A New Type of Adenovirus Vector That Utilizes Homologous Recombination To Achieve Tumor-Specific Replication

Kathrin Bernt, Min Liang, Xun Ye, Shaoheng Ni, Zong-Yi Li, Sheng Long Ye, Fang Hu, and André Lieber

Division of Medical Genetics, University of Washington, Seattle, Washington 98195, and Shanghai Sunway Biotech and Liver Cancer Institute/Zhong Shan Hospital, Fudan University, Shanghai, People’s Republic of China

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We have developed a new class of adenovirus vectors that selectively replicate in tumor cells. The vector design is based on our recent observation that a variety of human tumor cell lines support DNA replication of adenoviruses with deletions of the E1A and E1B genes, whereas primary human cells or mouse liver cells in vivo do not. On the basis of this tumor-selective replication, we developed an adenovirus system that utilizes homologous recombination between inverted repeats to mediate precise rearrangements within the viral genome resulting in replication-dependent activation of transgene expression in tumors (Ad.IR vectors). Here, we used this system to achieve tumor-specific expression of adenoviral wild-type E1A in order to enhance viral DNA replication and spread within tumor metastases. In vitro DNA replication and cytotoxicity studies demonstrated that the mechanism of E1A-enhanced replication of Ad.IR-E1A vectors is efficiently and specifically activated in tumor cells, but not in nontransformed human cells. Systemic application of the Ad.IR-E1A vector into animals with liver metastases achieved transgene expression exclusively in tumors. The number of transgene-expressing tumor cells within metastases increased over time, indicating viral spread. Furthermore, the Ad.IR-E1A vector demonstrated antitumor efficacy in subcutaneous and metastatic models. These new Ad.IR-E1A vectors combine elements that allow for tumor-specific transgene expression, efficient viral replication, and spread in liver metastases after systemic vector application.

Adenoviruses (Ad) are attractive for tumor gene therapy because of their potential to transduce all tumor sites after systemic application. They can be engineered both on the level of the capsid and genome to increase their tumor specificity and oncolytic efficacy. Two major approaches have been developed to achieve tumor-specific replication. In conditionally replicating Ad type 1 (CRAD-1), viral genomic deletions have been introduced that affect viral protein function(s) dispensable in cancer cells (1, 3, 7, 9, 13, 26, 27). In CRAD-2, adenoviral early gene expression is controlled by a tumor-specific promoter (12, 20, 23). Immediate-early viral genes are localized in the E1 region. E1A is the first transcription unit to be expressed upon Ad infection. E1A-289R functions as the main transactivator of other viral promoters. E1A-243R forces quiescent cells into the cell cycle by transactivating cellular genes (15, 17, 22) and by interacting with cell cycle regulatory proteins such as pRb and p300. To prevent early apoptosis induced by E1A-243R, Ad utilize the E1B-19K and E1B-55K proteins, whereby E1B-55K inactivates p53 and blocks p53-mediated apoptosis (35). Because inactivation of pRb and p53 is accomplished by Ad E1A and E1B proteins, respectively, adenoviral mutants that fail to accomplish these functions because of either deletion of E1B-55K (3, 14) or partial deletion of E1A (9, 13, 26) have been proposed to selectively replicate in tumor cells that lack p53 or pRb (CRAD-1). Ad with a deletion of E1B-55K have shown antitumor efficacy in preclinical models and after intratumoral injections in patients; however, their replication selectivity could not clearly be correlated with the status of p53 (10, 11, 29).

CRAD-2 vectors have been developed to control E1A and/or E1B expression with tumor-specific promoters. To drive E1 expression, multiple promoters have been used for specific cancers, including the prostate-specific antigen promoter for prostate cancer (45) and the α-fetoprotein promoter for hepatocellular carcinomas (12, 20). This approach is restricted to specific tumor types and complicated by the heterogeneity of cancer. Moreover, application of tumor-specific promoters in Ad vectors is problematic, because their activity and specificity can be affected by viral enhancers and promoters present in the viral genome (2, 40).

