J Gastroenterol 2005; 40:31–42
DOI 10.1007/s00535-004-1490-y

Journal of Gastroenterology

Promoter-controlled infectivity-enhanced conditionally replicative adenoviral vectors for the treatment of gastric cancer

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Background. Gastric cancer is the fourth most common malignancy worldwide. Adenoviral vectors (Ads) have been applied for gene therapy of various cancers because of their high transduction efficiency. However, the infectivity of gastrointestinal cancer cells is poor due to the limited expression of the Coxsackie-adenovirus receptor (CAR). In addition, few tumor-specific promoters (TSPs) have been characterized for this type of cancer. To overcome these problems, we proposed TSP-driven conditionally replicating adenoviruses (CRAds) with fiber modification for virotherapy of gastric cancer. ***Methods.*** We assessed the expression profile of eight TSPs in gastric cancer cell lines and evaluated promising candidates in the context of CRAd cytocidal effect. Next, infectivity enhancement by fiber modifications was analyzed in the gastric cancer cell lines. Finally, we combined the TSP-driven CRAds of choice with the fiber modifications to augment the killing effect. ***Results.*** Out of the eight TSPs, the midkine (MK) and cyclooxygenase-2 (Cox-2M and Cox-2L) promoters showed high transcriptional activity in gastric cancer cells. When these promoters were used in a CRAd context, Cox-2 CRAds elicited the strongest cytocidal effect. The greatest infectivity enhancement was observed with adenoviral vectors displaying 5/3 chimeric fibers. Likewise, Cox-2 CRAds with 5/3 chimeric fibers showed the strongest cytocidal effect in gastric cancer cell lines. Therefore, Cox-2 CRAds with 5/3 chimeric fiber modification showed good selectivity and infectivity in gastric cancer cells to yield enhanced oncolysis. ***Conclusions.*** Cox-2 CRAds with 5/3 chimeric fiber modification are promising for virotherapy of gastric cancer.

Key words: gastric cancer, conditionally replicative adenoviral vectors, tumor-specific promoter, fiber modification, cytocidal effect

Received: December 7, 2003 / Accepted: August 6, 2004
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Introduction

Gastric cancer is the fourth most common malignancy worldwide.[1] In 2000, 870000 people were diagnosed with the disease, while 650000 patients died from it.[1,2] In most countries, patients diagnosed with gastric cancer have advanced-stage disease[3,4] leading to a poor prognosis. While the overall 5-year survival rate of gastric cancer patients is about 20%,[1,3,4] that of patients with distant metastases is usually less than 5%.[3,4] For patients with advanced-stage gastric cancer, neoadjuvant or adjuvant chemotherapy with 5-fluorouracil, doxorubicin, and cisplatin is currently under investigation.[1,3] Despite the use of the latest chemotherapy, advanced gastric cancer is still difficult to treat. These issues indicate the necessity for the development of a new therapeutic modality, and gene therapy represents one of those developmental efforts.

Adenoviral vectors (Ads) have been applied for gene delivery of various cancers because of their high efficiency. However, in some fields, including gastrointestinal cancer, the efficiency of adenoviral vector-based gene delivery is extremely limited, due to low expression of the Coxsackie-adenovirus receptor (CAR). We have endeavored to overcome this problem by applying virotherapy with conditionally replicating adenoviruses (CRAds) and fiber modification.

CRAds were designed to selectively replicate in tumor cells.[5–8] Two basic strategies have been used to enable selective replication. Mutation-type CRAds, such as ONYX-015 or AdΔ24, can replicate in tumor cells that have p53[9,10] or retinoblastoma protein mutations, respectively.[11] Promoter-controlled CRAds are

controlled with tumor-specific promoters (TSPs) to drive the expression of an essential gene (e.g., *E1a*) required for Ad replication.[12–16] In terms of safety, it is important to avoid vector toxicity in normal organs caused by the ectopic expression of an effector gene, or nonspecific replication in the case of CRAds. Because the liver clears the majority of adenoviruses administered systemically, avoiding hepatotoxicity is very important. This dilemma is especially relevant in gastric cancer, because blood flow from the stomach goes directly into the portal vein and flows to the liver.[17] Thus, for promoter-controlled CRAds, a "tumor-on/liver-off" promoter profile is needed to achieve liver-untargeting and avoid liver toxicity.

We have reported eight "tumor-on/liver-off" TSPs for adenoviral gene therapy. Vascular endothelial growth factor receptor-1 (fms-like tyrosine kinase-1; Flt-1) promoter was reported in teratocarcinoma.[18,19] Midkine (MK) promoter was tested in pediatric solid tumors,[20,21] ovarian cancer,[22] pancreatic cancer,[23] and cholangiocarcinoma.[24] Cyclooxygenase-2 (Cox-2) promoters were reported in gastrointestinal cancers.[22–25] Secretory leukocyte protease inhibitor (SLPI) promoter was used in ovarian cancer.[26,27] Vascular endothelial growth factor (VEGF) promoter and gastrin-releasing peptide (GRP) promoter were reported in lung cancer and cholangiocarcinoma.[24,28] In adenoviral vectors, all of these promoters demonstrated "tumor-on/liver-off" selectivity for each target. Particularly for gastric cancer, only several TSPs have been reported. However, a side-by-side evaluation has not been done for these promoters. Precise comparison of these promoters is necessary to determine the optimal promoter for gastric cancer CRAd construction.

Another issue to address regarding the use of adenoviral vectors for gastric cancer is infectivity enhancement. We have achieved increased infectivity of CAR-negative cells, which is the case for gastric cancer, by modifying the fiber region of adenoviral vectors. We have reported that both an RGD-4C motif incorporation in the HI-loop of the adenoviral fiber-knob region[29–32] and replacement of the adenovirus type 5 knob with that of adenovirus type 3[24,33–37] showed improvement in infectivity for low-CAR expressing cell lines. These fiber modifications represent potential strategies to augment adenoviral transduction in gastric cancer cells and would therefore enhance the efficiency of CRAds against this type of tumor.

