ABSTRACT

The employment of conditionally replicative adenoviruses (CRAd) constitutes a promising alternative for cancer treatment; however, in the case of esophageal adenocarcinoma (EAC) the lack of an appropriate tumor-specific promoter and relative resistance to adenovirus infection have hampered the construction of CRAds with clinically applicable specificity and efficacy. By combining transcriptional targeting with infectivity enhancement for CRAds, we generated novel cyclooxygenase-2 (Cox-2) promoter-controlled replicative viral agents for the treatment of EAC. We used infectivity enhancement based on incorporation of an RGD-4C motif into the HI loop of the adenoviral (Ad) fiber knob domain as well as replacement of the Ad5 knob with the Ad3 knob. The Cox-2 promoter was highly active in EAC, whereas showing no significant activity in Cox-2-negative cell lines and primary cells isolated from normal mouse esophagus and stomach. Evaluation of infectivity-enhanced vectors revealed that the transduction and virus-cell binding ability of Ad5/Ad3-chimera were significantly more efficient than that of unmodified and Arg-Gly-Asp (RGD)-modified vectors. All of the Cox-2 CRAds demonstrated replication and subsequent oncology in EAC cells but not in Cox-2-negative cells in vitro, thus confirming the dependence of their replication on the Cox-2 promoter activity. Ad5/Ad3 CRAds exhibited significantly improved oncolysis and progeny production compared with unmodified and RGD-modified vectors without sacrificing tumor selectivity. Whereas unmodified and RGD-modified CRAds showed insignificant therapeutic effect in vivo, Ad5/Ad3 CRAds remarkably suppressed tumor growth of established xenografts in mice. Thus, our studies have demonstrated that Ad5/Ad3-chimeric Cox-2 promoter-driven CRAds are selective and potent agents for the treatment of EAC.

INTRODUCTION

The incidence of esophageal adenocarcinoma (EAC) has increased rapidly since the mid-70s and is now the fastest expanding type of cancer in Western countries (1–5). The highest rate of increase was nearly 10% per year in the United States (6). Although the reason for this rapid rise is unclear, it is well known that 10% of patients with gastroesophageal reflux disease eventually develop Barrett’s esophagus, a metaplasia of the lower esophageal epithelium, resulting with a 1% annual risk of developing adenocarcinoma (7, 8). Improvements in the diagnosis of EAC at earlier stages as well as the treatment of late-stage disease remain insignificant, whereas the overall 5-year survival rate is <10% (9, 10). In this regard, novel oncolytic virus approaches using the most advanced therapeutic vectors may offer more effective treatment of EAC.

Most initial cancer gene therapy protocols were performed with replication-incompetent adenoviruses (Ad), which by their nature are limited in their ability to infect cancer cells and spread throughout the tumor (11). To overcome these obstacles, the concept of conditionally replicative Ads (CRAds) has been proposed (12, 13). These agents are designed to replicate specifically in tumor cells, followed by the spread of the viral progeny to neighboring cancer cells (14). A variety of Phase I and II clinical trials have demonstrated the tolerability and safety of CRAd administration (15–22). One such Ad (d1520/ON-YX-015) has entered a Phase III clinical trial for recurrent head and neck carcinoma (22). However, clinical experience has indicated that a satisfactory balance between safety and efficacy of CRAds has not yet been achieved (23). The current-generation CRAds are safe, but due to their relatively poor ability to spread within the tumor mass, repeated viral injections at multiple sites and over a prolonged period of time are required (11, 16, 17, 23).

One common approach to target virus replication to a specific subset of cells is through the use of tissue- or tumor-specific promoters (24–32). Therefore, the design of a particular tissue- or tumor type-specific virus vector depends on the availability of a relevant tissue- or tumor-specific promoter. In the case of EAC, the lack of an appropriate tumor-specific promoter that is both selective and strong in EAC cells has hampered the construction of CRAds with clinically usable specificity and efficacy. Among the promoters successfully used to drive CRAd replication is the cyclooxygenase-2 (Cox-2) gene promoter (31). Cox-2 is the key enzyme required for the conversion of arachidonic acid to prostaglandins, normally undetectable in most normal tissues (33). Recent studies have highlighted the involvement of Cox-2 in carcinogenesis and tumor progression (34, 35). Increased levels of Cox-2 have been reported in carcinomas of the colon, stomach, breast, lung, liver, and pancreas (33, 36, 37). Most importantly, Cox-2 has been found in EAC and Barrett’s esophagus, suggesting that the Cox-2 promoter may be applicable to target EAC (38–43). Additionally, our studies have clearly shown that the Cox-2 promoter possessed strong activity in many gastrointestinal cancer cells among a variety of tested promoters, whereas activity in normal tissues, especially in the liver, was minimal (28–31). On the basis of these findings, we explored the feasibility of the Cox-2 promoter for transcriptional targeting of replicative Ads to EAC.

EAC cells are known to be very resistant to infection by conventional Ad vectors (44–46). The efficiency of Ad5-mediated gene transfer largely depends on the biology of the virus-cell interaction (47). The initial high-affinity binding of Ad5 to the primary coxsackie-Ad receptor (CAR) occurs via the knob domain of the Ad fiber (48, 49). A subsequent step of virus internalization depends on interaction between the Arg-Gly-Asp (RGD) motifs in the Ad5 penton base protein and the integrin molecules on the cell surface (50).茎干ness from these considerations, efforts to improve transduction efficiency of Ads have included changing viral tropism by using the strategy of genetic modification of the viral capsid proteins, which would enable CAR-independent entry. Specifically, it has been reported that incorporation of an RGD-4C peptide into the HI loop of the fiber knob domain remarkably increased the viral infectivity in CAR-negative cells (51). In a previous study, we demonstrated the suitability of an RGD infectivity-enhanced Cox-2 promoter-based CRAd for pancreatic cancer (31). This enhancement was mainly
mediated by the binding of the RGD-modified Ad fiber protein to integrins, which are overexpressed on the surface of target cancer cells (51, 52). Another strategy to achieve CAR-independent cell entry includes fiber-knob chimerism where the fiber knob of Ad5 is replaced with that of another Ad type (53, 54). Notably, the Ad type 3 (Ad3) receptor, the identity of which has not yet been established (55), has been shown recently to be a better target for Ad vectors designed to treat ovarian cancer, squamous cell carcinoma of the head and neck, and renal cancer (56–59).