In this study, we utilized first-generation Ad vectors with deletions of both E1A and E1B (AdE1−). AdE1− vectors have been widely used for gene transfer in vitro and in vivo, including clinical trials with systemic vector application (16, 28, 30). AdE1− vectors are considered replication deficient in healthy cells. The absence of AdE1− replication after systemic vector application has been demonstrated in the livers of mice (25) or nonhuman primates (41). However, we recently found that human tumor cell lines derived from colon, breast, cervix, liver, and lung cancer support AdE1− vector DNA replication (37). These findings are in agreement with observations made almost three decades ago (36). Although we showed that viral replication in these cell lines was causally associated with deregulated cell cycling and passing through the G2/M phase, no obvious correlation with the p53, pRb, and p16 status of tumor
cells was found. The cellular factors that transcomplemented AdE1- replication remained unclear, except for human papillomavirus-associated cervical carcinoma cells where the human papillomavirus E6 and E7 proteins were found to partially substitute for E1A or E1B proteins (38).

On the basis of the selective DNA replication of these vectors in tumor cells, we developed a new AdE1- -based vector system (Ad.IR) that utilizes homologous recombination between inverted repeats to mediate precise rearrangements within the viral genome (39). As a result of these rearrangements, a promoter is brought into conjunction with a reporter gene, creating a functional expression cassette. Genomic rearrangements are dependent upon viral DNA replication, which in turn occurs specifically in tumor cells. In a mouse tumor model with liver metastases derived from human tumor cells, a single systemic administration of replication-activated Ad.IR vectors achieved transgene expression in every metastasis, while no extratumoral transgene induction was observed. Here we used this system for replication-activated expression of E1A from first-generation vectors. We hypothesized that this system would accelerate viral DNA replication specifically within tumor cells and support virus-mediated tumor cell lysis and intratumoral dissemination. To test our hypotheses, we constructed an Ad.IR-expressing vector and analyzed viral DNA replication, transgene activation, and development of cytotoxic effects (CPE) in three human tumor cell lines in comparison to healthy human cells. The tumor specificity of transgene expression and viral spread was analyzed in subcutaneous tumors and after systemic vector application in mice bearing liver metastases derived from human tumor cells.

### MATERIALS AND METHODS

**Plasmid constructs and vectors.** pAd.IR-AP was derived from pAd.IR- BG (39) by deleting the β-galactosidase gene using Bpu102I/AvrIII and inserting the CNA for human placental alkaline phosphatase (AP) [EcoRI/NcoI fragment of pAPSN-9]. The bicistronic AP/E1A cassette was assembled in pIRESG2-F (Clontech, Palo Alto, Calif.). The green fluorescent protein (GFP) gene in pIRESG2-GFP was excised with BstUI/BglG1, and a blunt end E1A gene was inserted instead. The E1A gene was obtained by PCR using a wild-type E1A gene (pGFI410; Microbix, Toronto, Ontario, Canada) as a template and PCR primers 5'-CTCGAGTACGCAGCGCTCCAGGACTC [sense] and 5'-GAGGCTCATTGATATGAGAAC-3' [ antisense]. An EcoRI/NcoI fragment containing the AP CNA was inserted in front of the internal ribosomal entry site (IRES) and the E1A gene. The AP-IRESG2/EIA-SV40polyA cassette (EcorII/NcoI) was transferred into pAd.IR-BG after deleting the β-galactosidase gene using Bpu102I/AvrIII, pAd.IR-AP and pAd.IR-AP/E1A were linearized by XmnI digestion and cotransfected with pBHiG10 (Microbix) into 293 cells. The corresponding viruses were named Ad.AP, Ad.IR-AP, and Ad.IR-AP/E1A (see Fig. 1A). To minimize the expansion of wild-type, E1- defective vectors, a promoter is brought into conjunction with a reporter gene, creating a functional expression cassette. Genomic rearrangements are dependent upon viral DNA replication, which in turn occurs specifically in tumor cells. In a mouse tumor model with liver metastases derived from human tumor cells, a single systemic administration of replication-activated Ad.IR vectors achieved transgene expression in every metastasis, while no extratumoral transgene induction was observed. Here we used this system for replication-activated expression of E1A from first-generation vectors. We hypothesized that this system would accelerate viral DNA replication specifically within tumor cells and support virus-mediated tumor cell lysis and intratumoral dissemination. To test our hypotheses, we constructed an Ad.IR-expressing vector and analyzed viral DNA replication, transgene activation, and development of cytotoxic effects (CPE) in three human tumor cell lines in comparison to healthy human cells. The tumor specificity of transgene expression and viral spread was analyzed in subcutaneous tumors and after systemic vector application in mice bearing liver metastases derived from human tumor cells.