In this study, we evaluated promoters for CRAd replication control and fiber modifications for the enhancement of CRAd efficacy in gastric cancer. Our data establish the utility of infectivity-enhanced TSP-driven CRAds as candidate therapeutic agents for gastric cancer.

Materials and methods

Cell culture

MKN1, MKN28, MKN45, MKN74, NUGC4 (Human Science Research Resources Bank JCRB0252, JCRB0253, JCRB0254, JCRB0255, JCRB0834, Osaka, Japan), MKN7 (Riken Cell Bank RCB0999, Ibaraki, Japan), and STKM1 (generously provided by Shunsuke Yanoma (Kanagawa Cancer Center, Research Institute, Yokohama, Japan) gastric cancer cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Mediatech; Herndon, VA, USA) with 10% fetal calf serum (HyClone; Logan, UT, USA). BT-474 (Cox-2-negative breast cancer cell line; American Type Culture Collection [ATCC], HTB-20) was cultured in RPMI 1640 with bovine insulin (0.01 mg/ml; Life Technologies, Rockville, MD, USA). LS 174T (MK-negative colon cancer cell line; ATCC, CL-188) and Hep G2 (SLPI-negative hepatocellular carcinoma cell line; ATCC, HB-8015) were maintained with Dulbecco's modified Eagle's medium (Mediatech) containing 10% fetal calf serum. BEAS-2B (VEGF-negative and Flt-1-negative normal human bronchial epithelial cell line; ATCC, CRL-9609) was maintained with BEGM BulletKit (Cambrex, Walkersville, MD, USA). All media, except for BEGM BulletKit were supplemented with penicillin (100 IU/ml), and streptomycin (100 mg/ml). Cells were incubated at 37°C and 5% CO_2 under humidified conditions.

Adenoviral vectors

Non-replicative adenoviral vectors expressing the firefly luciferase gene (*Luc*) under the cytomegalovirus promoter (AdCMVLuc), cox2M promoter (AdCox2-MLuc),[23–25] cox2L promoter (AdCox2LLuc),[23–25] MK promoter (AdMKLuc),[20,21,23,24,38] GRP promoter (AdGRPLuc),[28,39,40] VEGF promoter (AdVEGFLuc),[23,41] SLPI promoter (AdSLPILuc),[26,27] and Flt-1 promoter (AdFlt-1Luc)[18,19] have been reported previously. CRAdCox2F, MKE1, and GRPE1 have E1 expression cassettes controlled by the cox2, MK, and GRP promoters, respectively. Ad5RGDLuc1 has an RGD motif in the HI-loop of the fiber-knob region, while Ad5/3Luc1 has a replacement of its adenovirus type 5 knob with an adenovirus type 3 knob. Both viruses are nonreplicative, and contain a cytomegalovirus (CMV)-driven Luc-like unmodified AdCMVLuc. Similarly, RGDCRAdCox2F and 5/3CRAdCox2F are cox2 promoter-controlled CRAds with RGD fiber modification and 5/3 chimeric fiber modification respectively. Wild-type Ad5 viruses without fiber modification (Ad5Wt), with RGD fiber modification (RGDWt), and with 5/3 chimeric fiber modification (AdMG553) were utilized as controls.

All adenoviral vectors were constructed by homologous recombination in *Escherichia coli*,[42] using shuttle vectors constructed from pShuttle and pGL3-basic (Promega, Madison, WI, USA). Thus, all vectors have the same backbone except for the TSPs, E1 region, or fiber.

The viruses were propagated in E1-transcomplementing 911 cells and 293 cells, purified by ultracentrifugation in double cesium chloride density gradient, and subjected to dialysis. The titers for the viruses were determined by optimal absorbance measurement of dissociated virus DNA at $A_{260\,nm}$ and by using a plaque-forming assay. The viral particle (vp)/plaque forming unit (pfu) ratios for these vectors were within the range of 20–100.

Analysis of promoter activity

To assess the activity of the TSPs in an adenoviral context, 5×10^4 cells of each gastric cancer cell line per well were plated on 24-well plates and cultivated overnight. The next day, cells were infected at 50 pfu/cell with AdMKLuc, AdCox2MLuc, AdCox2LLuc, AdVEGFLuc, AdSLPILuc, AdGRPLuc, Ad Flt-1Luc, or AdCMVLuc in Dulbecco's modified Eagle's medium with 5% fetal calf serum (infection medium). Two hours later, the infection medium was replaced with the appropriate growth medium. After 48 h of infection, the cells were lysed by Cell Lysis Buffer (Promega) and the resultant lysates were analyzed with the Luciferase Assay System (Promega). The protein concentration was determined with the DC protein assay (Bio-Rad, Hercules, CA, USA).

In vitro analysis of the cytocidal effect of TSP-driven CRAds

To analyze the cytocidal effect of TSP-driven CRAds, cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Gastric cancer cell lines, BT-474 (Cox-2-negative cell line), and LS 174T (MK-negative cell line) were plated on 96-well plates at a density of 3×10^3 cells per well. After overnight culture, cells were infected with nonreplicative control (AdCMVLuc), CRAds (CRAdsCox2F, MKE1, GRPE1), and Ad5 wild-type (Ad5Wt) in infection medium at 0.1 vp/cell for 2 h. Then, the infection medium was replaced with the appropriate growth medium. An MTS assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) was performed on days 0, 1, 3, 6, 10, and 15 after infection.