In this study, we applied these strategies of Ad tropism modification to derive novel Cox-2 promoter-based CRAds as therapeutic agents for EAC. Importantly, we demonstrate that the oncolytic potency of the Cox-2 CRAds transcriptionally targeted to EAC can be augmented significantly by directing them to the Ad3 receptor. This modality of combining transcriptional targeting and Ad5/Ad3 modification to increase the therapeutic index provides a powerful and promising approach to develop oncolytic viruses for EAC.

**MATERIALS AND METHODS**

**Cell Lines.** OE19 and OE33 human EAC lines were obtained from the European Collection of Cell Culture (CAMR Centre for Applied Microbiology and Research, Wiltshire, United Kingdom) and were grown in DMEM (Mediatech, Herndon, VA). TE7 (human EAC; Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan), A549 (Cox-2 positive lung cancer cell line; American Type Culture Collection (ATCC), Manassas, VA), and BT474 (Cox-2 negative breast cancer cell line; ATCC) cells were cultivated in RPMI 1640 (Mediatech, Herndon, VA). Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC (a kind gift from Dr. Janet Price, M.D. Anderson Cancer Center, Houston, TX) were propagated in DMEM/F12, 50:50 (Mediatech). Chinese hamster ovary cells (Ad3 receptor-negative control) were grown in HAM's F-12 medium. Human hepatoma cell line HepG2 (CAR positive control; ATCC), transformed human embryonic retina cell line 911 (a kind gift from Dr. Alex J. van der Eb, Leiden University, Leiden, the Netherlands), and 293 cells (ATCC) were maintained in DMEM. Each medium was also supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM l-glutamine and penicillin (100 IU/ml), and streptomycin (100 mg/ml). Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC and cultured in EGM-2 medium (Cambrex Biosciences, Walkersville, MD). All of the cells were incubated at 37 °C in a humidified environment with 5% CO2.

**Adenoviral Vectors.** Replication-incompetent adenoviral vectors (AdCox2L, AdCox2MLuc, AdCox2MLuc) encoding the luciferase (LacZ) reporter gene were constructed as described previously (30). AdCox2Luc and AdCox2MLuc carry the Cox2L (wild-type fiber) and the RGDw fiber gene, respectively. The vectors were propagated in E1-transcomplementing 911 or 293 cells, and purified virions using a Blood DNA kit (Qiagen). Total cellular RNA was extracted from cultured cells using the RNeasy mini RNA kit (Qiagen), and aliquots corresponding to 106 genomes were analyzed by PCR. Thermal cycling conditions were: initial denaturation (5 min at 95 °C), and 25 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C), and final extension (10 min at 72 °C). The regions detected and expected band size were as follows: wild-type Ad5 (485 bp), E1a (338 bp), Cox-2 promoter (405 bp), forward direction CRAd (392 bp), Ad5 fiber knob region (wild-type fiber: 247 bp and RGD fiber: 274 bp), and Ad5/Ad3 fiber knob region (293 bp). For the Ad5/Ad3 fiber knob region, the following primers were used: Ad5/Ad3F (sense) 5’-GCTGCTGCTCTGAAAG-3’ and Ad5/Ad3R (antisense) 5’-CGCTTGGTTATTGCTTATG-3’. The regions were amplified as described previously.

**Validation of Viral Constructs.** Several critical vector components of the CRAd genomes were confirmed by PCR. Viral DNA was isolated from purified virions using a Blood DNA kit (Qiagen), and aliquots corresponding to 107 genomes were analyzed by PCR. Thermal cycling conditions were: initial denaturation (5 min at 95 °C), and 25 cycles of amplification (30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C), and final extension (10 min at 72 °C). The regions detected and expected band size were as follows: wild-type Ad5 (485 bp), E1a (338 bp), Cox-2 promoter (405 bp), forward direction CRAd (392 bp), Ad5 fiber knob region (wild-type fiber: 247 bp and RGD fiber: 274 bp), and Ad5/Ad3 fiber knob region (293 bp). For the Ad5/Ad3 fiber knob region, the following primers were used: Ad5/Ad3F (sense) 5’-GCTGCTGCTCTGAAAG-3’ and Ad5/Ad3R (antisense) 5’-CGCTTGGTTATTGCTTATG-3’.

**Analysis of Cox-2 RNA Levels.** Total cellular RNA was extracted from semiconfluent cell cultures in 10-cm dishes using the RNeasy mini RNA extraction kit (Qiagen). Cox-2 and glyceraldehyde-3-phosphate dehydrogenase RNAs were detected in a semiquantitative manner using the Gene Amp Gold RNA PCR Core kit (Perkin-Elmer, Branchburg, NJ) as described previously.

**Flow Cytometry.** Cultured cells were trypsinized, washed with PBS, centrifuged, and resuspended in ice-cold PBS supplemented with 1% BSA and 0.1% NaN3. Cells (105) were incubated for 1 h at 4 °C with mouse antibodies Rm12B to CAR (1:1000; produced using hybridoma purchased from ATCC), I120 (1:1000), P166 to ov integrin (1:1000), P4G11 to β1 integrin (1:50), or HA5 to ov β1 integrin (1:1000; Chemicon, Temecula, CA). The cells were then washed twice with PBS/BSA/NaN3 buffer and incubated with secondary FITC-labeled goat anti-mouse IgG serum (1:50; Sigma). After another PBS/BSA/NaN3 rinse, 2.5 μg/ml propidium iodide (Sigma) was added to sort out dead cells from the sample. The cells (106) were analyzed immediately by flow cytometry at the University of Alabama at Birmingham FACS Core Facility.

