Transduction of Liver Metastases After Intravenous Injection of Ad5/35 or Ad35 Vectors With and Without Factor X-Binding Protein Pretreatment

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Abstract

Inefficient tumor transduction with targeted adenoviral vectors is largely due to unspecific virus sequestration by blood components, including coagulation factor X, and Kupffer cell scavenging. In this study, we show that preinjection of snake venom factor X-binding protein (X-bp) reduces hepatocyte transduction and increases the circulation time in blood of an intravenously injected, fiber-chimeric Ad5/35 vector. X-bp pretreatment resulted in improved Ad5/35 transduction of liver metastases and increased the antitumor efficacy of an Ad5/35-based oncolytic adenovirus. Furthermore, we demonstrate that a vector based on adenoviral serotype 35, which is less sequestered by factor X, is efficient in tumor targeting. This gives a rationale for using Ad35-based vectors in virotherapy of cancer.

Introduction

A MAJOR TASK IN CANCER VIROTHERAPY is to achieve targeted infection of metastatic tumors after intravenous application of viral vectors. In the past, adenoviral vectors based on serotype 5 (Ad5) have been used for *in vivo* gene transfer. However, because Ad5 vectors transduce predominantly hepatocytes after intravenous injection, and because tumor cells often do not express the Ad5 receptor (coxsackievirus–adenovirus receptor, or CAR), these vectors are unsuitable for tumor targeting. On the other hand, Ad5 vectors containing Ad35 fibers (Ad5/35) use CD46 as a receptor for infection of cells (Gaggar *et al.*, 2003). Because CD46 expression is upregulated in malignant tumors, Ad5/35 vectors efficiently infect malignant cells (Sova *et al.*, 2004). Studies in nonhuman primates and CD46 transgenic mice that express CD46 in a pattern and at a level similar to humans demonstrated that hepatocyte transduction was significantly less pronounced with Ad5/35 vectors compared with Ad5 vectors (Ni *et al.*, 2005, 2006). In models with preestablished liver metastases, intravenously injected Ad5/35 vectors achieved tumor-localized transgene expression; however, the transduction efficiency of tumor cells was generally less than 5% (Ni *et al.*, 2006). Inefficient tumor transduction with targeted adenoviral vectors is thought to be largely due to unspecific

sequestration and degradation as a result of adenovirus interaction with both cellular and noncellular blood components. Studies have shown that a high-affinity interaction between coagulation factor X (FX) and hexon for a number of adenoviral serotypes, including serotype Ad5, mediates adenovirus uptake into hepatocytes after intravenous adenovirus injection into mice (Kalyuzhnyi *et al.*, 2008; Vigant *et al.*, 2008; Waddington *et al.*, 2008). Uptake of the Ad5-FX complex into hepatocytes involves heparan sulfate proteoglycans (HSPGs).

Because Ad5/35 vectors contain Ad5 hexons, we hypothesized that sequestration of Ad5/35 vectors in mice is mediated through FX. Ad5 hexon binding to FX can be inhibited by X-binding protein (X-bp), a snake venom-derived protein that binds with high affinity to the Gla domain of human and murine FX (Atoda *et al.*, 1998). Waddington and colleagues showed that preinjection of mice with X-bp resulted in a 150-fold reduction of liver transduction after intravenous injection of an Ad5 vector into mice and rats (Waddington *et al.*, 2008). Binding affinity to FX varied greatly between human adenoviral serotypes. Whereas Ad5 bound to FX at picomolar affinities, binding of Ad35 was either undetectable (Kalyuzhnyi *et al.*, 2008) or significantly less than that of Ad5 (Waddington *et al.*, 2008). This, together with the fact that Ad35 uses CD46 as the primary receptor (Tuve *et al.*, 2006), makes Ad35 vectors interesting for tumor targeting.

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Materials and Methods

Cells

293 cells (Microbix, Toronto, ON, Canada), TC1-CD46 cells (Ni *et al.*, 2006), HT-29 cells (HTB-38; American Type Culture Collection [ATCC], Manassas, VA), and CHO-K1 cells (CCL-61; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 1 $\times$ minimal essential medium (MEM) non-essential amino acid solution (Invitrogen, Carlsbad, CA).

Proteins

Human factor X was from Haematologic Technologies (Essex Junction, VT) (HCX-0050, lot T1206). X-bp is a 29-kDa protein, isolated from *Deinagkistrodon acutus* (the hundred pace snake), that binds with high affinity to the Gla domain of human and murine FX. X-bp was purified as described elsewhere (Atoda *et al.*, 1998).