Flow cytometry for CAR and integrin expression in gastric cancer cell lines

Cells were dislodged with 1% trypsin/ethylene diaminetetraacetic acid (EDTA) and resuspended in SM buffer (phosphate-buffered saline [PBS], 0.1% sodium azide, 1% bovine serum albumin [BSA]) at 3×10^5 cells/mL. One million cells were incubated with 1.5 μg/ml of mouse anti-human CAR monoclonal antibody, RmcB[43](ATCC), mouse anti-human integrin αvβ3 monoclonal antibody, LM609 (Chemicon International, Temecula, CA, USA), and mouse anti-human integrin αvβ5 monoclonal antibody, P1F6 (Chemicon International), or mouse immunoglobulin (negative control) in 100 μl of SM buffer for 1 h at 4°C. Cells were washed with SM buffer twice and incubated with 1.5 μg/ml of secondary fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G serum (Jackson Labs, West Grove, PA, USA) in 100 μl of SM buffer for 30 min at 4°C. After washing the cells with SM buffer twice, 1×10^4 cells were analyzed by flow cytometry.

Infectivity enhancement with fiber modification

To analyze infectivity enhancement with fiber modification, luciferase expression adenoviral vectors with or without fiber modification were applied. The day before infection, 5×10^4 gastric cancer cells per well were plated in 24-well plates. The next day, cells were infected with Ad5Luc1, Ad5RGDLuc1, and Ad5/3Luc1 at 50 vp/cell in infection medium. After 2-h incubation, the infection medium was replaced with the appropriate growth medium. Forty-eight hours later, the cells were lysed and lysates were analyzed for luciferase activity. The protein concentration was determined with the DC protein assay (Bio-Rad).

In vitro analysis of cytocidal effect of fiber-modified TSP-driven CRAds

Cytocidal effect was analyzed in vitro by staining the cells with crystal violet. One day after 2.5×10^4 cells per well were plated on a 12-well plate, cells were infected with or without fiber-modified Cox-2 CRAds at 0.01 vp/cell and controls in infection medium. The viruses included the nonreplicative controls AdCMVLuc, Ad5RGDLuc1, and Ad5/3Luc1; the wild-type controls Ad5Wt, RGDWt, and AdMG553 (with 5/3 chimeric fiber); and CRAdCox2F, RGDCRAdCox2F, and 5/3CRAdCox2F. Two hours after infection, the infection medium was replaced with the appropriate growth medium. When total cell death was observed by microscope for cells infected with Cox-2 CRAds with or

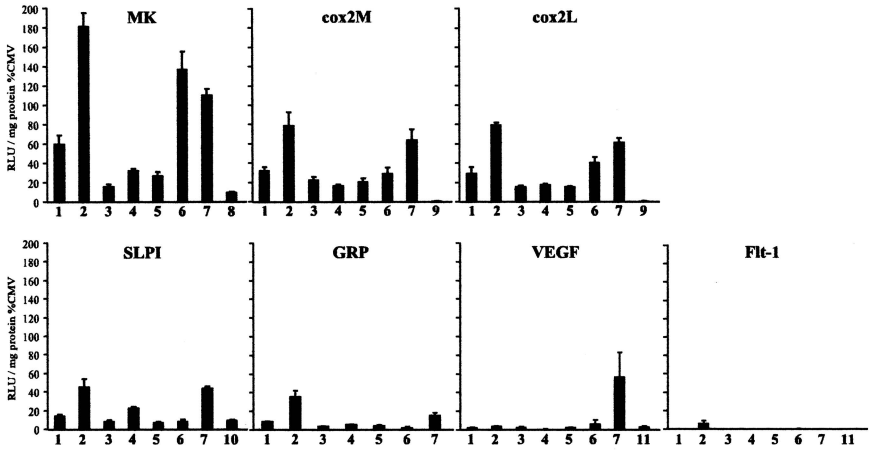

Fig. 1. Promoter activities in gastric cancer cell lines. Each cell line was infected with tumor-specific promoter (TSP)-driven luciferase vectors at 50 pfu/cell and the luciferase activity was analyzed 48 h after infection. The results are standardized by protein concentration and shown as percentages relative to cytomegalovirus (*CMV*) promoter luciferase activity. *1*, midkine [*MK*]N1; *2*, MKN7; *3*, MKN28 (gastrin-releasing peptide [*GRP*]-negative cell line); *4*, MKN45; *5*, MKN74; *6*, NUGC4; *7*, STKM-1 (gastric cancer cell line); *8*, LS174T (MK-negative colon cancer cell line); *9*, BT474 (cyclooxygenase 2 [*Cox2*] negative breast cancer cell line); *10*, HepG2 (secretory leucocyte protease inhibitor [*SLPI*]-negative hepatocarcinoma cell line); *11*, BEAS-2B (vascular endothelial growth factor [*VEGF*]- and fms-like tyrosine kinase-1 [*Flt-1*]-negative cell line)

without fiber modification, the cells were fixed with buffer-formaldehyde for 10 min and stained with 1% crystal violet in 70% ethanol. Stained cells were washed with tap water three times and dried.

In vivo antitumor effect analysis

Antitumor effect was analyzed in vivo by using MKN28 gastric cancer subcutaneous xenografts. The cells (3×10^6 per injection site) were inoculated into the flanks of female BALB/cAnNCr-nu/nu mice (Frederick Cancer Research, Frederick, MD, USA) (6–8 weeks of age). After tumor establishment (6- to 8-mm diameter), a single virus dose (5×10^9 vp) of CRAds and control vectors was injected intratumorally. The condition of mice was monitored daily and the tumor diameter was measured every 3 days. The tumor volume was calculated as: tumor volume = width^2 × length/2. The mice with PBS and AdCMVLuc were killed 30 days after viral injection, due to over-sized tumors which cannot be maintained according to animal protocol guidelines.

Results

Cox-2M, Cox-2L, and MK promoters are promising for gastric cancer cell lines

To optimize CRAds for gastric cancer, we first compared the transcriptional activites of the candidate TSPs. Compared to the luciferase activity of the CMV promoter, those of MK (80.6 ± 63.3%), Cox-2M (38.0 ± 24.0%), and Cox-2L (37.1 ± 25.0%) promoters showed high levels. On the other hand, SLPI (21.6 ± 16.8%), GRP (11.3 ± 11.9%), VEGF (10.9 ± 20.4%), and Flt-1 (1.0 ± 2.3%) promoters had minimal activities (Fig. 1).

