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Replacement of native adenovirus receptor-binding sites with a new attachment moiety diminishes hepatic tropism and enhances bioavailability in mice


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Abstract

The in vivo efficacy of adenovirus vectors (AdVs) in gene delivery strategies is hampered by the broad tissue tropism of the virus and its efficient binding to human erythrocytes. To circumvent these limitations, we developed a genetically targeted prototype AdV. We replaced the adenovirus fiber with a chimeric molecule consisting of the fiber tail domain, the reovirus $\sigma$1 oligomerization domain, and a polyhistidine tag as model targeting moiety. We also abolished the integrin-binding motif in the penton base protein. The chimeric attachment molecule was efficiently incorporated onto AdV capsids, allowed efficient propagation of AdV without requirement for complementing fiber and conferred highly specific tropism to the AdV. Importantly, the targeted AdV exhibited markedly reduced tropism for liver cells. In comparison to control AdV with native tropism, the targeted AdV showed 1,000-fold reduced transduction of HepG2 cells and 10,000-fold reduced transduction of mouse liver cells in freshly isolated liver slices. Following intravenous inoculation of C57BL/6 mice, the targeted AdV exhibited delayed clearance in comparison to the native AdV, leaving approximately 10-fold greater levels in the blood 2 h after inoculation. For all tissues analyzed, the targeted AdV displayed significantly reduced in vivo transduction in comparison to the native vector. Furthermore, in contrast to the native AdV, the targeted AdV did not bind human erythrocytes. Together, our findings suggest that the targeted AdV design described here provides a promising platform for systemic in vivo gene delivery.
8.1 Introduction

Human adenoviruses of serotype 2 and 5 are acknowledged as promising gene-delivery vehicles. However, in vivo utility of these adenovirus vectors (AdVs) is hampered by their promiscuous tropism. The broad array of cell types infected by adenovirus leads to AdV transduction of undesired tissues and reduces the fraction of the administered dose available for transduction of specific target cells. The liver is the major target of AdV transduction in mice following systemic administration and accounts for more than 99% of total transduction (1-4). The liver also is targeted in non-human primates, although in these animals a significant fraction of the administered AdV dose transduces the spleen (5). An additional complication of AdV administration in humans is the interaction of the vector with erythrocytes (6, 7). In contrast to murine erythrocytes, human erythrocytes are efficiently bound by AdVs, which prevents specific transduction of target cells. In blood samples obtained from a patient enrolled in a clinical gene therapy trial, greater than 98% of the virus was associated with erythrocytes (7). Thus, the native tropism of adenovirus diminishes the in vivo efficacy of AdV-mediated gene delivery. To circumvent this problem and enhance the specificity of AdV transduction, a new generation of AdVs is required. Such vectors should eliminate native adenovirus tropism and incorporate unique target-binding moieties.

The tropism of adenovirus types 2 and 5 is determined by at least three distinct receptor-binding sites on the viral capsid. These sites mediate attachment to the coxsackievirus and adenovirus receptor (CAR), \( \alpha_v \) integrins, and heparin sulphate glycosaminoglycans (HSG), respectively (8-13). Recently, also blood coagulation factors FVII, FIX, FX and protein C and complement component C4-binding protein (C4BP) were shown to play a role in adenovirus’ tropism by bridging adenovirus to cell-surface receptors (14, 15). Ablation of the direct receptor interactions can be accomplished by modification or elimination of the receptor-binding sequences, which induces a change in the in vivo biodistribution of AdVs in mice, rats, and non-human primates (2, 4, 5, 16-19). Remarkably, whereas CAR forms the primary receptor for AdVs in vitro, ablation of the CAR-binding site alone does not substantially affect the biodistribution of AdVs in vivo (2, 3). Instead, alteration of at least two binding sites is required to influence AdV biodistribution. AdVs lacking binding sites for both CAR and \( \alpha_v \) integrins exhibit significantly decreased transduction of the heart, kidney, liver, and lung (2, 4).
Ablation of the HSG-binding site in combination with alteration of the binding sites for either CAR or αv integrins also reduces transduction of many organs (4, 17). Not unexpectedly, the most substantial reduction in tissue transduction is obtained when all three binding sites are altered (4, 19).