Adenoviral vectors

Ad5-GFP and Ad5/35-GFP vectors have been described previously (Shayakhmetov *et al.*, 2000). Ad5/35.IR-E1A/TRAIL is an oncolytic adenoviral vector targeted to CD46 by the adenoviral serotype 35 fiber. This vector confers tumor-specific, replication-activated expression of E1A and TRAIL (TNF [tumor necrosis factor]-related apoptosis inducing ligand) (Sova *et al.*, 2004). To construct the Ad35 vector, we inserted the green fluorescent protein (GFP) gene into pAd35CMV (Gao *et al.*, 2003) downstream of the cytomegalovirus (CMV) promoter. This plasmid was then cotransfected together with pAd35Helper (Gao and Gambotto, 2008) into C7-Cre cells (Barjot *et al.*, 2002) to generate recombinant virus (Ad35). Adenoviral vectors were purified by ultracentrifugation in CsCl gradients. The ratio of viral particles to plaque-forming units was for all vectors 15:1. Each viral stock produced was tested for endotoxin contamination by the *Limulus* amebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA). For *in vivo* experiments, only virus preparations confirmed to be free of endotoxin contamination were used.

In vitro adenovirus attachment studies

Chinese hamster ovary (CHO) cells were detached from culture dishes by incubation with Versene and washed with phosphate-buffered saline (PBS). A total of 10^5 cells per tube were resuspended in 100 μ l of ice-cold adhesion buffer (DMEM supplemented with 2 mM $MgCl_2$, 1% FCS, and 20 mM HEPES) containing ^3H -labeled Ad5, Ad5/35, or Ad35 at a multiplicity of infection (MOI) of 8000 viral particles (VP) per cell in the presence or absence of factor X (8 μ g/ml). After 1 hr of incubation on ice, cells were pelleted and washed twice with 0.5 ml of ice-cold PBS. After the last wash, the supernatant was removed, and the cell-associated radioactivity was determined with a scintillation counter. The number of viral particles bound per cell was calculated on the basis of the virion-specific radioactivity and the number of cells.

In vitro transduction studies

The day before infection, 3×10^5 cells were seeded per well (24-well plate). The next day, attached cells were counted and

virus was added, at the MOI indicated in the caption to Fig. 1, in 1 ml of growth medium containing FCS either with or without FX (8 μ g/ml). After 2 hr of incubation with adenoviral vectors, medium was removed and cells were washed. GFP expression was analyzed by flow cytometry after 24 hr of incubation in complete medium. Cells were incubated with virus for 24 hr. The percentage of GFP-positive cells and mean fluorescence were determined by flow cytometry.

In vivo studies

All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington (Seattle, WA). All mice were housed in specific pathogen-free facilities. The CD46 transgenic C57BL/6 mouse line MCP8B (C57-CD46) was generously provided by B. Horvat (INSERM, Paris, France) (Marie *et al.*, 2002). (This line has been crossed into the C57BL/6 background for nine generations.) These mice are heterozygous for CD46 and express CD46 at levels similar to human cells. The transgene encodes CD46 isoform C1, and is under the control of the ubiquitously active hydroxymethylglutaryl-CoA reductase (HMGR) promoter. CD46 DNA-positive mice were identified by polymerase chain reaction (PCR) of tail DNA, using the following primers: F-CD46, 5'-GGTCAAATGTC GATTTCAGT-3'; R-CD46, 5'-AATCACAGCAATGACCC AAA-3'. To establish mouse models with liver metastases, animals were infused with 1×10^6 TC1-CD46 cells through a permanently placed portal vein catheter as described elsewhere (Ni *et al.*, 2006). For intravenous application, 2×10^9 plaque-forming units (PFU) of adenoviral vector in 100 μ l of PBS was injected through the tail vein. For analysis of adenoviral transduction, 3 days after adenovirus injection, liver metastases were microdissected and digested for 2 hr at 37°C with collagenase (1 mg/ml; Roche, Mannheim, Germany) plus 0.1 mM $CaCl_2$ in RPMI 1640. This was followed by incubation with Versene (1:1, v/v) for 1 hr. Protease digestion was stopped by adding FBS to a final concentration of 10%. Cells were pelleted, resuspended in 2 ml of RPMI-10% FBS-DNase I (1 mg/ml), and incubated for 30 min at 37°C. After washing, tumor cells were subjected to flow cytometric analysis for GFP fluorescence and CD46 expression, using an anti-CD46 antibody (clone E4.3) conjugated to phycoerythrin (PE) (Santa Cruz Biotechnology, Santa Cruz, CA).

