Survivin promoter-based conditionally replicative adenoviruses target cholangiocarcinoma

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Abstract. Cholangiocarcinoma is a highly malignant neoplasm with no effective treatment. Conditionally replicative adenoviruses (CRAds) represent a promising new modality for the treatment of cancer in general. A key contribution in this regard was the introduction of tumor-selective viral replication for amplification of the initial inoculum in the neoplastic cell population. Under ideal conditions following cellular infection, the viruses replicate selectively in the infected tumor cells and kill the cells by cytolysis, leaving normal cells unaffected. However, to date there have been two limitations to the clinical application of these CRAd agents, i.e. poor viral infectivity and tumor specificity. Here we report the construction of three new CRAd agents, CRAd-S.RGD, CRAd-S.F5/3 and CRAd-S.pk7, in which the tumor specificity is regulated by a tumor-specific promoter, the survivin promoter, and the viral infectivity is enhanced by incorporating a capsid modification (RGD, F5/3 or pk7) in the adenovirus fiber region. These CRAd agents effectively target cholangiocarcinoma cells, induce strong cytotoxicity in these cells in vitro and inhibit tumor growth in a murine xenograft model in vivo. In addition, the survivin promoter has extremely low activity both in the non-transformed cell line, HMEC, and in human liver tissue. Our results suggest that the survivin-based CRAds are promising agents for targeting cholangiocarcinoma with low host toxicity. Such results should provide important insights into the identification of novel therapeutic strategies for cholangiocarcinoma.

Introduction

Cholangiocarcinoma is a malignancy derived from the human biliary tract epithelium. It is a highly lethal disease with a poor long-term prognosis and few effective treatments (1-4). Cholangiocarcinoma represents 20% of all hepatobiliary malignancies in the United States, a number which is increasing every year (5). In these patients, only 30% are candidates for attempted curative surgical resection at present and another 70% are found to have occult metastatic or advanced local disease, precluding curative resection. The average 5-year survival is only 10%, with a median survival of 1.5 years. Even after resection, the five-year survival rate is only 20-40%, and chemotherapy and radiotherapy have limited efficacy (6). Clearly, novel treatment modalities which increase efficacy are needed.

Adenovirus (Ad)-mediated gene therapy has been demonstrated to be a powerful tool to target tumor cells and can infect a broad range of human cells, both dividing and non-dividing, with high gene transfer efficiency (7,8). In addition, wild-type adenoviruses have a low pathogenicity, typically causing only mild ‘cold-type’ symptoms in the human host. The use of conditionally replicative adenovirus (CRAd) represents a novel approach to treat neoplastic diseases, including malignant cholangiocarcinoma(9).

Conditionally replicative adenoviruses target tumor cells leading to their death via a direct consequence of viral replication (10). It is apparent that the specificity of the viral agent for achieving tumor cell killing via replication (‘oncolysis’) is the functional key to successful exploitation of these agents for therapy. To this end, an ideal viral agent would, thus, possess two characteristics: i) high infectivity with which viral vectors would have the capacity to infect tumor versus non-tumor cells; and ii) tumor specificity with
which viral vectors would possess a relative preference for replication in tumor versus non-tumor cells. However, both viral infectivity and specificity have been relatively poor when using current applicable CRAd vectors. To develop enhanced infectivity and tumor-specific CRAd agents for cholangiocarcinoma, better constructs are needed.