Ablation of native tropism (de-targeting) has to be combined with the introduction of new tropism to come to the ultimate AdV with beneficial properties for in vivo application. Generally, two strategies have been used to provide AdVs with new tropism: a one-component strategy, which is based on genetic modification of fiber or another capsid component, and a two-component strategy, which relies on the use of bi-specific adapter molecules (20). There are advantages and disadvantages of each. The one-component strategy is better defined and more feasible for generation of clinically applicable targeted vectors. It also is better suited for design of targeted oncolytic adenoviruses, which rely on efficient in vivo production of targeted viral progeny to produce anti-tumor effects. Since the fiber molecule principally defines adenovirus tropism, most targeting approaches exploit this capsid protein as a platform for display of novel binding entities. However, insertion of large and complex folded ligands into the fiber can alter its structure and preclude encapsidation onto the virus particle. Several groups including our own have developed chimeric attachment molecules in which the fiber knob is replaced with an exogenous trimerization domain (21-23). This strategy allows the introduction of complex receptor-binding moieties and simultaneously causes partial ablation of native AdV tropism by removal of the CAR-binding site. Until recently, application of such targeted AdVs was severely limited by their impaired propagation efficiency, which necessitated wild-type fiber complementation for their production.

We recently reported the generation of a new genetically targeted AdV, AdG.L.Tail-T(ii)-MH, which contains the chimeric attachment molecule Tail-T(ii)-MH replacing the fiber (24). Tail-T(ii)-MH consists of the tail domain of adenovirus fiber, the T(ii) oligomerization domain of reovirus attachment protein σ1, and C-terminal Myc and polyhistidine tags. Therefore, AdG.L.Tail-T(ii)-MH lacks binding sites for CAR and HSG, for the ‘bridging’ factors FIX and C4BP, and most likely for FVII, FX and protein C as well (14, 15). Instead, this AdV is targeted to an artificial His-tag binding receptor which allows efficient propagation on packaging cells expressing this receptor without fiber complementation (24). To further reduce native adenovirus tropism for systemic in vivo gene delivery, we generated a derivative of AdG.L.Tail-T(ii)-MH, AdG.L.p*Tail-T(ii)-MH, which lacks the integrin-
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binding site as well. In this study, we evaluated both Tail-T(ii)-MH-containing AdVs for targeting specificity, liver cell transduction, and biodistribution following intravenous inoculation of mice. In comparison to control AdV, AdG.L.p*Tail-T(ii)-MH displays substantially enhanced bioavailability and diminished tissue transduction. This new AdV thus offers a promising platform for the design of highly targeted AdVs for numerous gene-delivery applications.

8.2 Materials and Methods

Cell lines
The Ad5 E1-transformed human embryonic kidney cell line 293 and the human hepatoma cell line HepG2 were purchased from the American Type Culture Collection. The cell line 293.HissFv.rec is a derivative of the cell line 293 that stably expresses an artificial His-tag binding receptor (25). Cell lines were maintained in F12-supplemented Dulbecco modified Eagle medium (DMEM F-12) supplemented to contain 10% fetal calf serum and antibiotics (Gibco BRL, Life Technologies B.V., Breda, The Netherlands). Medium used for 293.HissFv.rec was supplemented to contain 300 \( \mu \)g/ml of G418.

Adenoviral vectors
The genetically targeted AdV, AdG.L.Tail-T(ii)-MH, and the control vector, AdG.L, were described previously (24). Both AdVs contain GFP and luciferase reporter genes in the E1 locus. AdG.L expresses wild-type fiber and penton base genes conferring native adenovirus tropism, whereas AdG.L.Tail-T(ii)-MH contains Tail-T(ii)-MH-encoding sequences in place of the fiber gene and consequently lacks CAR- and HSG-binding sites. To abolish the integrin-binding site of AdG.L.Tail-T(ii)-MH, the integrin-binding motif RGD in the penton base protein was replaced with RGE to generate the new AdV, AdG.L.p*.Tail-T(ii)-MH. Site-directed mutagenesis of the penton base gene was performed using primers 5’-GCCATCCGCGGCGAGACCTTTGCCCACAC-3’, 5’-TCACCTGACCCGTGGATGAGG-3’, 5’-GGCAAGATCCCTCTCGTG-3 and 5’-GTGCCCAGCTCTCGCCG-3 and pBHG11 (26) as template. The resulting PCR product containing a penton base gene with a mutated integrin-binding site, designated p*, was digested with Pmel and Ascl and inserted in pBHGI11∆Asc. This derivative of pBHGI11 was generated by digestion of pBHGI11 with Ascl and religation. After
insertion of p* into pBHGI11∆Asc, the Ascl fragment was re-introduced into that plasmid, generating pBHGI11p*. This construct was digested with Rsrl, and the penton base gene-containing fragment of 7707 bp was isolated and inserted into the 27,246 bp, Rsrl-digested fragment of pAdEasy.AdTail-σ1T(ii)-MH (24). The resultant pAdEasy.p*.AdTail-σ1T(ii)-MH construct was recombined with pAdTrack.CMV.Luc (24) to generate pAdG.L.p*.Tail-T(ii)-MH, which contains the full-length adenovirus genome with GFP and luciferase reporter genes in place of E1, the Tail-T(ii)-MH-encoding sequences in place of the fiber gene, and an RGE-encoding sequence in the penton base gene.