For therapy studies, NOD.CB17-*Prkdc*^{scid}/J (Jackson Laboratory, Bar Harbor, ME) were used. Mice underwent intraportal transplantation of 1×10^6 human colon adenocarcinoma HT-29 cells. Twenty-three days later, when metastases had established, mice were intravenously injected with X-bp and/or Ad5/35.IR-E1A/TRAIL as described in the caption to Fig. 5. Serum was collected on day -1 and on days 4, 8, and 12 after adenovirus injection. On day 12 after adenovirus administration, animals were killed and tumors were microdissected and weighed.

Quantitative PCR for adenoviral genomes present in serum

Heparinized blood, collected from animals at the indicated time points, was centrifuged and the viral load in serum was analyzed by quantitative PCR (qPCR). Samples were diluted 1:1000 and qPCR was performed with primers (Ad5F, 5'-GCCCCAGTGGTCATACATGCACATC-3'; and Ad5R,

FIG. 1. *In vitro* adenovirus attachment and transduction with CHO cells. **(A)** Attachment of viral particles. ^3H -labeled adenoviruses were added to CHO cells in the absence or presence of factor X (FX, $8\text{ }\mu\text{g/ml}$) and the number of viral particles (VP) bound per cell was determined after 1 hr of incubation on ice. **(B)** Adenoviral transduction. CHO cells were infected with Ad5-GFP, Ad5/35-GFP, and Ad35-GFP at an MOI of 20 and 100 PFU/cell in the presence or absence of human factor X ($8\text{ }\mu\text{g/ml}$) in complete medium containing FCS. After 2 hr of incubation with adenoviral vectors, medium was removed and cells were washed. GFP expression was analyzed by flow cytometry after 24 hr of incubation in complete medium. $n = 3$, $*p < 0.05$. ns, nonsignificant.

5'-GCCACGGTGGGGTTTCTAAACTT-3') against adenoviral hexon (Heim *et al.*, 2003). All reactions, performed in triplicate in a total reaction volume of $15\text{ }\mu\text{l}$, using ImmoMix (Bioline USA, Taunton, MA), SYBR green (Bioline USA), and a $3\text{ }\mu\text{M}$ concentration of each primer, were carried out in a GeneAmp 5700 instrument (Applied Biosystems, Foster City, CA). The following parameters were used for amplification and melting curve analysis: 95°C for 10 min (95°C for 15 sec, 60°C for 1 min) 40 cycles, 60°C for 2 min, 95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec. For quantitative analysis, viral genomes were purified from fixed numbers of viral particles for each adenovirus (assessed spectrophotometrically), and serially diluted to generate a standard curve.

Serum cytokines

Concentrations of interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1/CCL-2, interferon (IFN)- γ , TNF- α , IL-10, and IL-12 were measured by cytometric bead array assay according to the manufacturer's instructions (BD Biosciences, San Jose, CA).

Serum carcinoembryonic antigen levels

The enzyme-linked immunosorbent assay (ELISA) for human carcinoembryonic antigen (CEA) (Calbiotech, Spring Valley, CA) was used according to the manufacturer's protocol. For analysis, mouse serum samples were diluted 1:10 with PBS.

Results and Discussion

In this study, we tested whether the combination of X-bp and an Ad5/35 vector or a vector derived from serotype 35 would result in less sequestration by hepatocytes and better transduction of CD46^{high} liver metastases in CD46 transgenic mice. We used E1/E3-deleted Ad5/35 and Ad35 vectors that contained the same CMV-GFP expression cassette inserted into the E3 region. For comparison, we also employed an Ad5-GFP vector.