Cox-2, MK, and GRP CRAds show significant cytocidal effect in gastric cancer

We investigated the cytocidal effect of TSP-driven CRAds in gastric cancer cell lines. Viable cells infected with MKE1, CRAdCox2F (promising promoter-driven CRAd), and GRPE1 (weak promoter-driven CRAd) were quantitated with an MTS assay. Ad5Wt (adenovirus type 5 wild-type) and AdCMVLuc (nonreplicative adenovirus) were used as positive and negative controls, respectively. CRAdCox2F showed significant cytocidal effect in four gastric cancer cell lines (MKN28, MKN45, NUGC4, and STKM1) while MKE1 showed cell killing in three cell lines (MKN28, MKN45, and STKM1). GRPE1 caused a cytocidal effect in two gastric cancer cell lines (MKN45 and STKM1). None of the tested CRAds showed much cell killing in MKN1, MKN74, or MKN7 by MTS assay (Fig. 2). Cox-2 CRAd demonstrated no cytocidal effect in negative control cell lines, while MK and GRP CRAds killed negative cells on day 10 (data not shown). In the context of the in vitro cytocidal effect, Cox-2 CRAd was the most promising for

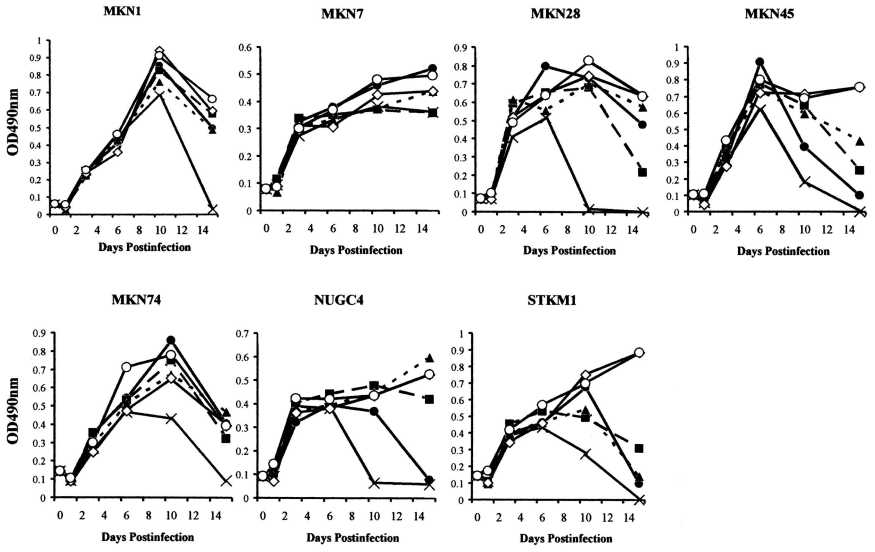

Fig. 2. Cytocidal effect of conditionally replicating adenoviruses (CRAds). Cytocidal effect was analyzed by quantitating viable cells, using an MTS assay. Each cell line was infected with TSP-driven CRAds at 0.1 vp/cell and assayed over the course of 15 days. The conversion of MTS into formazan was measured by absorbance (optical density; *OD*) at 490 nm. CRAdCox2F (*closed circles*) showed a cytocidal effect in four out of seven cell lines (MKN28, MKN45, NUGC4, and STKM1). MKE1 (*closed squares*) demonstrated cell killing in three out of seven cell lines (MKN28, MKN45, and STKM1). GRPE1 (*closed triangles*) showed a cytocidal effect in only two cell lines (MKN45 and STKM1). There was no significant difference between no-virus (*open circles*) and CRAds in MKN1 and MKN74. No killing effect was seen in MKN7 until day 15. *Crosses*, AdWt300; *open diamonds*, AdCMVLuc

gastric cancer, while MK and GRP CRAds also showed considerable cytocidal effect, but less selectivity.

Various levels of CAR expression in gastric cancer cell lines

Because the expressions of CAR and integrins are the major determining factors of adenoviral gene transfer efficiency, their levels were analyzed by flow cytometry in gastric cancer cell lines. MKN1 (1.36%), MKN7 (5.57%), MKN28 (9.16%), MKN74 (4.20%), NUGC4 (2.89%), and STKM1 (8.06%) showed low CAR expression (thick solid lines in Fig. 3a), while MKN45 (44.72%) indicated intermediate CAR expression (Fig. 3a). Expression of αvβ3 integrin (thin solid lines in Fig. 3b) was shown to be low in MKN7 (1.65%), MKN28 (7.45%), MKN45 (1.76%), MKN74 (1.64%), NUGC4 (1.29%), and STKM1 (1.25%), while expression was moderate in MKN1 (21.33%). MKN1 (0.78%), MKN45 (3.37%), MKN74 (6.68%), and NUGC4 (3.80%) showed low expression of αvβ5 integrin (thick solid lines in Fig. 3b), while MKN7 (16.10%), MKN28 (19.94%), and STKM1 (11.60%) showed moderate expression (Fig. 3b).

5/3 Chimeric fiber modification improves infectivity in gastric cancer

Infectivity enhancement in gastric cancer cells by fiber modification (RGD modification and 5/3 chimera) was analyzed by a luciferase assay. The luciferase activity of Ad5/3Luc1 was dramatically higher than that of Ad5Luc1 in seven out of seven gastric cancer cell lines ($P < 0.05$). On the other hand, enhancement by RGD modification was minimal. While the luciferase activity of Ad5RGDLuc1 was significantly higher in MKN45 (1.95-fold) and NUGC4 (1.93-fold; $P < 0.05$), there was no significant increase in other gastric cancer cell lines (Fig. 4). These results suggest that 5/3 chimeric fiber modification can significantly increase infectivity in gastric cancer compared to both RGD-modification and no modification.