AdG.L.p*.Tail-T(ii)-MH was generated by transfecting PacI-linearized pAdG.L.p*.Tail-T(ii)-MH into 293.HissFv.rec cells using Lipofectamine Plus (Invitrogen Life Technologies). Resultant virus was propagated using 293.HissFv.rec cells to reach a scale of twenty T182 flasks. Harvested AdVs were purified by two successive rounds of CsCl centrifugation, followed by dialysis. Infectious titers of AdVs were quantified on the basis of virus particles (vp) and infectious units (IU) (24).

**Immunoelectron microscopy**

Immunoelectron microscopy was performed as described (27). CsCl-purified virions were spotted onto carbon-coated copper grids and incubated with anti-knob monoclonal antibody (MAb) 1D6.14 (28), anti-Myc MAb 9E10, or anti-(His)_5. Penta-His antibody (Qiagen, Hilden, Germany). Grids were subsequently incubated with rabbit anti-mouse serum (Dako Cytomation A/S, Copenhagen, Denmark), followed by gold-labeled protein-A (Amersham Biosciences Europe GmbH, Freiburg, Germany). After fixation (1.5% glutaraldehyde) and negative staining (1% uranyl acetate), virions were visualized using a Philips EM 410-LS transmission electron microscope.

**Immunoblot analysis**

CsCl-purified virions of each AdV (5 x 10^9 vp) were incubated at 95°C for 5 min in denaturating sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, and 2.5% β-mercaptoethanol) and resolved by SDS-10% PAGE. Viral proteins were transferred to PVDF membranes and incubated with anti-fiber tail antibody Ab4 (Neomarkers, Fremont, CA, USA) or anti-Myc MAb 9E10 as primary antibody. Bound viral proteins were visualized by chemiluminescence using rabbit anti-mouse immunoglobulin G conjugated to horseradish peroxidase (RαM HRP;
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Dako, Glostrup, Denmark) as secondary antibody and Lumilight\textsuperscript{TM} reagent (Roche, Almere, The Netherlands).

**AdV infections**

293, 293.HissFv.rec, and HepG2 cells were seeded at a density of 5 x 10\(^4\) cells/well in 96-well plates one day prior to infection. AdG.L, AdG.L.Tail-T(ii)-MH, and AdG.L.p\(^+\)Tail-T(ii)-MH were either untreated or pre-incubated with 330 ng 1D6.14 MAb or 300 ng Penta-His antibody at room temperature for 1.5 h. After 2 h adsorption to cells, the inoculum was removed, fresh medium was added, and cells were incubated for 48 h. GFP expression was assessed using fluorescence microscopy, or cells were lysed in 50 \(\mu\)l reporter lysis buffer (Promega, Madison, WI, USA), and luciferase activity was measured using the Chemiluminescent Luciferase Assay (Promega) and a Berthold luminometer (Berthold, Bad Wildbad, Germany).

Infection of liver tissue was assessed using liver slices prepared from freshly isolated mouse livers. Livers were resected from C57BL/6 mice, placed in ice-cold Krebs buffer (25 mM D-glucose, 25 mM NaHCO\(_3\) and 10 mM HEPES), and immediately processed by drilling cores of 8 mm in diameter. Cores were sliced at a thickness of 200-250 \(\mu\)m using a Krumdieck tissue slicer (Alabama R&D, Muntfort, AL, USA) in ice-cold Krebs buffer. Liver slices were transferred to 12-well plates and pre-incubated for 1 h in Williams' medium E (WME) supplemented to contain 25 mM D-glucose and antibiotics (Gibco BRL) at 37\(^\circ\)C in a 95% \(\text{O}_2\) and 5% \(\text{CO}_2\) atmosphere. Slices then were incubated with 10\(^8\) vp/slice (~100 vp/cell) in oxygenated WME medium at 37\(^\circ\)C under continuous rocking in a 95% \(\text{O}_2\), 5% \(\text{CO}_2\) climate chamber. After 72 h incubation, slices were lysed in 50 \(\mu\)l CCLR lysis buffer (25 mM Tris-phosphate, 20 mM CDTA, 200 mM DTT, 10% glycerol, 1% Triton X-100) at room temperature for 15 min, followed by three freeze-thaw cycles. Lysates were cleared by centrifugation, and luciferase activity was assessed using 10 \(\mu\)l of the supernatant. Luciferase activity was normalized on the basis of protein concentration as determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