In vitro adenovirus attachment and transduction studies

First we studied the effect of FX on adenovirus attachment and infection *in vitro* on CHO cells. CHO cells do not express the Ad5 receptor CAR, or the Ad5/35 and Ad35 receptor CD46, but possess heparan sulfate proteoglycans (HSPGs) on their surface (Tuve *et al.*, 2008). Notably, HSPGs are involved in the uptake of Ad-FX complexes in the liver (Shayakhmetov *et al.*, 2005; Waddington *et al.*, 2008). Attachment studies were performed on ice with [^3H]thymidine-labeled Ad5, Ad5/35, and Ad35 viral particles (Fig. 1A). We found only inefficient attachment of viral particles in the absence of FX, because of the lack of CAR and CD46 expression on CHO cells. Addition of FX ($8\text{ }\mu\text{g/ml}$) to cells together with ^3H -labeled adenoviral vectors increased the number of attached [^3H]Ad5 and [^3H]Ad5/35 particles 60.1- and 22.3-fold, respectively, whereas [^3H]Ad35 attachment increased only 6.6-fold. The lesser

FIG. 2A-D. (continued next page).

FIG. 2. Transduction of liver metastases derived from TC1-CD46 cells in CD46 transgenic mice. (A) *In vitro* transduction of TC1-CD46 cells with Ad5/35 and Ad35. After 2 hr of incubation with adenoviral vectors, medium was removed and cells were washed. GFP expression was analyzed by flow cytometry after 24 hr of incubation in complete medium. $n = 3$. Shown are mean values. Standard deviation was less than 10%. (B) TC1-CD46-derived liver metastases stained for CD46 expression. A total of 1×10^6 TC1-CD46 cells were injected into CD46 transgenic mice via a portal vein catheter. Three weeks later, livers with established metastases were analyzed for CD46 expression, using an anti-CD46-PE antibody (Abcam). CD46 staining appears in red. (C) Three weeks after TC1-CD46 cell transplantation, mice received an intravenous injection of 2×10^9 PFU of Ad5/35 or Ad35 vectors via the tail vein. Half of the mice were intravenously injected with 200 μ l of X-bp (4.8 μ g/kg in PBS) 30 min before adenovirus injection. The other half received PBS preinjection. Three days later, liver sections were analyzed for GFP fluorescence. Representative sections that contained either tumor tissue only or liver tissue only are shown. Original magnification, $\times 20$. (D) Higher magnification ($\times 40$) of GFP-expressing hepatocytes (left, liver-Ad5/35) and GFP-expressing TC1-CD46 tumor cells (right, tumor-Ad35 + X-bp). Scale bar: 100 nm. (E) Three weeks after TC1-CD46 cell transplantation, mice received an intravenous injection of 2×10^9 PFU of Ad5, Ad5/35, or Ad35 vectors via the tail vein. Half of the mice were intravenously injected with 200 μ l of X-bp (37.5 μ g/mouse in PBS) 30 min before adenovirus injection. The other half received PBS preinjection. The number of tumor-localized GFP-positive cells on liver sections was counted (10 sections per tumor, 5 mice per group). $*p < 0.05$. ns, nonsignificant. (F and G) Quantification of GFP-positive cells in cell suspensions of micro-dissected liver metastases. Tumor tissue was digested with collagenase and Versene and cell suspensions were analyzed by flow cytometry for GFP and CD46 expression. Representative samples are shown. Transduced TC1-CD46 cells appear as GFP⁺/CD46^{high} cells (upper right quadrant). Controls are cell suspensions from mock-injected mice stained with an isotype control antibody ("isotype control") and cell suspensions from mock-injected mice stained with an anti-CD46-PE antibody ("no virus"). [(F) is the graphical representation of the flow cytometric data shown in (G)]. $n = 5$. $*p < 0.05$. ns, nonsignificant.

impact of FX in [³H]Ad35 attachment is probably due to the lower affinity of the Ad35 hexon-FX interaction (compared with the Ad5 hexon-FX interaction). Adenoviral transduction studies were performed on CHO cells infected with Ad5-GFP, Ad5/35-GFP, and Ad35-GFP at MOIs of 20 and 100 PFU/cell for 2 hr in the presence and absence of FX (8 μ g/ml) (Fig. 1B). Cells were then washed and GFP expression was analyzed by flow cytometry 24 hr later. In agreement with earlier studies (Dechecchi *et al.*, 2001; Tuve *et al.*, 2008), we found low-level transduction of CHO cells with all vectors despite the absence of primary attachment receptors. Interestingly, however, when infections were performed in the presence of FX (8 μ g/ml), the percentage of GFP-expressing cells significantly decreased (2.3-fold) in Ad5- and Ad5/35-infected cells but not in Ad35-infected cells. No difference between Ad5/35 and Ad35 in transduction efficiency was seen on CHO cells that were modified to express CD46 (Gaggar *et al.*, 2003) (data not

shown). Our transduction data on CHO cells are in conflict with studies on cultured hepatocytes, where FX increased Ad5 transduction (Kalyuzhnyi *et al.*, 2008). We speculate that different types of HSPGs are present on CHO cells and on hepatocytes. Furthermore, it cannot be excluded that during viral infection postattachment steps are different for FX-mediated infection of CHO and hepatocytes.