**Promoter and Gene Delivery Analysis with Luciferase Expression Ads.** EAC cells grown in 24-well plates were infected with Ad vectors at a multiplicity of infection of 50 pfu/cell. The infection medium was replaced.
with the appropriate growth medium 2 h later. Two days after infection, the cells were lysed with cell culture lysis buffer (Promega, Madison, WI), and Luc activity was determined with the Luciferase Assay System (Promega). Experiments were performed in triplicate and standardized with protein concentration quantitated by the DC protein assay (Bio-Rad, Hercules, CA).

The Cox-2 promoter selectivity was also analyzed in mouse esophageal and gastric primary cells. The esophagus and stomach of a Balb/C mouse were aseptically isolated and cut by scissors into small pieces, followed by wash with PBS twice. The tissues were dispersed with Liver Digestion Medium (Life Technologies, Inc.) for 3 h at 37°C, rocking every 15 min. After adding 25% volume of FBS to stop digestion, the cells were centrifuged and resuspended in 20% FBS DMEM/F12 containing 1 μM dexamethasone. The cells were plated and infected as described above.

In Vitro Analysis of Cytoidal Effect by Crystal Violet Staining. To analyze virus-mediated cell killing, 25,000 cells/well were plated in 12-well plates and infected with the viruses in 200 μl of growth medium containing 5% FBS at an multiplicity of infection of either 0.01 or 0.1 vp/cell. After 3 h, 1 ml of the growth medium was added. Two days later, the infection medium was replaced with 1% FBS medium. After 12 days (18 days for TE7 cell line) 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 with water and drying.

Virus Binding Assay. To analyze virus-cell binding, 50,000 cells/well cultivated in 24-well plates were infected with 2000 vp/cell in 100 μl of DMEM and incubated for 1 h at 4°C. At this temperature, virus internalization would not occur. After washing with PBS three times, cells were scraped from the plates and processed with a QIAamp Blood Mini kit (Qiagen). The isolated DNA was analyzed by real-time PCR analysis to determine the Ad DNA copy number with E4-specific primers at the University of Alabama at Birmingham Gene Therapy Center Correlative Laboratories as described before (31). For the standard curve, E4 template DNA with known copy numbers (10^8, 10^6, 10^4, and 10^2) was used. All of the PCR reactions were carried out using the Light Cycler System (Roche Molecular Biochemicals, Indianapolis, IN) as recommended by the manufacturer. Thermal cycling conditions were: initial denaturation for 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed with the Light Cycler software. The primers were designed to detect a 68 bp-long sequence within E4 region: forward primer (5′GGGAGTGCGCGGAGACAC3′, nucleotides 34007–34024), reverse primer (5′ACTAAGTCGGCGTTCAT3′, nucleotides 34074–34056) and 6-carboxyfluorescein labeled probe (6-carboxyfluorescein-TGGCATGACACTAC-FAM). Absorbance was measured at a wavelength of 490 nm in an E-Max spectrophotometer (Molecular Device Corporation, Sunnyvale, CA), and standard curves were generated by analyzing the known number of live cells. On the basis of this curve, the number of living cells was calculated for the experimental groups using the SOFTmax computer software (Molecular Device Corporation). All of the experiments were repeated in triplicate.

Viral Progeny Production Assay. To determine production of virus progeny, 250,000 cells seeded in 12-well plates were infected with Ad vectors at a multiplicity of infection of 1 vp/cell in 200 μl of 5% FBS medium. After 3 h, the infection medium was replaced with 1 ml of the appropriate growth medium. Twelve days later, cells and medium were harvested, freeze/thawed three times, and centrifuged, and the supernatant was titered by a standard plaque assay on 911 cells. All of the experiments were performed in triplicate.

In Vivo Antitumor Effect in an EAC Xenograft Model. Female ncr/nu nude mice (Frederick Cancer Research, Frederick, MD; 6–8 weeks of age) were used to establish EAC xenografts. OE19 cells (2.4 × 10^6 per injection site) were inoculated into the flanks of mice. When the nodules reached a size of 8–10 mm in maximum diameter, a single virus dose (10^10 vp in 100 μl PBS) of CRAds or control viruses was injected intratumorally. The condition of the mice was monitored daily, and the tumor diameter was measured twice a week with calipers. The tumor volume was calculated using the formula: tumor volume = width^2 × length/2. In accordance with institutional approved animal experimental protocols, mice were euthanized 16 or 21 days after viral injection due to the overgrowth of tumors in the control group. All of the animals received humane care based on the guidelines set by the American Veterinary Association. All of the experimental protocols involving live animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Statistical Methods. Statistical analysis of CRAd efficacy in vitro and in vivo was performed with a two-tailed t test. Data are expressed as a mean ± SD of at least three sets of results. Results were considered statistically significant when P < 0.05.

RESULTS

Confirmation of CRAd Structure. All of the CRAd vector structures were confirmed by PCR analysis of the viral DNA. None of the vectors was contaminated with nonselective replication-competent Ad resulting from spontaneous homologous recombination during vector propagation in E1-transcomplementing cell lines (Fig. 2A). The CRAds possessed both the E1a gene and the Cox-2 promoter sequence as part of their E1 expression cassettes (Fig. 2, B and C). A 392-bp fragment corresponding to the forward direction cassette was amplified from CRAdCox2F, RGDCRAdCox2F, and 5′/3′CRAdCox2F with a sense primer recognizing the left inverted terminal repeat sequence and an antisense primer recognizing the Cox-2 promoter sequence (Fig. 2D). In the analysis of the fiber region, vectors with wild-type Ad5 fiber (CRAdCox2F and CRAdCox2R) produced a 247-bp signal, whereas vectors with the RGD-modified fiber (RGDCRAdCox2F and RGDCRAdCox2R) yielded a 274-bp band (Fig. 2E), the increased size of which represents the 9 amino acid insertion in the HI loop. Ad5/Ad3-chimeric CRAds (5′/3′CRAdCox2F and 5′/3′CRAdCox3R) amplified with 5′F primers showed a 293-bp signal, which confirms the presence of the Ad3 knob in the fiber (Fig. 2F). These data validate the structure and stability of the CRAd constructs.
Cox-2-negative BT474 and normal HUVEC cell lines.