In order to overcome these two disadvantages, poor infectivity and specificity, many approaches have been described. For example, cells may be resistant to Ad infection due to their lack of the coxsackie adenovirus receptor (CAR) on the surface of tumor cells resulting in poor infectivity (11). To circumvent this, genetic and immunologic alterations to the virus fiber which utilize CAR-independent pathways have been identified. An example of this is the utilization of the RGD motif in the fiber knob of the Ad. This capsid modification appears to facilitate Ad binding and entry into tumor cells via integrin receptors that are abundantly expressed on tumor cells (12). Additional capsid modifications have been explored to obtain infectivity enhancement of Ads including AdF5/3 (13), Ad5-pk7 (14) and Ad5-CK (15). Transcriptional targeting exploits promoters that display preferentially in tumor cells but not in normal host cells. This has been described as an efficient strategy to increase viral replication specificity (16,17). An ideal tumor-specific promoter (TSP) for transcriptional targeting exhibits selective high activity in tumor cells (termed a ‘tumor on’ phenotype). To mitigate hepatotoxicity upon systemic delivery, candidate promoters additionally need to exhibit low activity in liver (termed a ‘liver off’ phenotype). To develop TSP-CRAds, one of the most widely used methods is to drive Ad E1 gene expression with a selected promoter, as Ad E1 is the main element which drives viral replication. In this method, a CRAd replicates only in tumor cells, killing cells by oncolysis, but not in normal host cells, thereby avoiding the toxicity of the CRAd agent. Many TSPs have been explored for specific cancers, such as prostate-specific antigen (PSA) for prostate cancer, and α-fetoprotein (AFP) promoter for hepatocarcinoma (18,19). Recently we reported a novel TSP, the survivin promoter, which showed a tumor on/liver off phenotype in vitro and in vivo (17). This promoter has also been reported to exhibit radiation response elements and cisplatin sensitivity capabilities (20,21). Therefore, the survivin promoter is an excellent candidate to drive E1 expression in development of a new CRAd agent. Kamizono et al (22) and our group (23) have described the survivin-CRAd in which the Ad E1 is driven by the survivin promoter as a multiple tumor-specific promoter target.

We have previously subcloned and characterized the cholangiocarcinoma cell line, SK-ChA-1. Tumorigenicity studies have demonstrated that a subclone of SK-ChA-1 can grow as tumors in nude mice (24). Therefore, as a proof-of-principle we developed Ad agents to target these tumors.

We further constructed CRAd vectors in which the Ad E1 gene was regulated by using the survivin promoter as a TSP, viral infectivity was enhanced with a capsid modification (RGD, pk7 or F5/3), and the Ad vector targeting to cholangiocarcinoma cells occurred via a CAR-independent pathway. We verified that these infectivity-enhanced and tumor-selected vectors, specifically CRAd-S.F5/3, replicate in cholangiocarcinoma cells, kill tumor cells and inhibit tumor growth in a xenograft animal model. We also showed that the survivin promoter had extremely low activity compared to the CMV promoter in an in vitro human liver slice hepatotoxicity model although they have similar oncolytic activity in cholangiocarcinoma cells. The data indicated that CRAd-S.F5/3 is an excellent candidate for translation into a clinical trial for treatment of cholangiocarcinoma in patients.

Materials and methods

Cells, tissues and animals. Human cholangiocarcinoma cells derived from SK-ChA-1 were characterized and described previously (25). Cells were grown in RPMI-1640 complete supplemented with 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO2 environment under humidified conditions. The normal cell line, human mammary epithelial cells (HMEC), as control cells, was purchased from the Cambrex BioScience Company (Walkersville, MD) and cultured in medium purchased from the same company.

Human liver samples were obtained from hepatectomy remnants (donor) not needed for diagnostic purpose after liver transplantation following IRB approval. To generate liver tissue slices, tissue was cut in consecutive 0.5-mm thick slices using the Krumdeck 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, 100 U/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% CO2. Three tissue slices were examined per group.

Female BALB/c nude mice (6-8 weeks of age, Charles River, Wilmington, MA) were used in the in vivo experiments. All animals received humane care based on the guidelines set by the American Veterinary Association. All experimental protocols involving live animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Recombinant adenoviruses. Ad5-CMV, Ad5-Cox-2, Ad5-CXCR4, Ad5-EGP-2, Ad5-HPR, Ad5-SLP1, Ad5-MsLn, and Ad5-Survivin (Ad5-S) are replicative-defective Ads containing a luciferase reporter gene driven by the tumor-specific promoters, Cox-2 (26), CXCR4 (16), HPR (27), SLP1 (28), MsLn (29) and survivin (23) and a control CMV promoter, respectively, in the E1 region and have been described previously. These Ads were used in this study for evaluating the transcriptional activity of the TSPs by means of the expression of the luciferase reporter gene in cholangiocarcinoma cells. Ad5.RGD, Ad5pk7, Ad5F5/3, and Ad5pk7.RGD are replicative-defective Ads containing a luciferase reporter gene driven by the CMV promoter in the E1 region and incorporating a capsid modification, RGD (30), pk7 (14), F5/3 (31), or pk7.RGD (14) respectively as described previously. These Ads were used in this study for evaluating the transizational activity of the capsid modifications by means of the expression of a luciferase reporter gene in cholangiocarcinoma cells.