**Cells.** Cells were grown on the American Type Culture Collection (Manassas, Va.) unless otherwise indicated. 293 cells (Microbix, Toronto, Ontario, Canada) were grown in Dulbecco modified Eagle medium with 10% fetal bovine serum, 2 mM l-glutamine (Gln), 100 U of penicillin per ml, and 100 μg of streptomycin. SAEC (primary nontransformed human small airway epithelial cells) were obtained from BioWhittaker (Walkersville, Md.) and grown in SAGM medium (SABM medium [BioWhittaker] supplemented with 0.3 mg of bovine pituitary extract per ml, 0.5 μg of hydrocortisone per ml, 0.5 mg of human epidermal growth factor per ml, 0.5 μg of epinephrine per ml, 10 μg of transferrin per ml, 5 μg of insulin per ml, 0.1 ng of retinoic acid per ml, 6.5 μg of tridiethyleneamine per ml, 50 μg of gentamicin per ml, 50 ng of amphotericin B per ml, and 0.5 mg of bovine serum albumin per ml)

**Cytotoxicity assay.** To stain the cells with crystal violet, the medium was removed, and the cells were fixed for 3 min in 3% paraformaldehyde at room temperature. The cells were then washed with phosphate-buffered saline (PBS) and incubated for 3 min in 1% crystal violet in 70% ethanol. After the cells were stained, they were rinsed three times with water and air dried for photography.

**Staining for AP.** Cultured cells or tissue sections were fixed in 0.5% glutaraldehyde–PBS for 30 min at room temperature. After the samples were washed with PBS, they were incubated at 65°C for 1 h to inactivate endogenous AP. Staining was performed in a solution containing 0.1 M Tris (pH 9.5), 0.1 M N-acetyl-0.5 mg of NBT (5-bromo-4-chloro-3-indolylphosphate) (Roche Diagnostics) per ml, and 0.1875 mg of BCIP (5-bromo-4-chloro-3-indolylphosphate) (Roche Diagnostics) per ml. The reaction was stopped by adding PBS containing 1 mM EDTA.

**Replication assays.** Methylated AdE1- vectors were produced by the addition of a methyl group onto the N6 position of the adenine base of XhoI sites, CTCGAG during propagation of the viruses in 293 cells expressing the XhoI isoschizomer PaeR7 methyltransferase (PMT) (25). Loss of methylation during the second and succeeding rounds of viral DNA replication restores XhoI cleavage and can be detected by Southern blotting of XhoI-digested genomic DNA from infected cells. Cells were infected with methylated virus for 3 h in duplicate. At 3 and 72 h after infection, cells were harvested and counted. DNA extraction was performed as previously described (21). To determine the ratio of replicate viral DNA to nonreplicate viral DNA, the extraternal total DNA (107 cells in 1 ml of Dulbecco modified Eagle medium with 10% fetal bovine serum, 2 mM l-glutamine (Gln), 100 U of penicillin per ml, and 100 μg of streptomycin. SAEC (primary nontransformed human small airway epithelial cells) were obtained from BioWhittaker (Walkersville, Md.) and grown in SAGM medium (SABM medium [BioWhittaker] supplemented with 0.3 mg of bovine pituitary extract per ml, 0.5 μg of hydrocortisone per ml, 0.5 mg of human epidermal growth factor per ml, 0.5 μg of epinephrine per ml, 10 μg of transferrin per ml, 5 μg of insulin per ml, 0.1 ng of retinoic acid per ml, 6.5 μg of tridiethyleneamine per ml, 50 μg of gentamicin per ml, 50 ng of amphotericin B per ml, and 0.5 mg of bovine serum albumin per ml)