Fig. 3. Analysis of cyclooxygenase (Cox)-2 mRNA levels in EAC cell lines. Total RNA of human EAC cells (OE19, OE33, and TE7), Cox-2-positive A549 cells, and Cox-2-negative BT474 cells was reverse-transcribed with oligo primer and amplified by PCR with Cox-2- (top) and glyceraldehyde-3-phosphate dehydrogenase- (GAPDH; bottom) specific primers. The signal for COX-2 mRNA was detected as a 723-bp band.

Fig. 4. Activity and selectivity of the cyclooxygenase (Cox-2) promoter in EAC. A, human EAC (OE19, OE33, and TE7), Cox-2-positive (A549), Cox-2-negative (BT474 and human umbilical vein endothelial cells; HUVEC) cells were infected with AdCMVLuc, AdCox2MLuc, and AdCox2LLuc. Luc activity was measured 2 days after infection. Data are shown as percentages of relative light units (RLU) per mg protein in relation to that of cytomegalovirus promoter-driven luciferase expression. The Cox2M and Cox2L promoters exhibited high levels of promoter activity in EAC and minimal activity in Cox-2-negative BT474 and normal HUVEC cell lines. Bars, ±SD calculated from four replicates. B, in primary cells isolated from normal mouse esophagus and stomach, Cox2M and Cox2L promoter-driven vectors showed no significant activity compared with the cytomegalo virus-controlled vector.

Transcriptional Status of Cox-2 in the EAC Cell Lines. The Cox-2 mRNA status of the cell lines used for this experiment was analyzed by reverse transcription-PCR (Fig. 3). Because the primers were designed to amplify a region with three introns, a 723-bp signal would represent a cDNA sequence reverse transcribed from the Cox-2 mRNA. All three of the EAC cell lines (OE19, OE33, and TE7) showed high levels of Cox-2 mRNA comparable with that of the Cox-2-positive control (A549 lung cancer). BT474 (breast cancer) Cox-2-negative control cell line was negative for Cox-2 mRNA. Glyceraldehyde-3-phosphate dehydrogenase mRNA levels were the same among all of the cells tested, serving as a control for RNA isolation and reverse transcription-PCR procedure (Fig. 3, bottom).

The Cox-2 Promoter Is Strong and Selective for EAC. To analyze the activity of the Cox-2 promoter in Ads, two Luc expression vectors with two different lengths of the Cox-2 5' upstream control region (Cox2M and Cox2L) were tested in OE19, OE33, and TE7 EAC cells, Cox-2-negative HUVEC and BT474 cells, and primary cells isolated from normal mouse esophagus and stomach (Fig. 4). The Cox2M promoter showed significantly higher activity in EAC cells compared with the Cox2L promoter. In all three of the EAC cell lines, Cox2M and Cox2L showed high transgene expression levels relative to CMV promoter-driven Luc expression, whereas showing no significant activity in HUVEC, BT474, and primary mouse esophageal and gastric cells.

Expression of CAR and Integrins αvβ3, αvβ5, αv, β1, and α5β1 on Human EAC Cell Lines. To establish the biological basis for the relative resistance of EAC to Ad5 infection, we evaluated the level of CAR and integrins on the cell surface using antibodies specific for CAR and integrins. We found that EAC cell lines were clearly negative for CAR expression (Fig. 5). Flow cytometry also demonstrated that EAC cells express low levels of αvβ3 and αvβ5 integrins (Fig. 5), whereas αv, β1, and α5β1 integrins were present on the cell surface in rather large amounts (Table 1).

Analysis of Transduction, Binding, and Oncolytic Efficiency of RGD- and 5/3-Modified Vectors in EAC. To determine whether incorporation of an RGD-4C motif into the HI loop of the Ad fiber knob domain or replacement of the Ad5 knob with the Ad3 knob would enhance the infectivity of Ad vectors in EAC cells, three identical replication-incompetent CMV promoter-driven Luc expression vectors with wild-type (Ad5Luc1), RGD-modified (Ad5RGDLuc1), and Ad5/Ad3-chimeric (Ad5/3Luc1) fibers were used (Fig. 6). OE19, OE33, and TE7 EAC cell lines demonstrated significantly higher levels of transgene expression with the RGD-modified vector (2.7-5.7-fold higher than that of Ad5Luc1) and even greater levels with the Ad5/Ad3 chimera (5.4-87.2-fold higher transgene expression in comparison with Ad5Luc1 and 2.0-15.2-fold higher relative to Ad5RGDLuc1). The infectivity enhancements of RGD-modified and Ad5/Ad3 vectors in each cell line were consistent regardless of the viral dose during infection (50 and 500 vp/cell; data not shown).

Transduction of EAC cells with these vectors resulted in an average of 10^8 infective viral particles per cell (Table 1). For evaluation of virus-cell binding, the cells were incubated with non-selective wild-type Ad5 vectors, including AdWT (unmodified fiber), RGDWT (RGD-modified fiber), and AdMG553 (Ad5/Ad3-chimeric fiber) for 1 h at 4°C. The isolated total cellular DNA was analyzed by real-time PCR to determine the bound adenoviral DNA copy number.