All the Ads are listed in Table I. The viruses are all isogenic and were propagated in 293 cells and purified by double CsCl density centrifugation. Of note, the firefly luciferase gene
incorporated into the Ads contained a modified coding region for firefly luciferase (pGL3; Promega) that had been optimized for monitoring transcriptional activity in transfected eukaryotic cells. The luciferase activity of cells infected with one of the Ads was normalized to fold-activity of cells infected with Ad5-CMV.

Physical particle concentration (v.p./ml) was determined by O.D. 260 nm reading. All experiments were based on v.p. numbers, although a plaque assay was performed to ensure sufficient quality of the virus preparation. The ratios of v.p./pfu (plaque forming units) were 19-40 among all Ad vectors.

Quantitative real-time PCR (QRT-PCR) for detection of the expression of the survivin, Ad5 E1 and E4 genes. For survivin gene expression, total cellular RNA was extracted from 5x10⁵ cholangiocarcinoma cells or HMEC cells using the RNeasy mini kit (Qiagen, Valencia, CA), followed by treatment with DNase to remove any possible contaminating DNA from the RNA samples. The fluorescent TaqMan probe (6FAM-TGACGACCCCATAGAGGAACATAAAAAGCAT) and the primer pair (forward primer, TGGAAGGCTGGGAGCCA; reverse primer, GAAAGCGCAACCGGACG) used for QRT-PCR in analysis of the survivin mRNA were designed using Primer Express 1.0 (Perkin Elmer, Foster City, CA), and synthesized by Applied Biosystems (Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. A negative control with no template was performed for each reaction series. QRT-PCR reaction was carried out using a LightCycler™ system (Roche Molecular Biochemicals, Indianapolis, IN). The thermal cycling conditions were 2 min at 50°C, 30 min at 60°C, 5 min at 95°C, then 40 cycles of 20 sec at 94°C, and 1 min at 62°C. Data were analyzed with LightCycler software.

For quantization of the Ad E1 and E4 genes, total cellular RNA or DNA were extracted from cells or cell cultures in 12/24-well plates using the RNeasy mini RNA extraction kit or a blood DNA kit (Qiagen), respectively. Both RNA and DNA samples were treated with RNase free DNase and DNase free RNase, respectively, to remove possible contamination. The Ad5 E1 gene was detected in RNA samples by using oligo pair forward primer-5’AACCAGTTGCCGTAGAGTTG and reverse primer-5’CTDGTTAAGCAAGTCCTCGATACA, and probe ORF6-CACAGCTGGCGACGCCCA; and the Ad5 E4 gene was detected in DNA samples by using oligo pair forward primer-5’GGAGTGCGCCGAGACAAC, and reverse primer-5’ACTACGTCCGGCGTTCCAT, and probe ORF6-TGGCATGACACTACGACCAACAGCATC. Negative controls and an internal control were performed for each reaction series as described above.

Transcriptional and transductional evaluations in vitro. Cholangiocarcinoma cells (5x10⁴ cells/well) were plated on 24-well plates in 1 ml of medium as described previously. On the following day, cells were infected with recombinant Ads [Ad5-CMV, Ad5-Cox-2, Ad5-CXCR4, Ad5-EGP-2, Ad5-HPR, Ad5-SLPI, Ad5-MsLn, and Ad5-S for transcription (Fig. 1) or Ad5.RGD, Ad5.pk7, Ad5.F5/3, and Ad5.pk7.RG for transduction (Fig. 2)] at 100 v.p./cell for 2 h in 200 μl of the medium containing 2% of FBS. 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 activity was standardized using the Reporter lysis buffer and luciferase assay system of Promega (Madison, WI). The transcriptional levels of the TSP of interest in cholangiocarcinoma cells were evaluated by measuring the expression activity of the luciferase reporter gene driven by different promoter. The transductional levels of the Ads in cholangiocarcinoma cells were evaluated by measuring the

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**Table I. The characteristics of adenoviral vectors used in this study.**