**Biodistribution of AdVs**

Female C57BL/6 mice weighing 20-25 g were obtained from Harlan-CPB (Harlan, Horst, The Netherlands), fed a standard laboratory diet, provided water ad libitum, and allowed to acclimatize for at least one week prior to the initiation of
experiments. Mice were randomly divided into three groups of at least 5 animals each and inoculated with $10^{10}$ vp of AdG.L, AdG.L.Tail-T(ii)-MH, or AdG.L.p*Tail-T(ii)-MH in 200 µl PBS into a lateral tail vein. At 2, 5, 10, 20, 30, 60 and 120 min after inoculation, blood was obtained by tail vein venipuncture and placed into heparinized micropipettes (Marienfeld, Lauda-Königshofen, Germany). AdV concentration in blood samples was quantified by luciferase expression after transduction of 293.HissFv.rec cells. One day prior to assay, 293.HissFv.rec cells were seeded at a density of 5 x $10^4$ cells/well in 96-wells plates. Cells were inoculated with a mixture of 2 µl whole blood and 50 µl DMEM-F12. After 48 h incubation, 293HissFv.rec cells were lysed in reporter lysis buffer (Promega), and luciferase activity was determined. AdV concentration was calculated using a standard curve, which was generated with known concentrations of the corresponding AdV.

Transduction of mouse tissues by AdVs was determined 48 h following intravenous administration. Mice were euthanized, and liver, spleen, kidneys, lungs and heart were resected. Tissues were snap-frozen in liquid nitrogen and homogenized using a mortar and pestle. A 25 mg aliquot of tissue was lysed in 100 µl CCLR lysis buffer by incubation at room temperature for 10 min, followed by two freeze-thaw cycles. Lysates were cleared by centrifugation, and transduction of each tissue was determined by luciferase activity in 20 µl lysate. Luciferase activity was normalized for protein concentration as determined by the Bio-Rad protein assay. Differences in tissue transduction by the three AdVs were evaluated with a one-tailed Mann-Whitney test using GraphPad Instat 3.0 (GraphPad Software, Inc., San Diego, CA). The experimental protocols adhered to the rules outlined in the Dutch Animal Experimentation Act and the "Guidelines on the Protection of Experimental Animals" by the council of the European Community and were approved by the Committee on Animal Research of the VU University Medical Center.

**Interaction of AdV with human erythrocytes**

Blood of mice, rats, or humans was collected in EDTA-tubes and mixed with 1 vol equivalent of Alsever solution (23 mM Tri-sodium citrate, 114 mM glucose, 55 mM NaCl, and 3 mM citric acid [pH 6.1]). Cells were centrifuged at 1,200 g for 10 min and washed three times by repeated resuspension in 2 vol equivalents of Alsever solution and centrifugation at 1,200 g for 10 min. The final pellet was resuspended in Alsever solution to generate a 30% packed-cell suspension.
Hemagglutination was assayed using a 1% erythrocyte suspension, which was generated by dilution of the 30% packed-cell suspension in HA-buffer (PBS, 0.005% BSA). A volume of 50 µl 1% erythrocyte suspension was prelayed in wells of a concave-bottom-shaped 96-well plate and gently mixed with 50 µl of a dilution series of each AdV (stock concentration, 10^{12} vp/ml). After 2 h gravitational sedimentation, plates were photographed and analyzed for hemagglutination.

Binding of AdV virions to erythrocytes was quantified by determining the number of adherent AdV genomes using quantitative PCR (qPCR). A 30% packed-cell suspension of human erythrocytes was diluted in PBS to a physiological concentration of 8.4 x 10^8 erythrocytes per 250 µl. Erythrocytes were incubated with 8.4 x 10^7 vp at 37ºC for 60 min. Virions bound to erythrocytes were separated from unbound virions by centrifugation at 1,200 g for 14 min. The erythrocyte pellet was washed twice with 10 vol equivalents of PBS. Adenovirus DNA in bound and unbound virus fractions was isolated using the QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturer’s protocol. Content of viral genome was quantified using the LightCycler® 480 (Roche Diagnostics, Mannheim, Germany) with the LightCycler® 480 SYBR Green I Master kit, 20 pmol of forward hexon primer 5’-ATGATGCGCAGTGGTCTTA-’3, and 20 pmol of reverse hexon primer 5’-GTCAAAGTACGTGGAAGCCAT-’3. A standard curve was generated with 10-fold serial dilutions of adenovirus DNA.