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RESULTS

Construction of a vector with a deletion of E1 or E3 that expresses E1A only upon DNA replication. On the basis of our recent findings that a variety of tumor cell lines support the DNA replication of vectors with E1 or E3 deleted and that recombination between homologous elements can be used to render expression of a transgene strictly dependent on viral DNA replication, we designed a vector expressing E1A in a replication-activated manner. In this vector (Ad.IR-AP/E1A), E1A is linked via an IRES to the reporter AP gene. Both transgenes are in the 3'→5' orientation relative to the Rous sarcoma virus (RSV) long terminal repeat promoter and are flanked by two introns in the inverse orientation (Fig. 1A). In cells that do not support replication of vectors with E1 deleted, AP and E1A are not expressed. However, in cells that allow for DNA replication of vectors with E1 deleted, recombination between the inverted repeats mediates the formation of a recombinant (delta) product, which carries a copy of the left half of the vector genome including the RSV promoter on each side of the transgene cassette (Fig. 1B). In this delta product, the promoter is linked to the 5' end of the AP-IRES-E1A cassette, resulting in the expression of both transgenes. The replication-activated expression of E1A should subsequently trigger a positive-feedback loop in which E1A enhances the replication of the parental vector. This should lead to a higher frequency of recombination events, resulting in an increase in delta product formation and E1A or transgene expression. The functionality of the constructed vectors was tested on cervical carcinoma cells (HeLa), which support DNA replication of AdE1− vectors (37–39) and on primary human cells, which do not support efficient AdE1− DNA replication (38, 39). HeLa cells and SAEC were infected with Ad.AP, Ad.IR-AP, or Ad.IR-AP/E1A at a multiplicity of infection (MOI) of 30 PFU/cell and stained for AP expression 48 h later (Fig. 1C). After infection with Ad.AP, AP expression was detected in >90% of HeLa cells and SAEC. In contrast, infection with the replication-activated vectors Ad.IR-AP and Ad.IR-AP/E1A resulted in detectable AP expression only in HeLa cells. AP staining (including the RSV promoter), flanking the transgene. In the delta product, the AP transgene is in the correct (5'→3') orientation toward the RSV promoter. The structure of Ad.IR-AP/E1A is similar to that of Ad.IR-AP but allows bicistronic expression of AP together with E1A from the delta product. E1A expression subsequently enhances viral replication of the parental vectors, which, in turn, leads to more recombination events and a further increase in E1A expression.

(C) AP expression in HeLa and SAEC after infection with Ad.AP, Ad.IR-AP, and Ad.IR-AP/E1A. HeLa cells and SAEC were infected with virus at an MOI of 30 PFU/cell for 3 h. After 48 h, cells were subjected to AP staining.

Statistical analysis. In the liver metastasis models, tumor sizes (as a percentage of total liver weight for Hep3B or as total liver weight for HeLa) in the different treatment groups were compared using the Student t test (Instat software). In the subcutaneous model, mean tumor sizes were compared at day 25 using the Student t test. Also, the mean time spans to reach statistically significant differences were considered borderline significant if P was <0.1 and significant if P was <0.05.

FIG. 1. Replication-dependent activation of E1A expression with positive-feedback mechanism. (A) Structures of Ad.AP, Ad.IR-AP, and Ad.IR-AP/E1A vectors. In Ad.AP, the AP reporter gene is under the direct control of the RSV long terminal repeat promoter. In Ad.IR-AP, the AP gene sequence is in the 5'→3' orientation relative to the RSV promoter and flanked by two homology elements in inverse orientation (IR). As IRs, two rabbit β-globin introns that do not contain any transcription stop sites and are spliced out upon transcription were used. A bidirectional simian virus 40 polyadenylation signal (pA) was used to terminate transcription of the AP or E1A genes and to prevent the formation of antisense RNA from the promoter 3' side of the cassette. (B) Schematic of E1A-enhanced Ad.IR replication. During replication in tumor cells, homologous recombination between the two inverted repeats mediates the formation of a delta product carrying two invested copies of the left half of the parental genome containing with a secondary anti-rabbit antibody labeled with fluorescein isothiocyanate (Molecular Probes Inc., Eugene, Oreg.). Cell nuclei were counterstained with 1 μg of 4',6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, Mo.) per ml.

Having defined a delta product formation and E1A or transgene expression. The functionality of the constructed vectors was tested on cervical carcinoma cells (HeLa), which support DNA replication of AdE1– vectors (37–39) and on primary human cells, which do not support efficient AdE1– DNA replication (38, 39). HeLa cells and SAEC were infected with Ad.AP, Ad.IR-AP, or Ad.IR-AP/E1A at a multiplicity of infection (MOI) of 30 PFU/cell and stained for AP expression 48 h later (Fig. 1C). After infection with Ad.AP, AP expression was detected in >90% of HeLa cells and SAEC. In contrast, infection with the replication-activated vectors Ad.IR-AP and Ad.IR-AP/E1A resulted in detectable AP expression only in HeLa cells. AP staining (including the RSV promoter), flanking the transgene. In the delta product, the AP transgene is in the correct (5'→3') orientation toward the RSV promoter. The structure of Ad.IR-AP/E1A is similar to that of Ad.IR-AP but allows bicistronic expression of AP together with E1A from the delta product. E1A expression subsequently enhances viral replication of the parental vectors, which, in turn, leads to more recombination events and a further increase in E1A expression. (C) AP expression in HeLa and SAEC after infection with Ad.AP, Ad.IR-AP, and Ad.IR-AP/E1A. HeLa cells and SAEC were infected with virus at an MOI of 30 PFU/cell for 3 h. After 48 h, cells were subjected to AP staining.