<table>
<thead>
<tr>
<th>Virus name</th>
<th>Promoter</th>
<th>Reporter</th>
<th>E1</th>
<th>E3</th>
<th>Modification</th>
<th>Replication competent</th>
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<tr>
<td>Ad5-CMV</td>
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<td>No</td>
</tr>
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<td>No</td>
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<td>No</td>
<td>No</td>
</tr>
<tr>
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<td>Luciferase</td>
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<td>No</td>
</tr>
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<td>RGD4C</td>
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<td>CRAd-S.FS/3</td>
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<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>F5/3</td>
<td>Yes</td>
</tr>
<tr>
<td>CRAd-S.pk7</td>
<td>Survivin</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>pk7</td>
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</table>
expression activity of the luciferase reporter gene driven with the same CMV promoter, but incorporating a different capsid modification into the Ad fiber region.

Development of CRAd agents. The CRAd genomes were constructed via homologous recombination in Escherichia coli (Fig. 3) as described previously (23). Briefly, DNA fragments containing nucleotides -230/+30 was cut with BamHI and HindIII restriction endonucleases from the clone pLuc-cyc1.2 which was a gift from Dr F. Li (Buffalo, New York), 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 XbaI/BamHI from a pGL3B vector (Invitrogen, Carlsbad, CA) and inserted into the pBSSK/Survivin by use of the same restriction sites. A generated clone named pBSSK/PA/Survivin was used to create shuttle vectors. DNA fragments containing SV40 PA and the survivin promoter (short) were cut with NotI/XhoI, and subcloned into a pScsE1 plasmid [gift from Dr D. Nettelbeck (Erlangen, Germany)] that contains the E1 gene with the same restriction sites. 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removed, the cells were washed three times with PBS to remove uninternalized viruses. DNAs were isolated from cells of each well using the DNeasy tissue kit (Qiagen) and quantitative RT-PCR was performed as described above. Ad E4 gene copy numbers were detected and normalized with human actin gene.

Analysis of replication of CRAd agents in tumor cells. Cholangiocarcinoma cells 10^5 well were cultured as discussed above, infected with 100 v.p./cell of Ad5-S, CRAd-S.RGD, CRAd-S.F5/3, CRAD-S.pk7 or Adwt in infection medium containing 2% FBS, and incubated at 37°C in a 5% CO2 environment. After 3-h incubation, the infection medium was removed, and the cells were washed three times to remove uninternalized viruses and placed in fresh culture medium with 10% FBS. Media from triplicate wells were collected 1, 3, and 9 days later, DNA was extracted from 200 μl of media with the DNeasy tissue kit (Qiagen) and quantitative RT-PCR was performed as described above. Ad E4 gene copy numbers were detected and normalized with human actin gene.

In vitro analysis of cytocidal effects. The in vitro cytocidal effects of the CRAd agents were analyzed by determining the viability of the cells with crystal violet staining after infection. Briefly, 2.5x10^4 cholangiocarcinoma cells/well were plated on a 24-well plate. Cells were infected at 500, 100, 20, 4, 0.8 or 0 v.p./cell with CRAd-S.pk7, CRAd-S.F5/3, CRAd-S.RGD, or Ad5-S in infection medium. Two hours later, the infection medium was replaced with the appropriate complete medium. After 10 days of incubation, 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 elsewhere (23).

Replication of CRAd agents on murine xenografts. The tumor cells were verified to have 95% viability by Trypan Blue exclusion. BALB/c nude mice were subcutaneously inoculated in their flanks with 2.5x10^4 cholangiocarcinoma cells (n=3/group). When the tumors reached 5 mm in widest diameter, 5x10^5 v.p. of each viral vector (Adwt, Ad5-S, or CRAd-S.F5/3) were injected intratumorally. The mice were sacrificed at day 1 and day 7 and the tumor samples were harvested. The DNAs were isolated from the tumor samples as described previously. DNA samples from xenografts were stored at -80°C until use. Ad E4 gene copy numbers were detected by QRT-PCR and normalized with human actin, respectively.

