10-15-fold higher doses of Adwt when compared to CRAd-C.F5/3. As expected, cytotoxicity of the two CXCR4-based CRAds is much weaker than that of Adwt in human normal fibroblasts.

The evidence for CRAd agent replication in SCCHN tissue slices. To verify the replication of the test CRAd agent under near-clinical conditions, we examined the replication of the CXCR4-based CRAd agents in SCCHN tumor specimens from three patients. The samples were sectioned as described in Materials and methods, and infected with 500 vp/cell of Ad5-CLuc, CRAd-C.RGD, CRAd-C.F5/3 or Adwt. The slices were collected after 24 and 72 h, respectively, and DNA was isolated from tissue as described in the Materials and methods. The E4 copy numbers were determined by real-time PCR which served as a surrogate for the levels of adenoviruses released from infected tumor cells. The data showed (Fig. 6) that E4 DNA copy number per ng DNA of CRAd-C.F5/3 was similar to that of Adwt on day 1 and day 3 in three patient tumor samples. The replication rates of CRAd-C.F5/3 were 170%, 170%, and 320% (E4 DNA copy number on day 3 divided by the E4 DNA copy number of day 1) compared to 130%, 150% and 120% for Adwt in the

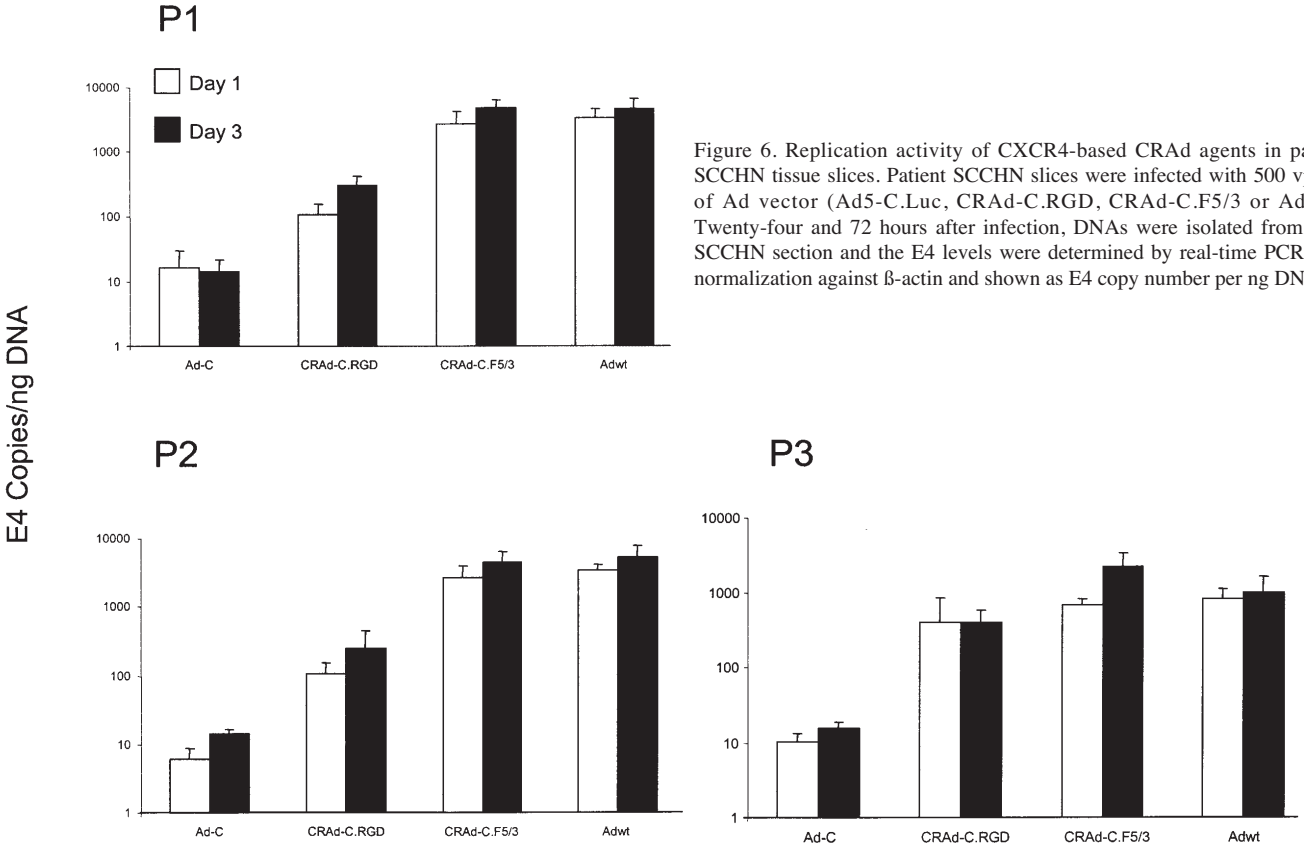

Figure 6. Replication activity of CXCR4-based CRAd agents in patient SCCHN tissue slices. Patient SCCHN slices were infected with 500 vp/cell of Ad vector (Ad5-C.Luc, CRAd-C.RGD, CRAd-C.F5/3 or Adwt,). Twenty-four and 72 hours after infection, DNAs were isolated from each SCCHN section and the E4 levels were determined by real-time PCR after normalization against ß-actin and shown as E4 copy number per ng DNA.