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was more intense in Ad.IR-AP/E1A-infected HeLa cells, indicating E1A-enhanced replication and activation of transgene expression.

Kinetics of viral DNA replication in HeLa cells. We have previously reported that the onset of (detectable) DNA replication of AdE1− vectors in HeLa cells is delayed for several hours compared to that of wild-type vectors (37). Since replication is a prerequisite for the expression of E1A, we hypothesized that DNA replication of Ad.IR-AP/E1A should start at about the same time as that of AdE1− vectors, but the replication efficacy should be greater due to the E1A-mediated positive feedback. To test this, we utilized a demethylation technique (25) that can distinguish input virus (methylated, 8-kb band) from replicated virus (demethylated, 6.5-kb band) by Southern blot analysis (Fig. 2). DNA replication of wild-type virus (Ad.WT) started near 8 h postinfection. DNA replication of both first-generation vectors (Ad.IR-AP and Ad.IR-AP/E1A) became detectable near 14 h postinfection. Importantly, once replication of the first-generation vectors was initiated, the amount of replicated Ad.IR-AP/E1A genomes was greater than that of Ad.IR-AP genomes and reached wild-type levels 8 to 10 h after replication initiation.

Replication rate in tumor cell lines and SAEC. To study E1A-enhanced replication in detail, HeLa (cervical carcinoma), Hep3B (hepatoma), SK-Hep1 (hepatoma-derived endothelial cells), and SAEC were infected with Ad.IR-AP/E1A or control viruses (Ad.IR-AP and Ad.WT) (Fig. 3). From a recent study, it was known that the selected tumor cell lines support DNA replication of AdE1− vectors to different degrees, with HeLa cells being the most permissive and SK-Hep1 cells being the least permissive for AdE1− replication (25, 38). To better compare replication levels in these cell lines, we chose a lower MOI (30 PFU/cell) (compared to Fig. 2) and analyzed replication 48 and 72 h after infection (replication). The amount of input (8-kb [dashed arrow]) and replicated (6.5-kb [solid arrow]) genomes was determined by Southern blotting. The corresponding ethidium bromide-stained agarose gel is shown below each blot to demonstrate equal loading (chromosomal DNA [dotted arrow]). Blots with uptake lanes were exposed twice as long as the other blots. The comparable infectivities of the different cell lines are reflected by the similar intensities of the uptake lanes.

already reached about half of the level of wild-type Ad replication. Seventy-two hours after infection of HeLa cells, the levels of Ad.IR-AP/E1A and wild-type Ad, but not Ad.IR-AP replication had plateaued. Similar results were obtained in Hep3B cells, except that the plateau level of replicated genomes was less than in HeLa cells. The replication of Ad.IR-AP in SK-Hep1 cells was too low to be detectable by this assay. This low replication level was, however, sufficient to induce recombination and E1A-mediated amplification of Ad.IR-AP/E1A replication. SAEC supported the replication of wild-type virus at levels comparable to those of the tumor cell lines. In contrast to the tumor cell lines, there was no evidence for either Ad.IR-AP or Ad.IR-AP/E1A replication at 48 h and only a faint band reflecting replicated genomes at 72 h for Ad.IR-AP/E1A.
CPE induced by Ad.IR-AP/E1A. Next, we tested the ability of Ad.IR-AP/E1A to induce CPE in tumor cells (Fig. 4). HeLa, Hep3B, SK-Hep1, and SAEC were infected with Ad.IR-AP, Ad.IR-AP/E1A, and Ad.WT at MOIs ranging from 0.001 to 100 PFU/cell. Ad.IR-AP/E1A was 2 to 3 orders of magnitude more efficient than Ad.IR-AP in inducing CPE in all three tumor cell lines. In contrast, in SAEC, the toxicity of Ad.IR-AP/E1A was only marginally higher than that of Ad.IR-AP and about 3 orders of magnitudes lower than that of wild-type virus.