8.3 Results

Vector generation and characterization
To achieve complete ablation of native adenovirus tropism, we generated a new AdV, AdG.L.p*Tail-T(ii)-MH, which lacks all known adenovirus receptor-interaction sites. AdG.L.p*Tail-T(ii)-MH was derived from AdG.L.Tail-T(ii)-MH (24). Both vectors encode the chimeric attachment molecule Tail-T(ii)-MH in place of fiber. However, AdG.L.p*Tail-T(ii)-MH incorporates the non-binding sequence, R_{340}GE_{342} in it’s penton base (29), in place of the integrin-binding motif, R_{340}GD_{342}. AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH were generated using 293.HissFv.rec cells (25), which express an artificial receptor comprising an anti-His-tag single-chain antibody. Both vectors replicated efficiently following infection of these cells without requirement for fiber complementation. In fact,
their particle titers were similar to that of the native control vector AdG.L following infection of 293.HissFv.rec cells (Table 8.1).

<table>
<thead>
<tr>
<th>Virus vector</th>
<th>Receptor binding site</th>
<th>Virus particles (vp/ml)</th>
<th>Infectious units (IU/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdG.L</td>
<td>+</td>
<td>5.3 x 10^{12}</td>
<td>2.6 x 10^{10}</td>
</tr>
<tr>
<td>AdG.L.Tail-T(ii)-MH</td>
<td>-</td>
<td>9.7 x 10^{12}</td>
<td>5.4 x 10^{9}</td>
</tr>
<tr>
<td>AdG.L.p*Tail-T(ii)-MH</td>
<td>-</td>
<td>3.8 x 10^{12}</td>
<td>1.7 x 10^{9}</td>
</tr>
</tbody>
</table>

* determined on 293.HissFv.rec cells

Tropism mediated by the His tag allowed us to assess targeted transduction independent of native infection pathways. AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH preparations exhibited similar vp-to-IU ratios, which were approximately ten-fold higher than the vp-to-IU ratio of the native control vector (Table 8.1). The lower functional titers of the targeted vectors might be attributable to differences in the expression levels of CAR and the His-tag binding receptor on the surface of 293.HissFv.rec cells or differences in vector affinity for these receptors. The similar vp-to-IU ratios observed for the targeted AdVs with and without an intact RGD sequence suggest that His-tag-mediated infection does not require penton base interactions with integrins.

To assess physical incorporation of Tail-T(ii)-MH in the capsids of the targeted AdVs and to study accessibility of the Myc and His-tags for binding, CsCl-purified virions were analyzed by immunoelectron microscopy (figure 8.1A). Immunogold labeling demonstrated anti-Myc MAb association with AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH virions but not with control vector AdG.L. Similar results were obtained using an anti-His MAb (data not shown). In contrast, incubation of the AdVs with an anti-fiber knob MAb did not reveal any association of gold particles with AdG.L.Tail-T(ii)-MH or AdG.L.p*Tail-T(ii)-MH, whereas AdG.L was clearly stained (figure 8.1A). Together, these findings confirm that wild-type fiber in the native vector has been replaced with the chimeric attachment protein in AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH.

The attachment protein incorporated in the AdVs was further characterized by immunoblotting (figure 8.1B). In a lysate of purified AdG.L particles, a fiber tail-specific antibody detected a 64 kDa protein, which corresponds to wild-type
Targeted adenovirus lacking native tropism

fiber. In AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH lysates, a 22kDa protein was detected that corresponds to the expected size of Tail-T(ii)-MH. To corroborate the identity of this protein, we reprobed the blot with a Myc-specific MAb. This antibody bound to the 22 kDa protein of AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH and did not recognize any capsid component of the control vector. These findings confirm the exclusive incorporation of Tail-T(ii)-MH in the capsids of AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH.

Transduction profile of targeted AdVs
To assess the effect of ablation of native adenovirus tropism and introduction of a binding sequence that engages an artificial His-tag binding receptor, we compared AdV transduction of parental 293 cells and 293.HissFv.rec cells (figure 8.2A). Cells were inoculated with AdVs at varying MOIs, incubated for 48 h, and assayed for luciferase activity. We observed dose-dependent transduction of both cell lines by all three viruses. As expected, native control vector AdG.L transduced both cell lines with almost equivalent efficiency. In contrast, the chimeric AdVs exhibited reduced transduction of 293 cells in comparison to 293.HissFv.rec cells.
Over the range of MOIs tested, AdG.L.Tail-T(ii)-MH exhibited an average reduction of ~ 400-fold. Additional alteration of the RGD sequence in AdG.L.p*Tail-T(ii)-MH yielded a decrease in transduction of ~ 1,200-fold. However, in comparison to AdG.L, AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH exhibited only 16- and 25-fold reductions, respectively, in transduction of 293.HissFv.rec cells. Thus, ablation of native adenovirus binding sites and incorporation of a His-tag binding moiety substantially diminishes AdV infection of 293 cells but only modestly reduces infection of 293.HissFv.rec cells.