Fig. 5. Flow cytometry of coxsackie-adenovirus receptor (CAR), αvβ3 integrin, and αvβ5 integrin in EAC cells. Cytofluorometry is shown for the expression of CAR (■) and integrins αvβ3 (□) or αvβ5 (△) in EAC (OE19, OE33, and TE7) and two control cell lines, A549 (integrin positive) and HepG2 (CAR positive). Unstained cells were used as a control (■).
Table 1  Expression of αv, β1, and α5β1 integrins on surface of EAC

<table>
<thead>
<tr>
<th>Integrin</th>
<th>E19</th>
<th>OE33</th>
<th>TE7</th>
<th>293*</th>
<th>A5490</th>
</tr>
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<tbody>
<tr>
<td>αv</td>
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<td>34.79</td>
<td>36.03</td>
<td>74.54</td>
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</tr>
<tr>
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<td>64.92</td>
<td>10.24</td>
<td>72.33</td>
<td>78.20</td>
<td>77.94</td>
</tr>
</tbody>
</table>

αv and α5β1 integrins positive control.
αv and α5β1 integrins positive control.

![Fig. 6](Image)

In all three of the EAC cell lines, the numbers of viral DNA copies with AdMG553 were significantly higher than that of AdWt and the RGD counterpart (Fig. 7), correlating with the gene transfer data. The RGD-modified vector showed insignificant binding in OE19 and TE7 cells, whereas no binding improvement was observed in OE33 cells.

Furthermore, we analyzed the oncolytic potency of the above nonselective wild-type vectors. We infected OE19, OE33, and TE7 cells with 0.01 vp/cell of each virus and stained the fixed surviving cells with crystal violet 12 days after infection (Fig. 8). In all of the EAC cell lines, infectivity-enhanced viruses demonstrated a stronger cytocidal effect compared with unmodified AdWt. The enhancement in cell killing effect was much more evident in cells infected with the Ad5/Ad3 chimera.

**Increased Oncolytic Efficiency of Infectivity-Enhanced Cox-2 CRAds in Vitro.** The established concept of Cox-2 promoter transcriptional targeting and Ad tropism modification were combined into a replicative virus by constructing RGD-modified and Ad5/Ad3-chimeric CRAds with a Cox-2 promoter-driven E1 expression cassette. To assess the selectivity oncolytic effect of Cox-2 CRAds, we infected EAC cell lines, Cox-2-negative cell line BT474, and human umbilical vein endothelial cells with a low titer (0.1 vp/cell) of each virus to allow multiple cycles of viral replication. In all three of the EAC cell lines, crystal-violet staining showed oncolysis after infection with all six of the CRAds, whereas the Cox-2-negative control cell line and HUVEC remained intact (Fig. 9A). CRAds with the left to right direction E1 expression cassette (CRAdCox2R, RGDCRAdCox2F, and 5/3CRAdCox2F) exhibited stronger oncolytic effect than CRAds with the reverse direction cassette (CRAdCox2R, RGDCRAdCox2F, and 5/3CRAdCox2R). RGD-modified Ads outperformed unmodified Ads, whereas chimeric vectors performed even better. The cytotoxicity of Ad5/Ad3 CRAds was similar to that of the replicative control viruses containing the wild-type early genes (Ad5Wt, RGDWt, and AdMG553); however, these Ad5/Ad3 Cox-2 CRAds maintained their replication specificity because they did not affect BT474 and HUVEC. Nonreplicative controls (Ad5Luc1, RGDCrLuc1, and 5/3Ad5Luc1) did not cause any oncolysis.

The replication and cytocidal effects of CRAds were additionally confirmed with a quantitative cell viability assay (Fig. 9B). In EAC cell lines, the quantitative cytotoxicity assay showed oncolysis with all of the Cox-2-based CRAds, whereas no cytocidal effect was observed in BT474 cells and HUVEC. In OE19, OE33, and TE7 cell lines, the percentages of surviving cells after infection with RGDCRAdCox2F compared with uninfected cells were 64.7%, 50.6%, and 81.4% (P = 0.0369, 0.0019, and 0.0338, respectively), whereas viability percentages after treatment with RGDCRAdCox2R were 77.5%, 70.4%, and 90.9% (P = 0.0231, 0.0045, and 1.746, respectively). When Ad5/Ad3 CRAds were used for infection, only 26.0%, 18.5%, and 25.1% (5/3CRAdCox2F; P = 0.0001, 0.0002, and 0.0002) and 20.8%, 37.6%, and 25.2% (5/3CRAdCox2R; P = 0.0006, 0.0002, and 0.0003) of cells remained alive, respectively. In comparison with unmodified CRAds, RGD CRAds showed unremarkable increase in killing in two of three EAC cell lines (OE19 and OE33).

In contrast, Ad5/Ad3 vectors demonstrated significantly improved oncolysis in all three of the cell lines, and this effect was superior to that of RGD-modified CRAds.

**Increased Viral Progeny Production and Selective Replication of Infectivity-Enhanced CRAds in Vitro.** We next determined whether the increased cytopathic effect of infectivity-modified CRAds correlated with increased Ad replication and progeny production. We infected OE19 (EAC cells) and BT474 (Cox-2 negative cells) with 1 vp/cell of each virus. Twelve days after infection, the cells and media were harvested, and the viral titer was determined by a plaque assay on 911 cells (Fig. 10). In OE19, RGDCRAdCox2F and RGDCRAdCox2R produced progeny titers of 1.27 × 10^6 and 2.42 × 10^6 pfu/ml, which were 3.0 and 5.7 times higher than those of their unmodified counterparts CRAdCox2F and CRAdCox2R, respectively. The final titers of the Ad5/Ad3 CRAds (5/3CRAdCox2F and 5/3CRAdCox2R) were 2.27 × 10^6 and 2.53 × 10^7 pfu/ml, respectively, which were 53.3 and 59.4 times higher relative to fiber-unmodified CRAds, and 17.9 and 10.4 times higher in comparison with RGD CRAds. This experiment also demonstrated that 5/3CRAdCox2F replicated in OE19 with an efficiency similar to that of the wild-type replicative control. In the BT474 Cox-2-negative cell...
Three cell lines: exhibited significantly improved oncolysis in all P/H11021 OE19 (modified CRAds, RGD CRAds demonstrated unremarkable samples from at least three experiments performed on each cell line. Infectivity-enhanced viruses demonstrated stronger oncolytic effect than unmodified AdWt. The increase in cell killing effect was superior with Ad5/Ad3-chimeric AdMG553.