three patient samples, P1, P2, and P3, respectively. The replication rates of CRAd-C.F5/3 were slightly higher than that of Adwt with no significant difference in the three patient samples. The Ad E4 DNA copy numbers of CRAd-C.RGD at day 3 were 309, 243 and 401, respectively, in P1, P2 and P3 samples and more than one log lower than that of CRAd-C.F5/3 and Adwt. As predicted, Ad5-C.Luc had low viral copy levels (three logs lower than that of CRAd-C.F5/3 and Adwt) and low viral replication rates in the three SCCHN tumor samples because of non-replicative control.

Discussion

Recent advances in our understanding of growth factors, molecular oncology, tumor immunology and gene therapy have provided the rationale for cancer gene therapy (42). Some of these approaches, including adenovirus-mediated p53 gene transfer and adenovirus-mediated herpes simplex virus thymidine kinase/ganciclovir gene therapy, are being tested in clinical trials in patients with lung cancer and malignant mesothelioma (14,43,44). In addition, Ad-p53 gene transfer in patients with advanced, locoregionally recurrent SCCHN has been translated into Phase I and II clinical trial studies (45), and a Phase II trial of intratumoral administration of ONYX-015, a replicative-selective adenovirus, in patients with SCCHN has been reported (46). The major challenges of gene therapy remain inefficient gene delivery (9,10), toxicity (13) and immune responses (47), although we did not focus on this latter issue in this study.

Recently, replication competent adenoviruses have come into focus as promising novel anti-tumor agents for viral oncolysis and enhanced transfer of therapeutic genes (40,48). Tumor specificity is the key to the realization of replicating viruses as cancer therapeutics. This is especially relevant in the context of systemic therapy, as anticipated for treatment of most malignant neoplasms. Despite the theoretical utility of conditionally replicative adenoviruses (CRAds), the overall tumor response in practice has been limited. This is based on the lack of native adenovirus receptor, CAR, on most tumor cell surfaces and the lack of TSPs to selectively drive Ad E1 expression. Ad E1 protein is critical for viral replication and deletion of the E1A coding region from the Ad genome makes the virus non-replication competent, allowing replication only in the E1A trans-complement cell lines, such as 293 and 911. In this study, we screened 7 tumor-specific promoters in SCCHN cell lines and found the CXCR4 promoter has the best activity in four SCCHN cell lines compared to the others. In addition, previous data showed that the CXCR4 promoter activity in murine liver *in vivo* and human liver tissue is low (28). Thus, the CXCR4 promoter has a phenotype of 'tumor on and liver off', and the CXCR4 promoter should prove to be a good candidate TSP for constructing the CRAd agents for SCCHN cancer gene therapy.

We successfully constructed the CXCR4-based CRAd agents, CRAd-C.RGD and CRAd-C.F5/3, in which E1 gene expression was under the control of the CXCR4 promoter, a TSP with high activity in SCHN cells. The RGD and F5/3 fiber modifications were carried out for enhancing viral infectivity via a CAR-independent pathway. The RGD targets α_v integrins (13) and F5/3 targets CD80/CD86/CD46 (49,50) on the surfaces of neoplastic cells. Based on data shown in Fig. 2, the RGD modification had lower transductional activity and the F5/3 higher transductional activity in the tested SCCHN cells. As tumor killing (oncolysis) is known to parallel viral infectivity on tumor cells, we evaluated both viral transductional levels and viral replication rates of CRAd-C.RGD and CRAd-C.F5/3 in four SCCHN cell lines (Figs. 3 and 4). The data clearly showed that CRAd-C.F5/3 had higher transductional activity and viral replication in the four SCCHN cell lines tested. Similar results were seen in the three patient tumor samples (Fig. 6), although there was no significant difference compared to Adwt. From these data compared to CRAd-C.RGD, we conclude that the CRAd-C.F5/3 had higher transductional activity or viral infectivity in both SCCHN cell lines and primary cells. One possible explanation for this is that the Ad3 receptor is highly expressed on SCCHN cells although this is not specifically determined in this compared to the viral 'replicative competent' control, Adwt, the CRAd-C.F5/3 had a slightly higher transductional activity and viral infectivity in both the SCCHN cell lines and primary cells, but they were not significantly different. An interesting finding was that the viral replication seen with all three Ad vectors, CRAd-C.RGD, CRAd-C.F5/3 and Adwt reached a plateau after a 3-day incubation in SCCHN cell lines. The reasons for this remain unknown. Transductional levels and viral infectivities were 3-4 logs lower in the Ad vectors if the E4 DNA copy number in day 3 reached 10^7 in the tumor cell lines (but only 10^3 levels in primary tumor slices). Possible explanations for these results include the receptor levels on the tumor cells, the slower spread of virus in tumor cells and the possibility that circulating antibody exists against adenoviruses. Finally, the viruses detected in the media were viable (Fig. 4) and were capable of being released from tumor cells with intact biological function.