To confirm that transduction by targeted AdVs is dependent on the binding activity of Tail-T(ii)-MH, we incubated the vectors with an anti-His-tag MAb prior to inoculation of 293.HissFv.rec cells. As controls, we used an anti-fiber knob MAb and native vector AdG.L. Following 48 h incubation, AdV-mediated GFP transduction was assessed by fluorescence microscopy (figure 8.2B). The anti-fiber knob MAb neutralized transduction by AdG.L but not by the two targeted AdVs. Conversely, the anti-His MAb neutralized transduction by the targeted AdVs but not by the native control vector. Therefore, replacement of native adenovirus binding sites with a new binding moiety ablates native tropism and results in highly stringent targeting specificity.
Fig. 8.2 Transduction specificity of targeted AdV. (A) 293 cells and 293.HissFv.rec cells were incubated with AdG.L, AdG.L.Tail-T(ii)-MH, or AdG.L.p*Tail-T(ii)-MH at the indicated MOIs (vp/cell). Forty-eight h after incubation, transduction efficiency was quantified by luciferase expression. The results are presented as mean luciferase activity in light units (LU)/1,000 cells for three independent experiments. Error bars indicate standard deviations. (B) AdG.L, AdG.L.Tail-T(ii)-MH, or AdG.L.p*Tail-T(ii)-MH were incubated with either fiber knob-specific MAb or His-specific antibody prior to infection of 293.HissFv.rec cells at an MOI of 0.5 IU/cell. Forty-eight h after incubation, transduction was visualized by analysis of GFP expression using fluorescence microscopy.

In vitro and ex vivo liver de-targeting
Since the majority of intravenously administered adenovirus is captured in the liver either via transduction of hepatocytes or uptake in Kupffer cells, the primary goal of adenovirus de-targeting for systemic administration is to limit liver sequestration. To determine whether the targeted AdVs display altered hepatic tropism, we first analyzed transduction efficiency using the human hepatoma cell line HepG2. HepG2 cells were inoculated with 1,000 vp/cell, and transduction efficiency was determined 48 h after incubation by quantifying luciferase activity (figure 8.3A). AdG.L was capable of efficient transduction of HepG2 cells, whereas transduction of these cells by both targeted AdVs was substantially reduced. In comparison to AdG.L, AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH displayed ~100- and 1,000-fold reductions in transduction of HepG2 cells, respectively.

We next determined whether the targeted AdVs were altered in transduction of fresh liver slices prepared from C57Bl/6 mice. Each slice was inoculated for 72 h with 10⁸ vp of AdG.L, AdG.L.Tail-T(ii)-MH, or AdG.L.p*Tail-T(ii)-MH. As internal control for liver cell viability, each slice was also inoculated with 10⁸ vp of an AdV
with native tropism expressing DsRed (AdDsRed). After 72 h incubation, AdDSRed transduction was equivalent in all slices (data not shown).

Fig. 8.3 Infection of human hepatoma cells and mouse liver slices by AdVs. (A) HepG2 cells were incubated with AdG.L, AdG.L.Tail-T(ii)-MH, or AdG.Lp*Tail-T(iii)-MH at an MOI of 1,000 vp/cell. Forty-eight hours after incubation, transduction efficiency was quantified by luciferase expression. The results are presented as mean luciferase activity for four independent experiments. Error bars indicate standard deviations. **, P < 0.01. (B) Freshly obtained mouse liver slices were incubated with AdG.L, AdG.L.Tail-T(ii)-MH, or AdG.Lp*Tail-T(iii)-MH at an MOI of 100 vp/cell. After 72 h incubation, transduction efficiency was quantified by luciferase expression. The results are presented as mean luciferase activity for at least six slices. Error bars indicate standard deviations. **, P < 0.01.
Transduction efficiency by the test vectors was quantified by luciferase activity in liver-slice lysates (figure 8.3B). As expected, AdG.L showed efficient liver cell transduction. In contrast, ablation of native binding sites in AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH was associated with a 10,000-fold reduction in liver transduction. In fact, the level of luciferase expression caused by the two targeted AdVs did not differ significantly from that of control slices transduced by AdDsRed, which does not express luciferase. These findings suggest that the targeted AdVs display markedly diminished tropism for the mouse liver.