线，the replication of Cox-2 CRAds was 200–450-fold less than in the EAC OE19 cell line, whereas nonselective wild-type viruses (AdWt, RGDWt, and AdMG553) generated higher titers of virus progeny in BT474 than in EAC cells.

Therapeutic Efficacy of Infectivity-Enhanced CRAds in Vivo. In vivo analysis of antitumor efficacy of CRAds was performed using an OE19 s.c. xenograft model in nude mice. Established tumors were treated with a single intratumoral injection of 10^10 vp of each virus, and the tumor size was monitored. On day 16 after injection, the CRAdCox2F group (relative tumor volume 5.83 ± 0.89; n = 6) and the CRAdCox2R group (5.96 ± 1.85; n = 6) showed tumor growth suppression, but the effect was not statistically significant in comparison to the untreated group (9.37 ± 6.68; n = 5) and the group that received a nonreplicative Luc expression vector (7.80 ± 3.02; n = 7; Fig. 11). Although RGDCRAdCox2F (day 16; 5.49 ± 2.06; n = 7) and RGDCRAdCox2R (day 16; 5.75 ± 2.72; n = 7) showed slightly stronger oncolytic effect than unmodified CRAds, no statistically significant difference was found between these groups and the nonreplicative Ad groups. In contrast, the sizes of the tumors treated with chimeric 5/3CRAdCox2F (day 16; 3.11 ± 2.12; n = 8) and 5/3CRAdCox2R (day 16; 4.30 ± 1.39; n = 6) were significantly reduced compared with Ad5Luc1, unmodified CRAds, and their RGD counterparts. On day 21, both Ad5/Ad3 CRAds yielded therapeutic effects that were superior to other CRAds [relative tumor volumes: 5/3CRAdCox2F (2.52 ± 0.82; n = 8) versus CRAdCox2F (7.26 ± 1.05; n = 5; P < 0.00001) and RGDCRAdCox2F (6.22 ± 3.1; n = 7; P < 0.01); and 5/3CRAdCox2R (5.17 ± 2.3; n = 6) versus CRAdCox2R (7.23 ± 4.08; n = 5; P > 0.05) and RGDCRAdCox2R (6.03 ± 1.85; n = 4; P > 0.05)]. Notably in this experiment, 5/3CRAdCox2F was even more effective than the wild-type replicative control AdMG553, which also contains the Ad5/Ad3 surface modification (4.35 ± 1.85; n = 7; P < 0.05). Thus, the in vivo data indicate that whereas the unmodified Cox-2 CRAds and the RGD counterparts demonstrated minimal in vivo therapeutic effect, the

- **Fig. 8.** Oncolytic potency of tropism modified vectors in EAC. Human EAC cell lines (OE19, OE33, and TE7) were infected with 0.01 vp/cell of AdWt, RGDWt, or AdMG553. Twelve days later, the cells were fixed and stained with crystal violet. Data are representative samples from at least three experiments performed on each cell line. Infectivity-enhanced viruses demonstrated stronger oncolytic effect than unmodified AdWt. The increase in cell killing effect was superior with Ad5/Ad3-chimeric AdMG553.

- **Fig. 9.** In vitro cytotoxicity of infectivity-enhanced cyclooxygenase (Cox)-2 conditionally replicative adenoviruses (CRAds) in EAC. A, human EAC cell lines (OE19, OE33, and TE7), Cox-2-negative cell line BT474 and human umbilical vein endothelial cells (HUVEC) were infected with CRAds at a multiplicity of infection of 0.1 vp/cell. Adenoviral cytotoxicity was analyzed by crystal violet staining. CRAds with the forward direction E1 cassette (F) exhibited stronger oncolytic effect than reverse-direction CRAds (R). RGD-modified CRAds outperformed unmodified vector, whereas Ad5/Ad3 CRAds performed even better. Data are representative samples from at least three experiments performed on each cell line. B, in parallel, cell viability was determined with a colorimetric cell proliferation assay. The results are shown as percentages of living cells remaining relative to uninfected cells. All of the Cox-2-based CRAds demonstrated oncolytic killing in all three of the EAC cell lines, whereas BT474 and HUVEC remained intact. In comparison to unmodified CRAds, RGD CRAds demonstrated unremarkable increase in killing in two of three EAC cell lines (OE19 and OE33). In contrast, Ad5/Ad3 CRAds exhibited significantly improved oncolysis in all three cell lines: P < 0.05 (±), P < 0.005 (***), P < 0.001 (**). Bars, ±SD from four experiments.
was 200-fold higher than in BT474 Cox-2-negative cell line. Twelve days after infection, cells and media were harvested to determine the viral titer by a plaque assay. In OE19 cells the replication of Cox-2 CRAds was 200–450-fold higher than in BT474 Cox-2-negative cell line. Infectivity-enhanced CRAds produced significantly higher titers of infectious particles compared with unmodified CRAds. Ad5/Ad3-chimeric CRAds outperformed RGD-modified CRAds: P < 0.005 (**), P < 0.001 (***). Bars, ±5D from triplicate experiments.