In vivo transduction studies in CD46 transgenic mice with TC1-CD46 liver metastases

To study whether avoiding adenovirus-FX interaction *in vivo* can improve tumor transduction, we used TC1-CD46 cells (a C57BL/6-derived lung cancer cell line) that stably express CD46 at a level of 5×10^5 RNA copies per cell (Ni *et al.*, 2006). *In vitro* transduction studies showed that TC1-CD46 cells can be efficiently transduced by the Ad5/35 and Ad35

vectors with comparable efficiency (Fig. 2A). To create a tumor model, we injected 1×10^6 TC1-CD46 cells into the portal vein of the CD46 transgenic C57BL/6 mouse line MCP8B (Horvat *et al.*, 1996). Three weeks after transplantation of TC1-CD46 cells, clearly visible CD46^{high} tumor nests formed in the liver (Fig. 2B). In a previous study, we have reported that in this mouse model, CD46 mRNA expression levels are >50-fold less in liver tissue than in microdissected TC1-CD46-derived liver metastases (Ni *et al.*, 2006). Three weeks after TC1-CD46 cell transplantation, mice received an injection of 2×10^9 PFU of Ad5, Ad35, or Ad5/35 vector via the tail vein. Half of the mice were intravenously injected with 200 μ l of X-bp (37.5 μ g/mouse in PBS) 30 min before adenovirus injection (this X-bp dose was found to be safe in one study [Waddington *et al.*, 2008]). The other half received PBS preinjection. Three days later, sections of liver, spleen, lung, kidney, brain, skeletal muscle, heart, and intestines were analyzed for GFP expression. Significant GFP expression was found only in liver metastases, liver parenchyma, and spleen. Numbers of GFP-positive tumor cells were counted on 10 sections per tumor (Fig. 2C–E). GFP-expressing tumor cells and hepatocytes were distinguished on the basis of their morphology. For Ad5 and Ad5/35, X-bp preinjection increased the number of GFP-expressing tumor cells about 5.5-fold ($p < 0.05$); however, as expected, X-bp preinjection had no significant effect on Ad35 transduction of tumor cells. Importantly, the number of GFP-positive tumor cells in mice that received Ad35 was 42.9-fold higher than in mice injected with the Ad5/35 vector, which indicates a clear advantage of the Ad35 vector in comparison with Ad5/35 in this model. While $6.2 \pm 1.3\%$ and $3.1 \pm 1.5\%$ of hepatocytes expressed GFP after injection of Ad5/35 with and without X-bp, respectively, only sparse GFP-positive hepatocytes were found in Ad35-injected mice in both X-bp- and mock-treated groups. In Ad5-injected mice with and without X-bp treatment, $78 \pm 8.9\%$ and $41 \pm 4.3\%$ of hepatocytes were found to express GFP, respectively. Compared with Ad5/35 and Ad35 vectors, tumor transduction with Ad5 vectors was less efficient. This is in agreement with studies by us and others showing that CD46-targeting vectors are superior in tumor cell transduction and safety over CAR-targeting, Ad5-based vectors (Stone and Lieber, 2006; Sakurai *et al.*, 2007). Therefore, we did not include Ad5 vectors in our subsequent studies.

To better quantify the number of GFP-expressing cells in TC1-CD46-derived liver metastases after intravenous injection of Ad5/35 and Ad35 vectors (with and without X-bp pretreatment), microdissected tumors were digested with collagenase and Versene. Cell suspensions were analyzed for CD46 and GFP by flow cytometry (Fig. 2F and G). In cell suspensions, transduced TC1-CD46 tumor cells appear as CD46^{high}GFP⁺ cells. In mice injected with Ad5/35, the percentage of GFP⁺ cells in the CD46^{high} cell fractions was $3.8 \pm 2.0\%$. Pretreatment with X-bp increased transduction to $7.7 \pm 2.9\%$ ($p = 0.002$). Injection with Ad35-GFP resulted in $25 \pm 7.1\%$ GFP⁺CD46^{high} cells (Fig. 2F) and X-bp treatment did not increase this number (data not shown). We were not able to generate viable hepatocyte suspensions by direct digestion of liver tissue with collagenase and Versene.