Antitumor effect of CRAd agents on mouse xenograft model. BALB/c nude mice were inoculated in their flanks with 2.5x10^4 cholangiocarcinoma cells (n=5/group) as described previously. When the tumors reached 5 mm in widest diameter, 1x10^5 v.p. of each viral vector (Ad5-S, CRAd-S.RGD, CRAd-S.F5/3, CRAd-S.pk7 or Adwt) was injected intratumorally. The same dose was repeated after one week. The tumor volumes were monitored twice a week and calculated by the following formula: 1/2xy^2, where x is the longest distance and y is the shortest distance of the tumor. The tumor volume was shown as mm^3. The statistical analysis was performed by two-way ANOVA.

Analysis of promoter activity in human liver slices. Human liver was obtained from hepatectomy specimens (donor) following liver transplantation. To generate liver tissue slices, tissue was cut in consecutive 0.5-mm thick slices using the Krumdieck tissue slicer. Sequential slices were then cultured in 24-well plates in RPMI medium supplemented with 10% bovine fetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml insulin. The tissue slices were infected with 500 v.p./cell of Ad vector, Ad5-CMV or Ad-S with infection medium. After 3-h infection, the liver slices were washed once with PBS and 10% bovine fetal serum medium was added for 48-h incubation, at 37°C, in a humidified atmosphere of 95% air and 5% CO2. Two days following infection, luciferase activity was detected by conventional assay and shown as ULR (relative light units) of luciferase activity.

Statistic analysis. Student's test was employed for statistical analysis where P<0.05 was considered to be statistically significant. For tumor growth inhibition experiment, two-way ANOVA was employed.

Results

Evaluation of tumor-specific promoters in vitro. The activity of all 7 tumor-specific promoters; i.e. the cox-2, CXCR4, EGP-2, HPR, SLPI, MsLn, and survivin promoters, respectively, was evaluated in cholangiocarcinoma cells. The backbone structure of the Ad vectors was identical in all constructs which contained a luciferase reporter gene derived from the pGL3 plasmid (Promega), except for the promoter which specifically drove the luciferase gene expression. The activity of the Ad vectors in tumor cells was normalized to an Ad5-CMV vector which had the same backbone but utilized the CMV promoter. The results are shown in Fig. 1A. Three out of seven TSPs, the survivin, CXCR4 and SLPI promoters, exhibited higher promoter activity than the others in cholangiocarcinoma cells; 111%, 67% and 25%, respectively, in cholangiocarcinoma cells, compared to that of the CMV promoter. The activity of the remaining promoters was <5% of the CMV promoter. These data provide evidence that these three promoters have a tumor on phenotype. In addition, survivin gene expression was 7000 RNA copies (S.D.±778) in cholangiocarcinoma cells as compared to undetectable (0) levels in the non-transformed HMEC cells as detected by QRT-PCR, which was significant (P<0.01). Human GAPDH gene expression was detected at a level of 48 (S.D.±7) and 42 (S.D.±29) RNA copies in cholangiocarcinoma and HMEC cells, respectively (Fig. 1B).

Evaluation of capsid modification in vitro. Four capsid modifications, pk7, pk7.RGD, F5/3 and RGD, were generated for viral infectivity enhancement via a CAR-independent pathway as described elsewhere (8). All of the Ad vectors again had similar backbones as described above, the difference being the incorporation of a different modification in the Ad fiber region. The activity of the modified Ad vectors in tumor cells was normalized to an Ad5-CMV vector which had the same backbone as the native Ad5 fiber (Fig. 2). The two Ad vectors with the F5/3 and pk7 modifications exhibited higher reporter activity in the cholangiocarcinoma cells; 664% (F5/3
were isolated from the media and the Ad E4 gene was determined by using RT-PCR. After 3-h infection, the cells were washed 3 times with PBS to remove uninfected free adenoviral vectors. The DNAs were isolated from these cell lines at 100 v.p./cell in 24-well plates as described in the Materials and methods. Replication rates of modified CRAd agents in tumor cell lines at 100 v.p./cell in 24-well plates as described in the Materials and methods. Medium (200 μl) was collected from the medium and Ad E4 copy numbers were detected by QRT-PCR, which served as a biomarker for the copy numbers of adenoviruses in the medium. The data shown in Fig. 4B compared E4 copies of Ad vectors in the medium at 1000- to 1000-fold at both 10 v.p./cell and 1000 v.p./cells compared to non-modified Ad as the control. Also the data showed that the F5/3 and pk7 modifications had somewhat higher transductional levels than that of an RGD modification in tumor cells at all three different doses.