5/3CRAdCox2F shows significant cytocidal effect in gastric cancer cell lines in vitro

To analyze the enhanced oncolytic potency due to fiber modification, gastric cancer cell lines were infected with TSP-driven CRAds with and without fiber modification and then stained with crystal violet (Fig. 5). 5/3CRAdCox2F showed the strongest cytocidal effect in six out of seven gastric cancer cell lines (MKN1,

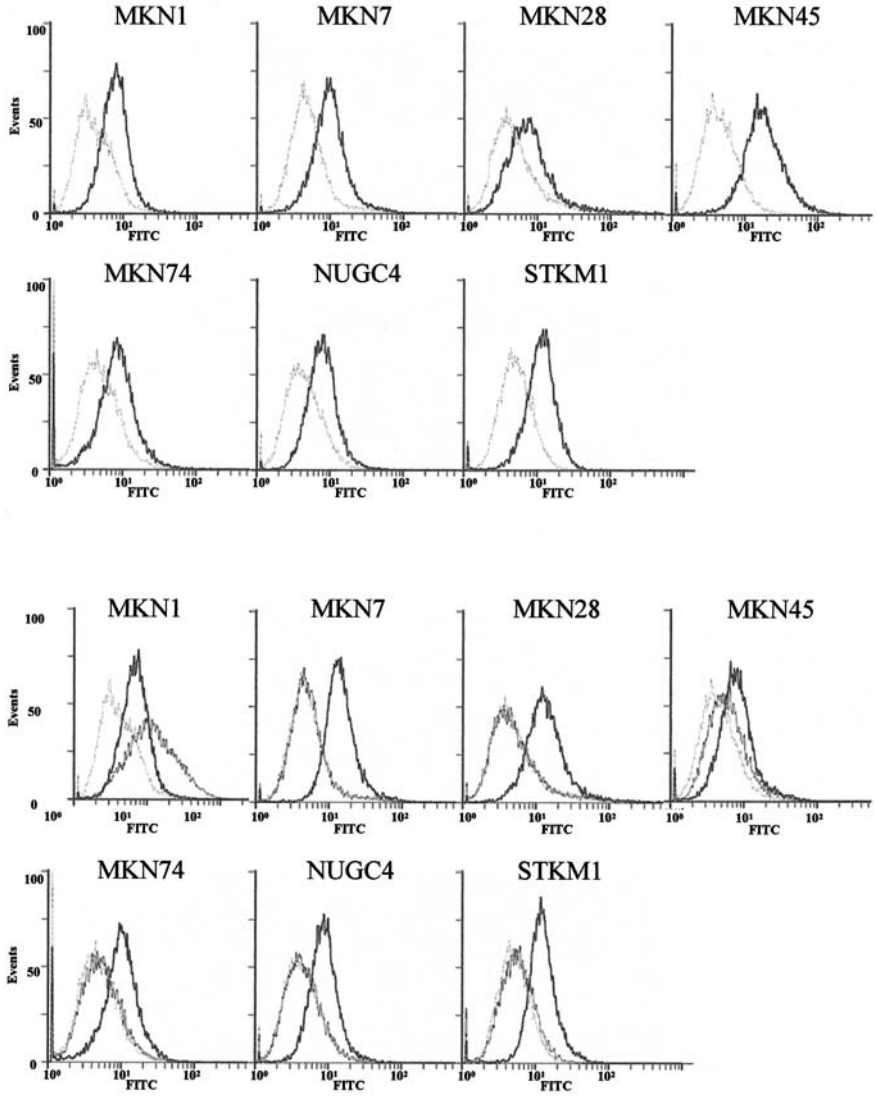

Fig. 3a,b. Expression of **a** Coxsackie-adenovirus receptor (CAR) and **b** αvβ3 and αvβ5 integrin in gastric cancer cell lines. Receptor expression levels were analyzed by flow cytometry. Cells were incubated with anti-CAR, anti-αvβ3, or anti-αvβ5 integrin monoclonal antibodies. **a** Expression of CAR (*solid lines*) was low (1.4%–9.2%) in six out of seven cell lines (MKN1, MKN7, MKN28, MKN74, NUGC4, and STKM1), but moderate (45%) in one cell line (MKN45). **b** Expression of αvβ3 integrin (*thin solid lines*) was extremely low (1.3%–7.5%) in six out of seven cell lines (MKN7, MKN28, MKN45, MKN74 NUGC4, and STKM1) but moderate (21.33%) in MKN1. Expression of αvβ5 integrin (*thick solid lines*) was low (0.8%–6.8%) in four cell lines (MKN1, MKN45, MKN74, and NUGC4) and moderate (12%–20%) in three cell lines (MKN7, MKN28, and STKM1). *Dotted lines*, negative control; *FITC*, fluorescein isothiocyanate

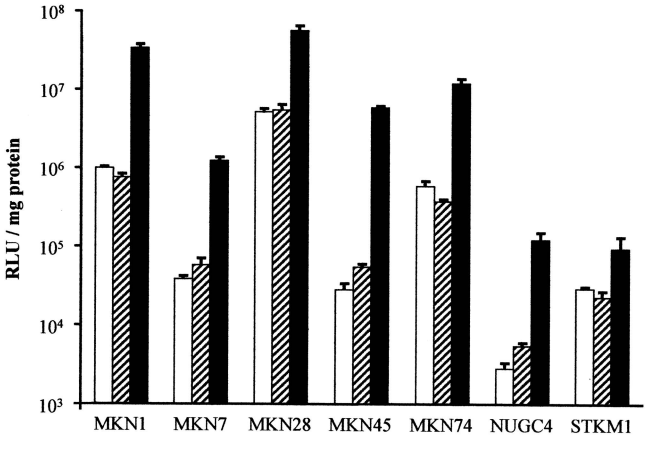

Fig. 4. Infectivity enhancement in gastric cancer cells by genetic fiber modification. The effect of infectivity enhancement was analyzed by using CMV promoter-driven luciferase expression vectors with unmodified, RGD modified, and 5/3 chimeric fiber. Two days after infection at 50 vp/cell, the cells were analyzed for luciferase expression. In all gastric cancer cell lines, the 5/3 chimeric vector (Ad5/3Luc1; *black bars*) showed 3.2- to 208-fold more transduction compared with fiber-unmodified vector (Ad5Luc1), while the RGD-modified vector (Ad5AGDLuc1; *striped bars*) demonstrated 0.7–1.9-fold increase in infectivity. *White bars*, Ad5Luc1