It is thought that reduction of unspecific adenovirus sequestration increases the circulation time of virus in the peripheral blood, which in turn contributes to better transduction of target cells (Wickham, 2000). We therefore mea-

FIG. 3. Blood clearance of adenoviral vectors. CD46 transgenic mice were injected intravenously with 2×10^9 PFU of Ad5/35 or Ad35 vector without or with X-bp pretreatment (as described in Fig. 2). Heparinized blood samples were collected by retroorbital bleeding 15 min, 1 hr, and 6 hr after adenovirus injection. Total DNA was extracted from serum fractions along with carrier DNA, and analyzed for the presence of adenoviral genomes by qPCR using universal, pan-serotype primers against hexon. $n = 5$ mice per group.

sured the amount of Ad5/35 and Ad35 vector genomes present in serum at 15 min, 1 hr, and 6 hr after adenovirus administration with and without X-bp pretreatment. We found that X-bp treatment, which prevents FX-mediated adenovirus sequestration, significantly delayed Ad5/35 viral genome clearance (Fig. 3). The number of Ad5/35 genomes present in serum 15 min and 1 hr after injection was 3.8- and 14-fold higher, respectively, in animals pretreated with X-bp. Clearance of Ad35 from blood was overall slower than that of Ad5/35 genomes and was not affected by X-bp treatment. For all experimental groups the amount of vector genomes present 6 hr postinjection was less than 1% of the amounts measured 15 min postinjection.

These data indicate that an approach that reduces adenoviral binding to FX (e.g., X-bp in combination of Ad5/35) or the use of an adenoviral serotype with hexons that bind less to FX (e.g., Ad35) reduces unspecific hepatocyte transduction and increases the circulation time in blood, which in turn results in increased gene transfer into liver metastases.

Safety of the combination approach of X-bp and Ad5/35 and Ad35 injection

We studied the biodistribution of gene expression and serum levels of proinflammatory cytokines and chemokines in treated CD46-transgenic mice. In the spleen, GFP-expressing cells were found for all groups in the peripheral zone of germinal centers. The number of GFP-positive cells was similar in all groups and we therefore speculate that Ad5/35 and Ad35 transduction of splenic cells is independent of FX. No significant GFP expression was observed with our vectors on lung sections of CD46 transgenic mice. In earlier studies, vector DNA has been detected in lungs early after intravenous injection of wild-type Ad35 into CD46 transgenic mice (Verhaagh *et al.*, 2006; Stone *et al.*, 2007). However, Ad35 sequestered in the lung is rapidly degraded (Stone *et al.*, 2007), which might

FIG. 4. Serum cytokines and chemokines. Mice received an intravenous injection of PBS (mock), X-bp (37.5 μ g/mouse in PBS), or 2×10^9 PFU of Ad35 and Ad5/35 vectors with and without X-bp pretreatment. Serum samples were harvested 6 hr later. $n = 5$. * $p < 0.05$. ns, nonsignificant.

explain the absence of transgene expression in lungs in our study. Notably, one study in cynomolgus monkeys also did not observe significant lung transduction with an intravenously injected Ad35 vector (Sakurai *et al.*, 2008). A question therefore arises concerning why the CD46-interacting vectors Ad5/35 and Ad35 transduce TC1-CD46 tumors but not other CD46-expressing tissues, particularly if FX-mediated liver sequestration is prevented. We speculate that (1) there is a threshold level of CD46 that must be present on cells to be efficiently infected with Ad35 fiber-containing vectors. As outlined previously, there is a more than 50-fold difference

in CD46 expression levels between liver parenchyma and metastases; and/or (2) CD46 is not accessible in most normal organs. Our preliminary data indicate that CD46 can be trapped in tight junctions in epithelial cells (Strauss *et al.*, 2008).

Proinflammatory cytokines and chemokines were measured in serum 6 hr after adenovirus infusion (Fig. 4). X-bp injection without subsequent adenoviral vector application did not increase cytokine/chemokine levels compared with mock (PBS, no Ad vector)-treated animals. Pretreatment of Ad5/35-injected mice with X-bp resulted in increased serum levels of IL-6 and IFN- γ compared with PBS-injected groups.