Replication of survivin-CRAds in tumor cell lines. After 18-24 h of incubation, the CRAd agents replicated in tumor cells and began to be released. Three CRAds plus a negative (Ad5-S) and positive control (Adwt) were used to infect two tumor cell lines at 100 v.p./cell in 24-well plates as described in the Materials and methods. Medium (200 μl) was collected from each well, and spun to remove cells. DNA was isolated from the medium and Ad E4 copy numbers were detected by QRT-PCR, which served as a biomarker for the copy numbers of adenoviruses in the medium. The data shown in Fig. 4B compared E4 copies of Ad vectors in the medium at days 1, 3, and 9 post-infection as described previously. The replication ratios (which were calculated from the E4 copies at day 1/E4 copies at day 1) were 1.3, 635, 2229, 220 and 3745 for Ad5-S, CRAd-S.RGD, CRAd-S.F5/3, CRAd-S.pk7 and Adwt, respectively. As expected, there was no significant replication in negative control, Ad5-S, because of its non-replicative nature. Not surprisingly, the positive (Adwt) control exhibited a higher replication rate compared to those of three CRAd agents.

Survivin-CRAds induce cytotoxicity (oncolysis) in cholangiocarcinoma cells. Conventional oncolysis analysis serves as one of the best modalities for monitoring tumor cell killing. Encouraged by the tumor-specific activity of the survivin expression, survivin-CRAds were explored for their ability to release tumor cells by inducing oncolysis of the CRAd agents; iv) a poly-A signal was inserted between the inverted terminal repeat (ITR) and the survivin promoter to stop the non-specific transcriptional activity of the ITR, and to retain the tumor specificity of the survivin promoter. All of the modified structures were verified by PCR and sequencing as described elsewhere (16-18). The yields of CRAd agents were 5.5x10^11, 2.6x10^12 and 1.5x10^12 v.p./ml with a v.p./pfu (plaque-forming units) ratio of 69, 41, and 60 for CRAd-S.RGD, CRAd-S.F5/3 and CRAd-S.pk7, respectively.

Transductional activity of capsid modified CRAds in tumor cells. Most human tumors contain low levels of the coxsackieadenovirus receptor (CAR), the natural endogenous receptor for human adenovirus serotype 2 and 5 (30,32). Capsid modification enhances viral infectivity via a CAR-independent pathway that has been described previously. In this study, we compared three capsid modifications, which were incorporated into the fiber region in the CRAd agents into the cholangiocarcinoma cells. In order to avoid viral replication, we infected tumor cells for 3 h and immediately isolated DNA from these cells. Between 18 and 24 h were needed to detect Ad DNA in medium, which corresponds to the life cycle of Ads from entry into tumor cells to release from them. The adenoviral copy number was determined by QRT-PCR and normalized to the house-keeping gene, actin. The data shown in Fig. 4A are the transductional levels (including viral binding and internalization) of three CRAd agents carrying different capsid modifications, i.e. RGD, F5/3 and pk7 in cholangiocarcinoma cells. The results indicate that modified CRAds enhanced the transduction by 100- to 1000-fold at both 10 v.p./cell and 1000 v.p./cells compared to non-modified Ad as the control. Also the data showed that the F5/3 and pk7 modifications had somewhat higher transductional levels than that of an RGD modification in tumor cells at all three different doses.