**DISCUSSION**

Advanced EAC is a highly aggressive disease with a poor long-term prognosis (3, 5, 8–10). Curative treatment consists mostly of surgery, which is not feasible for all patients; distant metastases, local irresectability, and poor condition of the patient allow only 20–30% to be potential candidates for surgical resection (63, 64). Therefore, new therapeutic approaches such as oncolytic viruses are needed to provide an effective treatment for irresectable EAC. Because EAC is easily accessible with the guidance of an endoscope, we developed our CRAd agents with the intention of applying direct intratumoral injection. However, even in the case of direct injection, ectopic transgene expression and liver toxicity remain major problems. In the setting of EAC, blood of the lower esophagus is collected into the portal vein, which flows directly to the liver. Therefore, mitigation of liver toxicity is extremely important. In this study, we applied CRAds based on Cox-2 promoter selectivity and enhanced their infectivity to develop novel therapeutic agents for EAC.

Tumor-specific promoters are actively being explored (24–32). However, many promoters, which show the desired properties in plasmid vectors, may become promiscuous in the context of a viral genome (23, 30). In our previous studies, the Cox-2 promoter was determined to be a promising tumor-specific promoter for many gastrointestinal cancers and demonstrated high levels of transgene expression in pancreatic, gastric, and colon cancers in vitro and in vivo, whereas maintaining low activity in normal tissues, including the liver (28–31). In this study, the utility of the Cox-2 promoter for EAC was initially determined by using two Luc expression vectors with two different lengths of the Cox-2 5′ upstream control region (AdCox2LLuc and AdCox2MLuc). Both vectors showed higher transgene expression levels comparable with the CMV promoter-driven vector in all of the EAC cell lines, whereas showing tumor specificity as indicated by minimal activity in HUVEC, the Cox-2-negative BT474 breast cancer cell line, and primary cells isolated from normal mouse esophagus and stomach (Fig. 4). These results were in agreement with the reverse transcription-PCR analysis, which indicated high Cox-2 mRNA transcription levels in EAC cells (Fig. 3). These findings clearly demonstrate the high activity and selectivity of the Cox-2 promoter in the tested EAC cells as well as their fidelity in an Ad backbone. Because the 1491-bp Cox2L promoter showed lower background expression in the liver compared with the 942-bp Cox2M promoter (30), we selected the Cox2L promoter for CRAd construction.

Viral replication and spread are the key factors of oncolytic efficacy. The most crucial parameter of efficient viral spread is the infection ability of the viral progeny, because transduction of surrounding cancer cells is required for amplification and oncolysis throughout the tumor (12, 52). In this regard, the natural CAR deficiency in various tumors (65) not only limits the initial infection event but also affects the potential therapeutic effect of CRAds afforded by viral replication downstream (66). We and others have reported that the efficacy of CRAds may be enhanced by incorporating a RGD-4C motif into the HI loop of the Ad fiber knob domain, which would allow the virus to bind to integrins overexpressed frequently on target cancer cells (31, 52). The native tropism of Ad vectors may also be altered by substituting the Ad5 knob with knobs from different Ad serotypes (53, 54). In particular, recent studies demonstrated that vectors displaying the Ad5 shaft/Ad3 knob were superior to Ad5.

**Fig. 10.** Increased and selective adenovirus progeny production of infectivity-enhanced conditionally replicative adenoviruses (CRAd) in EAC cells. Human EAC cell line OE19 and cyclooxygenase (Cox)-2 negative BT474 cells were infected at a multiplicity of infection of 1 vp/cell. Twelve days after infection, cells and media were harvested to determine the viral titer by a plaque assay. In OE19 cells the replication of Cox-2 CRAds was 200–450-fold higher than in BT474 Cox-2-negative cell line. Infectivity-enhanced CRAds produced significantly higher titers of infectious particles compared with unmodified CRAds. Ad5/Ad3-chimeric CRAds outperformed RGD-modified CRAds: P < 0.005 (**), P < 0.001 (***). Bars, ±5D from triplicate experiments.

**Fig. 11.** Superiority of adenovirus (Ad5/Ad3-chimeric cyclooxygenase (Cox)-2 conditionally replicative adenoviruses (CRAd) in an in vivo human esophageal adenocarcinoma model. OE19 s.c. xenografts in nude mice were treated with a single intratumoral injection of 10⁶ vp of each virus. The tumor size was measured and shown as relative volume compared with day 0. Mice from the untreated group and the groups treated with nonreplicative control viruses had to be sacrificed on day 16 due to excessive tumor size. Compared with the nonreplicative control on day 16, both Ad5/Ad3 CRAds (5/3CRAdCox2F and 5/3CRAdCox2R) significantly suppressed the size of established tumors whereas the unmodified CRAdCox2F and CRAdCox2R and RGD-modified (RGDCRAdCox2F and RGDCRAdCox2R) CRAds showed unremarkable therapeutic effect. On day 21 the chimeric 5/3Cox2CRAd showed significantly stronger antitumor effect than the unmodified, RGD-modified, and reverse-direction counterparts: P < 0.05 (*), P < 0.005 (**), P < 0.001 (***). Notably, 5/3CRAdCox2F demonstrated even stronger oncolytic effect than the wild-type replicative control AdMG553.

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vectors in infection of ovarian cancer, squamous cell carcinoma of the head and neck, and renal cancer (56–59).

Before analyzing whether RGD-modified or Ad5/Ad3-chimeric vectors would enhance Ad transduction efficiency in EAC, we first explored the biological basis of EAC refractoriness to Ad infection by evaluating human EAC cell lines for CAR and integrin expression. We found that the profound paucity of the primary Ad receptor in OE19, OE33, and TE7 cells is the major factor limiting transduction efficiency of Ad5 vectors in these cells (Fig. 5). Interestingly, the integrins αvβ3 and αvβ5, which have been described previously as predicting factors for RGD-modified virus infectivity in many gastrointestinal cancers (67), were found to be limited on the surface of EAC cells as well. On the other hand, αv, β1, and α5β1 integrins, also known to bind to the RGD motif of fibronectin (68–70), were highly expressed on EAC cells (Table 1). The presence of these receptors provides the rationale for the RGD-retargeting strategy based on the Arg-Gly-Asp motif for specific recognition of integrins. In fact, a subsequent gene transfer assay, comparing RGD-modified vectors to unmodified ones, demonstrated higher levels of transgene expression after infection with RGD vectors (Fig. 6).