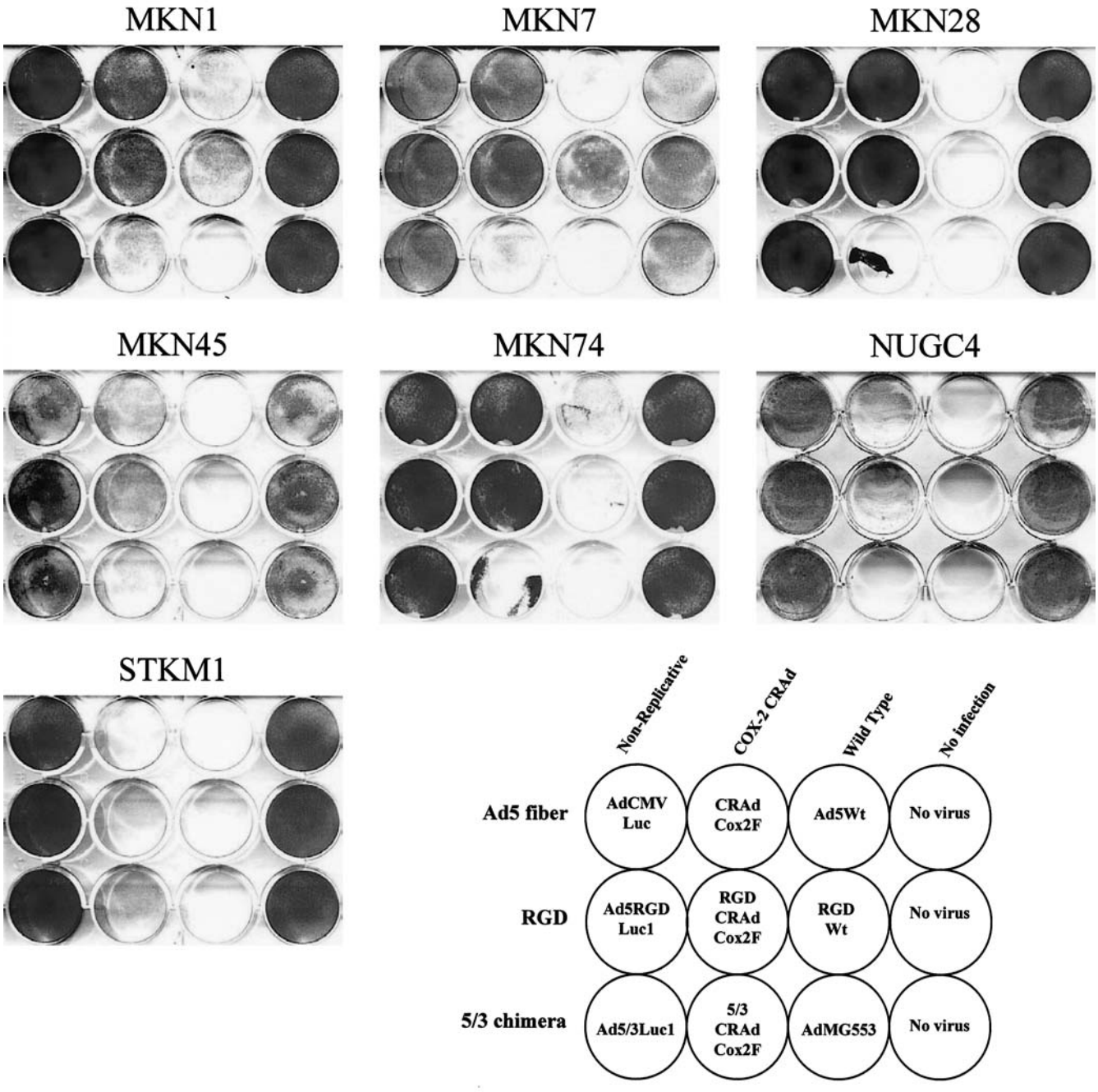

Fig. 5. Cytocidal effect of fiber-modified CRAds in gastric cancer cells. The cytotoxicity of Cox-2 CRAds was analyzed by crystal violet staining. E1-deleted vectors (AdCMVLuc, Ad5RGDLuc1, and Ad5/3Luc1; nonreplicative) did not show any cell killing. The wild-type vectors (Ad5Wt, RGDWt, and AdMG553) killed all cells. Cox-2 CRAds with 5/3 chimeric fiber showed the strongest cytocidal effect in six out of seven gastric cancer cell lines (MKN1, MKN7, MKN28, MKN45, MKN74, and NUGC4), while demonstrating weaker cytocidal effect in only one cell line (STKM1). Cox-2F CRAd with RGD-modified fiber showed a cytocidal effect similar to that of unmodified Cox-2F CRAd

MKN7, MKN28, MKN45, MKN74, and NUGC4). RGDCRAdCox2F demonstrated minimal enhancement of the cytocidal effect, which was comparable to unmodified CRAdCox2F, in all gastric cancer cell lines. With respect to infectivity enhancement to improve cell killing by TSP-driven CRAds, 5/3 chimeric fiber modification showed the strongest cytocidal effect in gastric cancer cell lines.