**Biodistribution of targeted AdVs in C57BL/6 mice**

To directly evaluate the effect of ablation of native adenovirus tropism in vivo, we inoculated C57BL/6 mice with $10^{10}$ AdV particles intravenously. During the first 2 h after inoculation, blood samples were drawn from the tail vein, and AdV titers were determined using 293.HissFv.rec cells (figure 8.4A). All three AdVs exhibited a biphasic clearance pattern with a rapid decrease during the first 30 min, followed by a slower decrease over the remaining 90 min. AdG.L exhibited the most rapid clearance with less than 1% of the administered dose detectable at 10 min and approximately 0.1% at 20 min. Although the clearance profile of AdG.L.Tail-T(ii)-MH suggests a somewhat improved bioavailability in the first 30 min after administration, clearance of this AdV did not differ significantly from that of AdG.L in the interval thereafter. In contrast, the half-life of AdG.L.p*Tail-T(ii)-MH immediately following inoculation was prolonged, with approximately 10% of the administered dose detected in the circulation at 10 min and 1% at 30 min. The virus then exhibited a rate of clearance identical to that of the other two vectors but at an approximately 10-fold higher concentration. Thus, AdG.L.p*Tail-T(ii)-MH displays enhanced bioavailability in the blood in the first 2 h after inoculation in comparison to the other AdVs.

To determine transduction efficiency of mouse tissues following intravenous administration of AdVs, mice were sacrificed 2 days post-inoculation, organs were resected and processed for luciferase assay (figure 8.4B). As expected, AdG.L preferentially transduced the liver, consistent with several previous reports (2, 4, 5, 16-19). Removal of the native adenovirus binding sites in the targeted AdVs significantly reduced liver transduction. In comparison to the control vector, AdG.L.Tail-T(ii)-MH exhibited a 70% ($P < 0.05$) reduction in liver transduction and AdG.L.p*Tail-T(ii)-MH exhibited an 83% ($P < 0.01$) reduction. Transduction of other tissues by AdG.L.Tail-T(ii)-MH also was reduced, reaching statistical
significance for the heart (75%; P < 0.05) and lung (50%; P < 0.05). In contrast, transduction by AdG.L.p*Tail-T(iii)-MH was significantly diminished for all tissues tested. Transduction of the heart and spleen by AdG.L.p*Tail-T(iii)-MH was reduced 75% (P < 0.05) and 66% (P < 0.01), respectively, and transduction of the kidney and lung were reduced to background levels (P < 0.01).

Fig. 8.4 AdV bioavailability and distribution after intravenous inoculation of mice. (A) Circulation half-life of AdVs. C57BL/6 mice were inoculated intravenously with the AdVs shown, blood was obtained by tail vein venipuncture at the indicated intervals post-inoculation, and AdV titers were determined by a quantitative infection assay using 293HissFv.rec cells. The results are presented as mean percent input virus for at least 5 independent experiments. Error bars indicate standard deviations. (B) Tissue distribution of AdVs. C57BL/6 mice were either mock-infected or inoculated intravenously with the AdVs shown. Forty-eight h after inoculation, luciferase activity in the tissues shown was determined. The results are expressed as mean luciferase activity in LU/mg protein for at least 5 independent experiments. Error bars indicate standard deviations. *, P<0.05; **, P < 0.01.
AdV interactions with red blood cells
AdVs with native tropism bind and agglutinate erythrocytes of human and rat but not murine origin (6, 18). Interactions with human erythrocytes constitute a major roadblock for therapeutic application of AdVs. Therefore, we tested the targeted AdVs for erythrocyte binding and agglutination (figure 8.5A and Table 8.2). Native AdV AdG.L produced hemagglutination of human and rat erythrocytes but not mouse erythrocytes, as anticipated. However, neither AdG.L.Tail-T(ii)-MH nor AdG.L.p*Tail-T(ii)-MH produced hemagglutination of any of the erythrocytes tested.

To corroborate these findings, we determined the fraction of each AdV bound to human erythrocytes. AdVs were incubated with human erythrocytes, and AdV genomes in cell (bound) and supernatant (unbound) fractions were quantified using qPCR (figure 8.5B). AdV with native tropism bound human erythrocytes efficiently, leaving less than 5% of the inoculum in the supernatant. In sharp contrast, the affinity of AdG.L.Tail-T(ii)-MH and AdG.L.p*Tail-T(ii)-MH for human erythrocytes was markedly reduced, with greater than 90% of the inoculum remaining in the supernatant. Thus, ablation of native adenovirus tropism substantially compromises the capacity of targeted AdVs to interact with human erythrocytes.