Infection with adenovirus causes profound changes in host-cell macromolecular synthesis that ultimately leads to cell death. Virion fiber protein inhibits macromolecular synthesis when applied directly to cells bearing the adenovirus receptor (51). Cell-specific DNA synthesis, export of cellular mRNAs from the nucleus to the cytoplasm, and cell-specific translation are all inhibited after infection, but the precise mechanisms are still not completely understood. Also, high expression of the CXCR4 gene in some blood elements, such as NK cells and lymphocytes, has been reported. However, the main damage to the host is that the majority (>95%) of viruses released into the blood stream and localized to the liver (13,52) which leads to host toxicity. We have reported that the activity of the CXCR4 promoter is 'off' in mouse liver and human liver tissues (27). To mimic the *in vivo* condition of the human host, we used human liver slices instead of murine liver, as we had in previous studies to rigorously evaluate the activity of the CXCR4 promoter. Compared to the three other promoters regularly used for tumor targeting, the activity of the CXCR4 promoter is the lowest among them in this organ (28). In other words, the CXCR4 promoter has the lowest toxicity to human liver among the readily available tested promoters.

Xenograft animal models are the conventional method to evaluate cancer therapeutics in the gene therapy field. However, this is not a perfect model system for several reasons.

Multiple studies have been performed in athymic nude mice and severely combined immonodeficient mice (scid) (53,54). Although nude mice lack functional T lymphocytes, they retain natural killer (NK) cell activity that can impede the normal patterns of growth and metastasis from xenograft implants (55). Thus, this model system, lacking a competent immune response, does not provide a reliable model for direct extrapolation to the clinical setting (51). For this reason, we used human SCCHN tumor tissue to evaluate the viral replication of the CRAd agent in this study from three patients with SCCHN. The patterns are similar in that the adenovirus E4 copy number and replication rates of the CRAd-C.F5/3 were higher than that of CRAd-C.RGD and slightly higher than that of Adwt in all three tumor samples tested. This argues that the higher DNA copy number might only reflect the infectivity potential of CRAd-C.F5/3 in primary tumor cells, but not replication.

In the CRAd-C.RGD vector, the Ad E1 is regulated by a TSP, the CXCR4 promoter. The E1A-induced activation of the apoptosis pathway(s) must be modulated by E1B protein to ensure efficient virus replication prior to cell death (56). Activation of the interferon-inducible RNase L pathway by an adenovirus-associated type I (VAI) RNA (57) may also contribute to the stimulating of apoptotic pathways in adenovirus-infected cells (58). The E3 11.6-kDa adenovirus death protein, which exists in CRAd-C.RGD, also has a role in cell killing and promotes the release of progeny virions from cells. Taken together, cell killing is related to virus replication which, in turn, is related to E1 expression level. As noted, the E1 expression of the CRAd-C.F5/3 is under the control of the CXCR4 promoter. We detected tumor cell killing with an oncolytic assay by staining with crystal violet. The data showed the CRAd-CXCR4 agent has strong cytoxicity to the SCCHN cancer cell lines, but not to the normal human skin fibroblasts. The oncolysis activities of the CRAd-C.F5/3 were 5-10-fold higher when compared to that of CRAd-C.RGD. As expected, the oncolysis was not seen in the negative control, Ad-C.Luc, a non-replication competent virus. From these data, the generated CRAd-C.F5/3 is able to target human SCCHN tumor cells. Because many studies have described a relationship between the CXCR4 gene and tumor metastasis, we anticipate evaluating whether metastatic SCCHN lesions will be modulated by this agent in a future study. We did not show tumor killing by CRAd agents in primary cells because it is technically not feasible. From our unpublished data we know that untreated primary tumor cells are alive for less than four days *in vitro*, thus, viability assays of virally infected primary cells would be essentially impossible to perform.

In conclusion, we identified the human CXCR4 promoter as a tumor-specific regulatory element for SCCHN tumors in human. Viral replication is active in established SCCHN cancer cell lines and primary tumor tissues. Thus, the CXCR4 promoter emerges as an especially promising transcriptional targeting gene approach for SCCHN cancer. It is clear the CXCR4 promoter in a CRAd context should be useful for future clinical applications for SCCHN and other cancer types.

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