5/3CRAdCox2F shows significant antitumor effect in vivo

The in vivo antitumor effect of fiber-modified CRAds was analyzed by using MKN28 gastric cancer subcutaneous xenografts. After establishment of the tumor (6- to 8-mm diameter), the CRAds and control viruses (5×10^9 vp) were injected into the tumors, and the tumor size was monitored. On day 30, the groups with RGDCRAdCox2F and 5/3CRAdCox2F showed significant antitumor effect ($P < 0.01$; analysis of variance [ANOVA]), while the therapeutic effect of Cox2CRAd without fiber modification (CRAdCox2F) was not statistically significant ($P > 0.05$; ANOVA) in comparison to the groups with untreated and nonreplicative luciferase expression vector. On day 60, both the RGDCRAdCox2F and 5/3CRAdCox2F groups showed more antitumor effect than the CRAdCox2F group. Relative to CRAdCox2F, 5/3CRAdCox2F demonstrated a stronger antitumor effect ($P < 0.01$; ANOVA) in contrast to RGDCRAdCox2F, which showed no significant difference ($P > 0.05$; ANOVA). Interestingly, the group with 5/3CRAdCox2F showed a stronger antitumor effect than that seen with the wild-type one, and this was the only group to show statistically significant shrinkage on day 60 in comparison with day 0 ($P < 0.01$; ANOVA; Fig. 6). The in vivo data indicate that, while the unmodified and RGD-modified Cox-2 CRAd demonstrated minimal killing effect, Cox-2 CRAd with 5/3 fiber modification showed a remarkable antitumor effect.

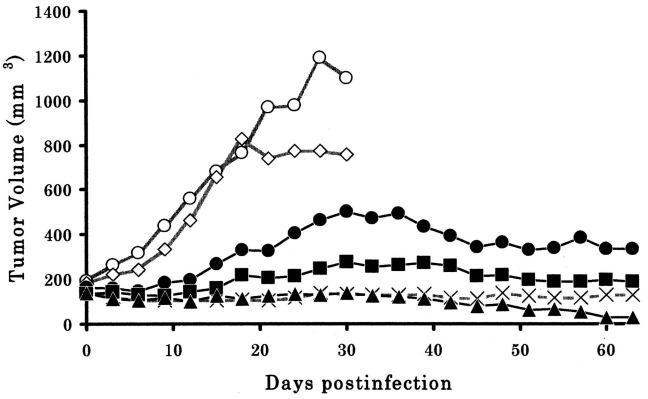

Fig. 6. In vivo antitumor effect of fiber-modified CRAds in a gastric cancer model. MKN28 subcutaneous xenografts in nude mice were treated with a single intratumoral injection of 5×10^9 vp of each virus. Mice treated with phosphate-buffered saline (PBS; *open circles*) and nonreplicative control viruses were killed on day 30 due to over-sized tumors. Compared to the nonreplicative control (AdCMVluc; *open diamonds*) on day 30, Cox-2 CRAds with unmodified fiber (CRAdCox2F; *closed circles*) showed no statistically significant effect ($P > 0.05$; analysis of variance [ANOVA]), while RGDCRAdCox2F (*closed squares*) and 5/3CRAdCox2F (*closed triangles*) demonstrated stronger antitumor effect, with statistical significance ($P < 0.01$; ANOVA). RGDCRAdCox2F showed a stronger antitumor effect ($P > 0.05$; ANOVA) compared to Cox-2 CRAds with unmodified fiber, while 5/3CRAdCox2F induced a much stronger, statistically significant antitumor effect ($P < 0.01$; ANOVA). The group with 5/3CRAdCox2F was the only group showing statistically significant tumor shrinkage on day 60 compared with day 0 tumor size ($P < 0.01$; ANOVA). *Crosses*, AdWt

Discussion

CRAds represent a novel and promising approach for treating neoplastic diseases.[7,44,45] One of the advantages of CRAds is the minimal initial dose required, because progeny virus produced by selective replication would spread throughout the tumor.[6] To achieve this effect, CRAds were designed to replicate only in tumor cells, leaving normal tissues intact. These strategies include mutation-type CRAds and promoter-controlled-type CRAds.[5] The Δ24 adenovirus and ONYX-015 are examples of the former type, which show tumor killing effect in some cancers.[10,11,46–57] However, the stringency of replication control in these CRAds is still under discussion.[58–61] In this study, because we aimed to increase the infectivity of adenovirus by fiber modification and, therefore, enhance cell killing, tight replication control was critical to maintain a good safety profile. For this purpose, promoter-controlled type CRAds may offer advantages, especially in light of the fact that well-characterized TSPs have been investigated in adenoviral cancer gene therapy to avoid damage to normal host tissues, especially the liver. While numerous studies have employed these TSPs in a CRAd context for various cancers, the application of TSP-CRAds for gastric cancer is limited.[62] In this sense, further investigation of CRAds as therapeutic agents is needed in this field.

In this study, we investigated candidate TSPs to control CRAd replication and fiber modifications to enhance the infectivity of CRAds for gastric cancer. The carcinoembryonic antigen and epithelial glycoprotein-2 promoters are the only TSPs in adenovirus reported for gastric cancer,[63–69] in the contrast to the many TSPs reported for other cancers. We have reported that the Flt-1, MK, Cox-2, SLPI, VEGF, and GRP promoters in adenovirus showed liver-off and tumor-on activity in malignant tumors. Our reported promoters therefore merited evaluation for the treatment of gastric cancer because of their strong activities and their documented overexpression in gastric cancer (Flt-1 in 84.6% of cases,[70] MK in 67% of cases,[71,72] and Cox-2 in 70% of cases[73–75]).

With regard to promoter activity in gastric cancer cell lines, the MK, Cox-2M, and Cox-2L promoters showed high expression levels, justifying further investigation of

these promoters in CRAds (Fig. 1). Notably, the Cox-2M and Cox-2L promoters demonstrated minimal activity in a Cox-2-negative cell line (BT474). CRAdCox2F showed strong cytocidal effect and selectivity in four gastric cancer cell lines, while MKE1 showed cell killing in three gastric cancer cell lines, based on MTS assay results. The cytocidal effect of TSP-CRAds was roughly correlated with the predetermined cell line specific promoter activity, except for MKN 45, which responded differently to the viruses, possibly due to high CAR expression (Fig. 3a). Crystal violet assays correlated with the MTS assay data for both CRAdCox2F (Fig. 5) and MKE1 (data not shown). It is important to note that although MKE1 was almost as effective as CRAdCox2F in terms of oncolytic effect in gastric cancer cells, the promoter selectivity of MK was poor relative to Cox-2, as evidenced by its higher activity in the liver[20,25] and by its nonspecific killing of MK-negative cells (data not shown). This observation rationalized our choice of Cox2 CRAds as the most promising TSP-driven CRAds for gastric cancer among those tested and, therefore, justified the further exploration of these agents for infectivity enhancement.