In conclusion, the in vitro studies demonstrated that DNA replication and cytotoxicity of the Ad.IR-AP/E1A vector are greater than those of the first-generation Ad.IR vector. This mechanism is efficiently activated in tumor cells but not in healthy human cells.

In vivo tumor specificity of Ad.IR-AP/E1A. We have previously reported that AdE1− vectors do not replicate in mouse liver and upon tail vein injection of a vector with a deletion of E1 or E3 into animals with liver metastases derived from human tumor cells, replication-activated transgene expression is confined to tumor tissue (39). Here we used a xenograft mouse model of liver metastasis to test whether this tumor specificity is maintained for Ad.IR-AP/E1A. Immunodeficient mice were transplanted with HeLa cells through the portal vein. Two weeks after transplantation, the mice received Ad.IR-AP/E1A (5 × 10⁹ PFU) via tail vein infusion. Six days after virus injection, mice were sacrificed, and liver sections were stained for AP expression. The lower panel shows a representative metastasis. The top panel shows a liver section of a naive mouse (without transplanted HeLa cells) that received i.v. Ad.IR-AP/E1A injection.

Viral spread in tumors over time. We have shown that the replication-activated expression of E1A enhanced viral replication and the ability to induce CPE in tumor cells in vitro. Now, we tested whether these favorable in vitro properties translate into enhanced viral replication, tumor cell lysis, and spread to neighboring tumor cells in vivo. To this end, mice bearing HeLa cell liver metastases were injected with Ad.IR-AP or Ad.IR-AP/E1A. At 2, 5, and 10 days after virus injection, animals were sacrificed, and replication-activated AP expression was analyzed in liver sections (Fig. 6). Initially, at day 2 postinfusion of Ad.IR-AP and Ad.IR-AP/E1A, AP expression was seen in a similar number of cells per metastasis in
which most AP-positive cells were single cells. Over the following days, the number of cells expressing AP remained fairly constant for Ad.IR-AP, indicating that AdE1- DNA replication in tumor cells did not culminate in efficient production of infectious particles. In animals treated with Ad.IR-AP/E1A, the size of AP-positive clusters increased over time, suggesting infection of neighboring cells by de novo-produced virus. To confirm dissemination of Ad.IR-AP/E1A within tumors, we employed a subcutaneous tumor model. This model also allowed us to assess what percentage of tumor cells had to be transduced initially in order to achieve a significant antitumor effect. HeLa cells were infected with Ad.IR-AP or Ad.IR-AP/E1A and mixed with nontransduced HeLa cells at different ratios. The mixture was injected subcutaneously into the inguinal region of immunodeficent hosts, and tumor growth was monitored over time (Fig. 7). As little as 0.1% infected cells with Ad.IR-AP/E1A were enough to exert an antitumor effect. The tumor growth kinetics were similar to that seen with a 100-fold-higher amount of Ad.IR-AP with E1 deleted (10% AP). A statistically significant reduction in tumor growth was achieved for cell mixtures containing 1% or higher of cells initially transduced with Ad.IR-AP/E1A (P < 0.05).

**Ad.IR-AP/E1A replication in liver metastases.** Groups of mice bearing hepatic metastases of HeLa or Hep3B cells received two injections of Ad.IR-AP/E1A, Ad.IR-AP, or virus dilution buffer on two consecutive days. (SK-Hep1 cells do not form metastatic tumors in mice.) To indirectly assess viral replication in tumors, three mice per group were sacrificed 10 days after the last virus injection, and tumor sections were analyzed by immunohistochemistry for viral hexon. Notably, hexon expression is initiated only late in infection, upon viral DNA replication (32, 35). In sections from animals treated with Ad.IR-AP/E1A, numerous hexon-positive tumor cells were detected in metastases derived from Hep3B cells (Fig. 8A to D) or HeLa cells (data not shown). Viral hexon was expressed only in tumor cells, not in liver tissue without tumors (Fig. 8F). Hexon staining was not detectable in Hep3B tumors of animals injected with Ad.IR-AP (Fig. 8E). This is consistent with our earlier observation that although DNA replication of AdE1- vectors occurs in tumor cells in vivo, late events in the viral life cycle and de novo production of virions are inefficient.