Attributes of the survivin CRAds incorporating capsid modifications. Based on these data, we choose the survivin promoter as the tumor-specific promoter to increase tumor specificity and the RGD, F5/3 and pk7 capsid modifications to enhance viral infectivity. The structures of the survivin-CRAd vectors shown in Fig. 3 were constructed by conventional methodologies, propagated in 911 cells, and expanded in D65 cells. The CRAd-survivin vectors have the following characteristics: i) the survivin-CRAds contain the human survivin promoter (nucleotide -230/+30) to drive E1 expression (23), the survivin-controlled E1 expression cassette was placed in the original E1 region of the Ad gene; ii) a capsid modification, RGD, F5/3 or pk7, was inserted into the Ad fiber knob region for enhancement of Ad infectivity; iii) the E3 gene was retained in the Ad genome for elevating the
promoter, we used the CRAd-S.RGD, CRAd-S.F5/3 and CRAd-S.pk7 constructs as oncolytic anti-tumor agents in the cholangiocarcinoma cells. Cytotoxicity was evaluated after 10 days of incubation via crystal violet staining (Fig. 5). While the replication-incompetent Ad5-S vector had no cytotoxic effect even at 100 v.p./cell, the survivin-based CRAds induced strong cytotoxicity in cholangiocarcinoma cells. Almost 50% of cells were killed, even at the smallest dose, 0.8 v.p./cell for CRAd-S.F5/3 and CRAd-S.pk7, and 50% of cells were killed with CRAd-S.RGD at 20 v.p./cell. As expected, however, no cytoxicity was observed in normal mammary epithelial cells, HMEC, even at the highest dose (500 v.p./cell).

Anti-tumor effect and replication of survivin-CRAds in vivo.

The CRAd anti-tumor effect was analyzed in vivo using cholangiocarcinoma cells injected subcutaneously into athymic mice. After establishment and growth of the tumor to 5 mm in diameter, 1x10⁹ v.p. of Ad5-S, CRAd-S.RGD, CRAd-S.F5/3, CRAd-S.pk7, Adwt or sham (same volume of PBS) was injected into the tumors, and the injections were repeated once more using the same dose one week later. The tumor growth was monitored over time. The tumor volume data (Fig. 6A) shows that there was a significant decrease in tumor volume between control and the treatment groups. At 21 days after treatment, the tumor growth ratio was calculated by dividing the tumor volume at day 21 by the tumor volume at day one x 100%. The tumor growth rates were 438%, 313%, 243%, 277%, 244% and 540% for treatment of Ad5-S, CRAd-S.RGD, CRAd-S.F5/3, CRAd-S.pk7, Adwt and sham, respectively. A statistically significant delay (P<0.05) in tumor growth was achieved for treatment of each of the CRAd agents compared to treatment with PBS or Ad5-S, the non-replicating adenoviral vector. The CRAd agents exhibited a strong anti-tumor effect similar to or better than Adwt, the replicative control in the cholangiocarcinoma xenograft murine model.

We also compared the replication rates of CRAd-S.F5/3 to that of the Adwt and Ad5-S vectors. Identical doses of the vectors were injected into the tumor nodules in the murine xenograft model as described in the Materials and methods. The Ad E4 copy number was determined by QRT-PCR as described previously. The E4 copy number was normalized as E4 copies/ng DNA.

Survivin promoter activity in human liver slices compared to the CMV promoter.

We compared the activity of the survivin
Discussion

Cholangiocarcinoma is a highly malignant tumor that arises from the bile duct epithelium (33). The tumor usually occurs in older patients and its occurrence in patients younger than 40 years is rare (34). Tumors originating from the large bile duct are discovered early due to their critical location; tumors originating from the small bile ducts do not cause significant biliary obstruction until the tumor itself or metastatic lesions cause obstruction of common bile duct (35). In terms of treatment, complete resection with negative histological margins provides the only hope for long-term survival. Currently, there is no effective adjuvant therapy for this tumor. Cholangiocarcinoma responds poorly to chemotherapy, and radiation therapy and conventional treatments are not adequate for the vast majority of patients with cholangiocarcinoma (36).

Novel therapeutic strategies are warranted for cholangiocarcinoma. Advances in the understanding of growth factor biology, molecular oncology, and tumor immunology have provided the rationale for several strategies for cancer gene therapy (37). Some of these approaches have been applied to the cholangiocarcinoma gene therapy field, including adenovirus-mediated mutant p27kip1 to induce apoptosis of cholangiocarcinoma cells (38), adenovirus-mediated cytosine deaminase suicide gene therapy and 5-fluorocytosine to enhance cytotoxicity in human cholangiocarcinoma (39).