The infectivity of CRAds is one of the most important factors determining therapeutic efficiency, because effective infection of the surrounding cancer cells by the progeny virus is crucial for the spread of CRAds. For adenoviral infection, the Coxsackie-adenovirus receptor (CAR) level of the cell is a crucial factor for virus binding. Most of the gastric cancer cell lines we tested showed very limited expression of CAR (Fig. 3a). Another factor that affects adenoviral infection is the availability of αvβ3 and αvβ5 integrins. In most of the gastric cancer cell lines examined, αvβ3 integrin expression was low, but αvβ5 integrin was moderately expressed in three gastric cell lines (Fig. 3b). For low-CAR expressing gastric cancer cells, infectivity improvement is needed to achieve effective tumor killing.

We have previously reported that adenoviral vectors with an RGD-4C motif in the HI-loop of the fiber knob region enhanced infectivity.[29–32] These data indicate that RGD fiber modification can possibly increase the infectivity of adenoviral vectors in gastric cancers. In two out of seven gastric cancer cell lines in the present study, an RGD-modified vector showed infectivity improvement, but the enhancement was minimal. The limited expression of αvβ3 and αvβ5 integrins in gastric cancer cells (Fig. 3b) may explain this observation.[31] Interestingly, the above results showed a discrepancy between the expression of integrins and the infectivity improvement of RGD-modified virus; however, there are some other factors (such as heparan sulfate glycosaminoglycans[76,77]) which may influence the infectivity of adenovirus. We have also demonstrated that adenoviral vectors with their type 5 knob replaced with a type 3 knob can greatly improve infectivity.[33–37] In contrast to RGD fiber modification, 5/3 chimeric fiber modification dramatically improved infectivity in all of the gastric cancer cells (Fig. 4).

Based on the encouraging results obtained with 5/3 chimeric fiber modification, we tested TSP-driven CRAds with and without fiber modification to achieve enhanced killing efficiency. In this experiment, 5/3CRAdCox2F showed a dramatic improvement in cytocidal effect in six out of seven gastric cancer cell lines (Fig. 5). STKM1 was the only cell line which showed low cytocidal effect with 5/3CRAdCox2F compared to RGDCRAdCox2F. This result is reasonable, because STKM1 was the only cell line which demonstrated less infectivity enhancement by 5/3 fiber modification compared to the other cell lines (Fig. 4).

We further investigated the 5/3 fiber modified Cox2 CRAds in vivo. In a subcutaneous xenograft model of gastric cancer, the therapeutic effect of unmodified, RGD-modified, and 5/3-modified CRAds was analyzed after a single intratumoral injection. On day 30, the group with RGDCRAdCox2F and 5/3CRAdCox2F showed significant therapeutic effect compared with unmodified CRAdCox2F. However, there were no significant differences between the fiber-modified Cox2CRAds and Ad5 wild type. On day 60, all replicative vectors suppressed the growth of established gastric cancer tumors. However, 5/3CRAdCox2F was the only vector which elicited significant tumor shrinkage, whereas none of the other vectors, including Ad5Wt, showed such an effect. In vivo, the oncolytic effect due to infectivity enhancement with 5/3 fiber modification was much more evident than that observed in the in vitro experiments. This suggests that 5/3 fiber modification confers a clear benefit for the antitumor effect of Cox2CRAds (Fig. 6).

Our strategy of using 5/3 fiber-modified Cox-2 CRAds addresses two key issues related to the clinical utility of adenoviral vectors; efficiency and safety. First, the use of the optimal Cox-2 promoter to drive CRAd replication yielded a strong killing effect in gastric cancer cells. This oncolytic effect was further enhanced by the use of 5/3 fiber modification to improve infectivity. Not only was the combination of Cox-2 CRAds and infectivity enhancement fruitful for tumor-killing efficiency but also the strategy may also resolve safety problems associated with adenoviral vectors. Common to all viral-based approaches is the challenge of overcoming the host innate immune response towards the vector itself.[78,79] With our strategy, the use of CRAds would require a much lower amount of administered virus relative to a nonreplicative strategy, because CRAds are designed to amplify themselves from the initial dose. Moreover, the improved cytocidal effect due to 5/3 fiber modification may decrease the necessary

amount of injected virus even further, avoiding a negative innate immune response towards the vector. Another safety issue to consider is toxicity in normal organs due to the ectopic replication of oncolytic adenoviruses. We have specifically chosen the Cox-2 promoter to avoid this situation. The Cox-2 promoter's selectivity would provide targeted adenoviral replication in gastric cancer cells and, hence, tumor killing, but minimal activity in normal organs. Combining this transcriptional targeting with 5/3 fiber modification would, therefore, yield an enhanced locoregional tumor killing effect.

In the present study, we optimized TSP-driven CRAds with fiber modification for gastric cancer. We have shown that Cox-2 CRAd can effectively kill gastric cancer cells and that 5/3 chimeric fiber modification can significantly increase this cytocidal effect. Thus, the combination of Cox-2 CRAd and 5/3 fiber modification represents a potentially effective and safe virotherapy agent for gastric cancer.

Acknowledgments. This work was supported by the National Institute of Health, R01 DK063615-01 (M.Y.), and R01 CA94084 (D.T.C.), and US Department of Defense, DAMD17-03-01 (M.Y.). We thank Long Le, Pedro Ramirez, Tatyana Gavrikova, Reiko Kunisaki, Syuich Hirai, and Shigeo Ohno for their excellent technical support and expert advice.

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