**Antitumor effect in liver metastasis models.** To investigate whether Ad.IR-AP/E1A would exert an antitumor effect after systemic application, mice with Hep3B or HeLa metastases were injected with virus as described above for Fig. 8. Four weeks (HeLa) or nine weeks (Hep3B) after the last virus injection, when mice injected with buffer began to exhibit signs of ascites, the mice were sacrificed, and the livers were analyzed macroscopically for tumor burden. Microdissection of tumor tissue from livers bearing Hep3B metastases showed that about 50% of the liver was taken up by tumorous tissue in mice treated with Ad.IR-AP or buffer (Fig. 9B). In contrast, tumor tissue accounted for only 10% of the total liver weight in Ad.IR-AP/E1A-treated mice. Importantly, the largest Hep3B tumor in an Ad.IR-AP/E1A-treated mouse was smaller than the smallest tumor in any control mouse (Fig. 9A). The difference between the Ad.IR-AP/E1A treatment group and both the Ad.IR-AP and buffer treatment groups was borderline statistically significant (P < 0.1). Systemic administration of Ad.IR-AP/E1A into mice bearing HeLa cell liver metastases resulted in a statistically significant (P < 0.05) reduction in tumor growth compared to Ad.IR-AP- or buffer-treated mice (Fig. 9B). From earlier studies, it was known that HeLa cell metastases, due to their faster growth, are more difficult to treat with oncolytic viruses (2a).

**Safety of systemic vector application.** In an attempt to assess the vector toxicity, increasing doses of Ad.WT, Ad.IR-AP, and Ad.IR-AP/E1A were injected intravenously (i.v.) into mice (Table 1). Injection of Ad.WT at a dose of 2 × 10^10 particles resulted in an 100% mortality within 7 days after infection, which is in agreement with earlier studies (8). In contrast, i.v. injection of both Ad.IR-AP and Ad.IR-AP/E1A at doses 30-fold higher was not associated with any signs of disease or mortality.
In conclusion, the in vivo data demonstrate that systemic Ad.IR-AP/E1A application into immunodeficient mice is safe, achieves transgene expression in liver metastases but not in healthy liver tissue, and results in viral spread within tumors.

DISCUSSION

We constructed a new type of CRAD whose tumor specificity does not rely on E1A or E1B mutants or the use of tumor-specific promoters. In our system, the discrimination between tumor cells and healthy cells is based on the selective DNA replication of first-generation vectors in tumor cells. We modified first-generation vector genomes with inversely oriented homology elements to mediate DNA replication-dependent homologous recombination resulting in activation of transgene expression. This replication-activated system achieves tumor-specific gene expression in liver metastases (39). Because the level of replication of first-generation vectors in tumor cells is low relative to that of wild-type Ad, efficient de novo produ-
tion of infectious virions and spread in tumors of Ad.IR vectors were limited. To convert these Ad.IR vectors into CRADs with antitumor efficacy, we have incorporated the E1A gene to provide a positive-feedback loop, inducing acceleration of viral replication and spread while maintaining the tumor specificity of the Ad.IR system. In these vectors, E1A expression should be activated only upon Ad-IR DNA replication, which should provide tumor specificity.

In vitro replication studies demonstrated that detectable DNA replication of Ad.IR-AP/E1A started at the same time as replication of the first-generation vector without E1A. However, Ad.IR-AP/E1A replicated more efficiently, plateauling much earlier than the first-generation vector. This indicates the dependence of E1A expression on the initiation of viral DNA replication and subsequent homologous recombination. As DNA replication and cytotoxicity studies demonstrated, the mechanism of E1A-enhanced replication of Ad.IR vectors was efficiently activated only in tumor cells. In comparison, replication and development of CPE were several orders of magnitudes less efficient in primary human cells (SAEC). Notably, SAEC were cycling at the time of infection, which may have supported the low background level of DNA replication detectable at late time points after infection with Ad.IR-AP/E1A. Background replication in proliferating primary human cells has also been observed with other CRADs at MOIs of ≥10 PFU/cell (13, 26). The low-level replication of Ad.IR-AP/E1A in normal human cells raises safety issues for systemic in vivo application in patients. This problem can be addressed by modifying the capsid to reduce infection of healthy proliferating tissue and target the virus specifically to tumor cells (33, 42).