Figure 7. Transcriptional activity of the CMV and survivin promoters in human liver slices. Human liver slices were infected with 500 v.p./cell of Ad vector, Ad5-CMV or Ad5-S (A) and CRAd-S.F5/3 and Adwt.F5/3 (B). Two days following infection, luciferase activity and E4 copy number were detected by using Luciferase assay and real-time PCR as described, respectively. ULR (relative light units) of luciferase expression (A) and E4 copies/ng DNA (B). **P<0.01.
express low levels of CAR, leading to poor infection rates (47). To circumvent this problem, the development of CAR-independent Ad vectors which enhance infectivity is critical. We constructed three survivin-CRAds incorporating the capsid modifications RGD, F5/3 or pk7 by which the CRAd agents target to $\alpha_i$ integrins (30), Ad3 receptors CD80/CD86/CD46 (48,49) or heparan sulfate-containing receptor (14) on the surfaces of tumor cells, respectively, via CAR-independent pathways. All of the CRAds which incorporated the capsid modifications had higher transductional activity, especially CRAd-S.F5/3 and CRAd-S.pk7 as compared to the Ad5-S which contained a native fiber with no modification (Fig. 4A). There was a 100- to 1000-fold increase of transductional activity when comparing modified vectors to a non-modified vector at both 10 v.p/cell and 1000 v.p./cell infection doses.

Survivin protein is a novel member of the inhibitor of apoptosis (IAP) protein family, which plays an important role in the survival of cancer cells and progression of malignancies (50). The survivin gene has been described as being selectively expressed in some of the most common human neoplasms, including cancer of the lung (51), pancreas (52), rectum (53), brain (54), and ovary (55). Although the survivin protein is undetectable in normal adult mouse tissue, trace amounts of survivin have been detected in human organs (50). Our data have shown that the survivin promoter has high activity in breast, ovarian cancer and melanoma tumor cells with an extremely low activity in mouse liver (17) and human liver slices (Fig. 7). These data argued that the survivin promoter had a tumor on/liver off phenotype, and thus was a good candidate to be chosen to drive the E1 gene of the CRAd agents in this study, and to restrict the replication of CRAd agents to cholangiocarcinoma tumor cells, not to normal host cells. The survivin promoter is an excellent tumor-specific promoter to target cholangiocarcinoma cells based on the following key points: i) survivin gene expression was detected (7000 RNA copies) in cholangiocarcinoma cells but was undetectable (0 copies) in the normal epithelial cell line, HMEC (Fig. 1B); ii) the CRAd agents exhibited strong cell killing in cholangiocarcinoma cells, but not in HMEC cells (Fig. 5); iii) anti-tumor growth experiments indicated that CRAd-S.F5/3 and CRAD-S.pk7 inhibit tumor growth similar to Adwt in in vivo experiments with a 50% tumor volume as compared to the untreated group (Fig. 6A); iv) there was very low survivin promoter activity in human liver slices as compared to that of the CMV promoter (81-fold) (Fig. 7). All this evidence leads to the incontrovertible conclusion that the survivin promoter has higher activity in cholangiocarcinoma cells but not in normal host cells, HMEC cells or human liver tissue. Thus, the survivin promoter is an excellent TSP to restrict viral replication in cholangiocarcinoma cells and minimize the toxicity to host cells.

Three capsid modifications, RGD, F5/3 and pk7, were used in this study to enhance the viral infectivity of CRAd agents via a CAR-independent pathway. From transductional activity analysis (Fig. 4A), all three capsid modifications were seen to enhance the infectivity in cholangiocarcinoma cells at the three tested doses. The viral infectivity levels were enhanced 100- to 1000-fold at both 10 v.p./cell and 1000 v.p./cell compared to the non-modified Ad5-S as control. Specifically, the F5/3 and pk7 had higher transductional activity compared to the non-modified vector when comparing modified vectors to a non-modified vector at both 10 v.p/cell and 1000 v.p./cell infection doses.

In conclusion, we identified the human survivin promoter as a tumor-specific regulatory element for targeting cholangiocarcinoma. CRAd agents armed with the survivin promoter and capsid modifications elevate both tumor specificity and viral infectivity in vitro and in vivo. The survivin-CRAds replicated in tumor cells and inhibited tumor growth in an in vivo murine model. In addition, the survivin promoter had extremely low activity in normal non-transformed epithelium and in human liver. It is apparent that the survivin-CRAds are positioned to be useful for experimental clinical applications for cholangiocarcinoma.

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