FIG. 5. Therapeutic effect of X-bp treatment in combination with an oncolytic Ad5/35 vector (Ad5/35-IR-E1A/TRAIL). Immunodeficient mice received an intraportal transplantation of human colon carcinoma HT-29 cells. HT-29 cells secrete carcinoembryonic antigen (CEA). Twenty-three days after tumor cell transplantation mice received an intravenous injection of either (1) PBS ("Co"), (2) 200 μ l of X-bp (37.5 μ g/mouse in PBS), (3) 2×10^9 PFU of Ad5/35-IR-E1A/TRAIL in 200 μ l of PBS ("Ad5/35-oncol"), or (4) 200 μ l of X-bp (37.5 μ g/mouse in PBS) 30 min before Ad5/35-IR-E1A/TRAIL injection (X-bp + Ad5/35-oncol). Serum was collected on day -1 and on days 4, 8, and 12 after adenovirus injection for analysis of CEA levels (A). On day 12 after adenovirus administration, animals were killed, tumors were microdissected, and weighed (B). $n = 5$ mice per group. * $p < 0.001$.

Overall, IL-6, MCP-1/CCL-2, IFN- γ , TNF- α , and IL-10 levels were higher for Ad35-injected than for Ad5/35-injected mice. For Ad35-injected mice, X-bp treatment had no effect on IL-6 levels. However, MCP-1/CCL-2, TNF- α , and IL-10 levels were decreased in X-bp-treated Ad35-injected mice compared with mock-treated mice, which might be the result of less FX-mediated uptake of adenoviral particles into hepatocytes or other cytokine-producing cells. There was no difference in IL-12 levels between the groups. Although Ad35 caused acute toxicity, when compared with earlier toxicity studies with other adenoviral serotypes in CD46 transgenic mice, levels of proinflammatory cyto- and chemokines were markedly lower in Ad35-injected mice than, for example, in mice injected with Ad3 or Ad4 (Stone *et al.*, 2007). Notably, less innate toxicity was seen in nonhuman primates administered Ad35 rather than Ad5 vectors (Sakurai *et al.*, 2008). In our study, none of the experimental groups displayed signs of distress or morbidity.

Therapeutic efficacy of Ad5/35-based oncolytic vector in combination with X-bp treatment

Our observation that X-bp increases transduction of CD46^{high} liver metastases with an Ad5/35 vector has implications for oncolytic virotherapy. Oncolytic viruses based on human adenoviruses, however, replicate only inefficiently in mouse tumor cells such as TC1-CD46 (Bernt *et al.*, 2005). We therefore decided to use an immunodeficient mouse model with liver metastases derived from human colon cancer HT-29 cells. HT-29 cells secrete carcinoembryonic antigen (CEA), and serum CEA levels in mice directly correlate with the tumor burden, as we have shown in one study (Kuhn *et al.*, 2008). As a model oncolytic adenovirus, we used Ad5/35-IR-E1A/TRAIL (Sova *et al.*, 2004). This vector is targeted to CD46 by the adenoviral serotype 35 fiber, allowing for tumor-

specific, replication-activated expression of E1A and TRAIL. Notably, there is no conditionally replicating oncolytic Ad35 vector available at this time.

Mice carrying preestablished liver metastases and displaying clearly detectable CEA levels received a single intravenous injection of 2×10^9 PFU of Ad5/35-IR-E1A/TRAIL with or without injection of X-bp (37.5 μ g/mouse in PBS) 30 min before adenovirus administration (Fig. 5). Serum CEA levels were measured by ELISA on day -1 and on days 4, 8, and 12 postinjection. On day 12 postinjection, liver metastases were microdissected and weighed. Ad5/35-IR-E1A/TRAIL injection alone resulted in significant lower CEA levels at all time points and lower tumor burden ($p < 0.001$). Importantly, pretreatment of mice with X-bp greatly increased the therapeutic effect of Ad5/35-IR-E1A/TRAIL as reflected in CEA levels and tumor burden ($p < 0.001$). Because, previously, an Ad5-based oncolytic vector (ONYX-015) did not exert a significant antitumor effect in the HT-29 liver metastasis model (Kuhn *et al.*, 2008), we did not include an Ad5-based vector in this study.

Our study shows that pretreatment of mice with X-bp increases transduction of liver metastases after intravenous vector administration and improves the antitumor efficacy of an Ad5/35-based oncolytic adenovirus. Our work also gives a rationale for using Ad35 vectors for tumor targeting.

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Author Disclosure Statement

No competing financial interests exist.

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