In our mouse models for liver metastases, replication-activated transgene expression was localized exclusively to tumor tissue after systemic application of Ad.IR-AP/E1A. Considering that the majority of systemically applied Ad5-based vectors is found in the liver (44) and that systemic injection of a 10-fold-lower dose of wild-type Ad resulted in efficient DNA replication in mouse liver (38, 39), our Ad.IR-AP/E1A vector achieved a remarkable tumor specificity. Furthermore, no toxicity was observed after i.v. injection of high doses of Ad.IR-AP/E1A (6 × 10^11 particles or ~3 × 10^10 PFU/mouse). This compares favorably with CRAD-1 vectors with the E1A CR-2 region or the E1B-55K protein deleted, which were reported to cause the death of half of the injected animals at a 10-fold-lower dose (50% lethal dose of 6 × 10^10 particles [13]).

In animals injected with Ad.IR-AP/E1A, the number of transgene-expressing cells increased over time. Also, the Ad.IR-AP/E1A vector demonstrated superior antitumor efficacy in subcutaneous and metastatic models compared to first-generation vectors without the E1A gene. This indicates that the E1A-mediated acceleration of DNA replication demonstrated in vitro resulted in enhanced de novo production of virus in initially infected tumor cells with subsequent dissemination to neighboring tumor cells.

In summary, this new CRAD allows for tumor-specific, replication-activated expression of E1A, which subsequently enhances viral replication and spread in tumors in vitro and in vivo. Compared to other CRADs, our system has a number of advantages or novel features. (i) It is based on first-generation vectors (deletion of E1 or E3), whose molecular virology and interaction with the host have been extensively studied in the past. (ii) Its tumor specificity does not rely on the use of mutations in E1 proteins, which could compromise the yield of production, viral replication, or spread (19). (iii) The system utilizes a strong viral promoter and achieves both high-level and tumor-specific transgene expression in tumors. Expression from tumor-specific promoters is generally lower than from viral promoters (31). Also, heterologous promoters exhibit considerable background transcriptional activity when placed into an adenoviral context due to interference with adenoviral enhancers (2, 40). (iv) In contrast to tumor-specific promoters, which are restricted to particular tumor types, this system is more universal. (v) In Ad.IR vectors, the transcriptionally active template is formed upon Ad DNA replication and hence, transgene expression from this template occurs only late in the Ad life cycle (5). This may be crucial for the expression of proapoptotic genes from CRADs, since the induction of apoptosis early after infection might abort viral replication and impede the formation of infectious particles that are able to spread to neighboring tumor cells. On the other hand, induction of apoptosis late in the infectious cycle may assist the release of progeny particles. In accordance with this theory, we have demonstrated that apoptosis induced after completion of virion assembly facilitates the release of de novo-produced virus from infected cells in vitro and supports viral spread in tumors in vivo (24). Taken together, this implies that the specific expression kinetics of Ad.IR vectors carrying proapoptotic genes instead of AP may promote efficient tumor killing while permitting efficient viral replication and supporting viral spread.

Our current efforts with this Ad.IR-E1A vector system are aimed towards replacement of the AP gene with genes encoding proapoptotic, prodrug-activating, or immunostimulatory proteins. We are also attempting to retarget Ad capsids to tumor cells (33). An issue that has to be addressed regarding CRADs is the influence of an antiviral immune response on viral replication and spread. Although an immune response will limit ongoing viral replication and spread eventually (14), immune responses might also lead to enhanced antitumoral effects (18).

This study presents a new concept to achieve tumor-specific replication of Ad. The ability to apply high viral doses without toxicity and the potential to include cytotoxic transgenes that are expressed in a tumor-specific and timed manner represent important steps towards the development of Ad vectors for the treatment of metastatic disease after systemic application.

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TABLE 1. Mortality after i.v. injection of increasing Ad doses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mortality at Ad dose^a:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × 10^10</td>
</tr>
<tr>
<td>Ad.WT</td>
<td>2/6</td>
</tr>
<tr>
<td>Ad.IR-AP</td>
<td>0/2</td>
</tr>
<tr>
<td>Ad.IR-AP/E1A</td>
<td>0/2</td>
</tr>
</tbody>
</table>

^a Mice were injected with the indicated virus vectors (Ad.WT, first-generation Ad.IR-AP, and Ad.IR-AP/E1A) in the tail vein with the indicated particle number in a total volume of 200 μl. The number of animals that died within a week after virus injection/total number of injected animals is shown for each condition.

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On July 9, 2017 by guest
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