The identification of the Ad3 cell surface receptor is still under investigation (55). Although it is possible to analyze the Ad3 receptor expression by flow cytometry using the Ad3 knob protein (59), a binding analysis performed with viral particles should represent functional binding during infection better than an assay using only the fiber knob of the virus. Therefore, we developed a novel approach to analyze the effect of infectivity enhancement in the context of virus-cell binding. The data obtained in this experiment demonstrated that whereas an RGD vector yielded remarkable improvements in binding, the number of bound Ad5/Ad3-chimeric virus was significantly higher than an unmodified vector and an RGD version in all three of the EAC cell lines (Fig. 7). These data suggest that the Ad3 receptor is highly expressed on human EAC cells and may be a better target for infecting EAC cells. Importantly, these binding data correlated with results from the Luc gene transfer assay (Fig. 6) and oncolytic potency experiment using the infectivity-enhanced vectors (Fig. 8).

Because promising results were obtained with both RGD and Ad5/Ad3 targeting strategies, we combined Cox-2 promoter transcriptional selectivity with infectivity enhancement by constructing RGD-modified and Ad5/Ad3-chimeric Cox-2-based CRAds. The Cox-2 promoter was incorporated into the E1 expression cassettes of these CRAds in both forward (F) and reverse (R) directions (Fig. 1). During vector amplification, the Cox-2 CRAds propagated consistently regardless of the presence of fiber modifications, and the yield was comparable with the E1-deleted vectors. The resulting viruses maintained their correct genomic structures and were free from nonselectively replicative recombinants (Fig. 2), indicating their feasibility for clinical grade vector production.

For any therapeutic agent to be clinically useful, both safety and efficacy are required. Therefore, to characterize the safety and selectivity of Cox-2 CRAds we used assays to compare their killing, replication, and progeny production potential in human EAC cells versus control Cox-2 negative cells BT474 and/or HUVECs. In vitro cytotoxicity studies revealed that the Cox-2 CRAds replicated and caused oncolysis in all three of the EAC cell lines but not in BT474 and HUVEC (Fig. 9), thus confirming the dependence of their replication on the Cox-2 promoter activity. The CRAds with the left to right direction E1 expression cassette (F) showed stronger cytotoxic effect than those with the reverse direction (R) version. Overall, the cytotoxicity of infectivity-enhanced CRAds was stronger than that of unmodified vectors. As predicted by the binding assay, the Ad5/Ad3-chimeric CRAds exhibited significantly improved oncolysis similar in degree to the wild-type control in all three of the EAC cell lines compared with the unmodified and RGD-modified vectors. We suspect that low expression of αvβ3 and/or αvβ5 integrins (known to be the secondary receptors for adenoviral internalization; Ref. 50) on EAC cells may be the reason why RGD-enhanced CRAds have been not so effective in killing EAC. To additionally characterize the oncolytic property of our CRAds, we performed a virus progeny production assay. The results indicate that in human EAC OE19 cells, the replication of Cox-2 CRAds was 200–450-fold greater than in BT474 Cox-2-negative cells, whereas nonselective wild-type replicative viruses generated similar high titers of virus progeny in both OE19 cells and in BT474 cells (Fig. 10). On the basis of the cytotoxicity and progeny production experiments, the Cox-2 CRAds maintained replication control specificity in vitro. Of note, our data seem to show a correlation between increased cytopathic effect of infectivity-enhanced CRAds and their replication and progeny production ability. Specifically, in OE19 EAC cells the RGD Cox-2 CRAds (F and R) produced 3.0 and 5.7 times higher pfu titers than their unmodified counterparts, whereas the final titers of the Ad5/Ad3 CRAds were 53.3- and 59.9-fold higher, respectively (Fig. 10). Altogether, these results clearly confirm that infectivity-enhanced Cox-2 CRAds were able to specifically replicate in and kill EAC cells in vitro and that the chimeric CRAds with Ad5 shaft/Ad3 knob were superior in transduction and replication in EAC cells.

To assess whether Cox-2 CRAds can cause oncolysis in cancer cells in vivo, the therapeutic effect of unmodified, RGD-modified, and Ad5/Ad3 CRAds was analyzed and compared in an EAC xenograft model. To allow multiple cycles of replication along with destruction of tumor cells to occur, we treated OE19 xenografts with a single s.c. injection of each virus. In vivo data revealed that on day 16, both Ad5/Ad3 CRAds (F and R) significantly suppressed the tumor growth of established OE19 xenografts, whereas their RGD counterparts showed only slightly stronger oncolysis compare to unmodified vectors, which exhibited remarkable therapeutic abilities (Fig. 11; day 16). Impressively, on day 21 the chimeric Cox-2 CRAd with the forward direction E1 cassette (5/3CRAdCox2F) statistically outperformed both unmodified CRAdCox2F and RGD-modified RGDAdCox2F, reflecting achievement of better infection with the Ad5/Ad3 fiber. Furthermore, the anti-tumor effect of the forward-direction 5/3CRAdCox2F was greater than that of the reverse-direction 5/3CRAdCox2R or wild-type replicative control virus AdMG553 (Fig. 11; day 21). The evident superiority in cytopathic effect of 5/3CRAdCox2F in vivo suggests the therapeutic utility of this agent for the treatment of EAC.

In summary, we have shown the feasibility of the Cox-2 promoter and the applicability of RGD- and Ad5/Ad3-based fiber modification for EAC. We have combined transcriptional targeting with infectivity enhancement for CRAds and demonstrated that Ad5/Ad3-chimeric Cox-2 CRAds are effective and selective therapeutic agents for EAC. Thus, these novel CRAds have great clinical potential for the treatment of EAC.

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Infectivity-Enhanced Cyclooxygenase-2-Based Conditionally Replicative Adenoviruses for Esophageal Adenocarcinoma Treatment

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