Table 8.2 Hemagglutination properties of AdG.L, AdG.L.Tail-T(ii)-MH, and AdG.L.p*Tail-T(ii)-MH*

<table>
<thead>
<tr>
<th>Virus vector</th>
<th>Species of origin RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>AdG.L</td>
<td>++</td>
</tr>
<tr>
<td>AdG.L.Tail-T(ii)-MH</td>
<td>-</td>
</tr>
<tr>
<td>AdG.L.p*Tail-T(ii)-MH</td>
<td>-</td>
</tr>
</tbody>
</table>

*Hemagglutination scored according to the scale described by Cichon et al. (6): -, no hemagglutination; +, short-range hemagglutination; ++, medium-range hemagglutination; ++++, long-range hemagglutination
Fig. 8.5 Interaction of AdG.L, AdG.L.Tail(T(ii)-MH, and AdG.L.p*Tail-T(ii)-MH with human erythrocytes. (A) Agglutination of human erythrocytes by AdVs. A suspension of 1% packed erythrocytes was incubated with an equal volume of virus, serially diluted two-fold from $10^{12}$ vp/ml, for at least 1.5 h. (B) Binding of AdVs to human erythrocytes. AdVs ($6.4 \times 10^7$ vp) were incubated with a physiologic concentration of washed human erythrocytes in PBS at 37°C. After 60 min incubation, the cellular (bound) and supernatant (unbound) fractions were separated by centrifugation, and AdV genomes present in each fraction were quantified by qPCR. The results are presented as mean percentage of AdV genomes in each fraction for three independent erythrocyte donors. Error bars indicate standard deviations.
8.4 Discussion

Targeting AdVs to specific cells by complete abrogation of native adenovirus tropism and introduction of a unique binding moiety should improve the in vivo applicability and efficacy of these vectors. We previously generated a genetically targeted AdV, AdG.L.Tail-T(ii)-MH, by replacing the adenovirus fiber molecule with a prototype chimeric attachment protein, which lacks CAR- and HSG-binding sites and confers His-tag-dependent tropism (24). In addition, deletion of the fiber knob removed putative binding sites for coagulation factors FVII, FIX, FX, protein C and complement component C4BP, which are shown to link adenovirus to alternate cell-surface receptors (14, 15). This vector design has the potential to incorporate large and complex proteins as ligands and allows vector propagation without the need for complementing fiber.

In this study, we substantially improved the AdG.L.Tail-T(ii)-MH platform to facilitate systemic in vivo administration by ablating the final known adenovirus attachment site, the integrin-binding motif, from the viral capsid. Importantly, this modification did not affect efficient propagation of the vector using cells that express an artificial His-tag binding receptor. In fact, the new genetically targeted vector, AdG.L.p*Tail-T(ii)-MH, replicates in His-tag receptor-expressing cells efficiently and achieves viral particle yields that approximate those of an AdV with native tropism. We conclude that binding of the chimeric attachment protein to the artificial His-tag binding receptor is sufficient for AdV internalization and delivery of the viral genome to the nucleus, thus allowing efficient virus replication and transgene expression. This suggests that retention of the presumably physiologically inert His-tag in derivative targeted AdVs incorporating selective binding ligands of choice will allow their efficient production using His-tag receptor-expressing cells. Therefore, production of a variety of genetically targeted AdVs with different binding specificities should be possible using a single packaging cell line.

Many studies have shown that intravenous administration of native AdV expressing luciferase usually results in very high luciferase activity per mg protein in the liver with approximately 100-fold lower levels in other tissues (2-4, 19, 30). However, we found approximately 70% of AdG.L activity in the liver and 10-15% in lungs and spleen. This was more in line with a previous observation by Seki et al (31) who found a similar transduction efficiency of the liver and an even more efficient transduction of the spleen. We can only speculate as to why the
biodistribution profile of AdV with native tropism differed considerably between individual studies using similar methods. Perhaps this could be partially explained by differences in the injected AdV dose. Seki et al and we injected $10^{10}$ particles per animal (31). In most studies the injected dose was higher, reaching up to $10^{11}$ particles per animal. It has been reported that liver cell transduction efficiency after systemic AdV administration in mice is nonlinear, exhibiting a viral dose threshold effect at approximately $3 \times 10^{10}$ particles (32). Thus, less effective gene expression by the mouse liver as seen by us might not be unexpected after low dose AdV infusion.

Targeted vector AdG.Lp*Tail-T(ii)-MH exhibited His-tag receptor-specific transduction and substantially diminished infectivity of liver cells in vitro. Most importantly, intravenous inoculation of mice with the targeted vector resulted in reduced transduction of all tested tissues in comparison to the native vector. Interestingly, transduction of kidney and lung by AdG.Lp*Tail-T(ii)-MH was even diminished to undetectable levels. In contrast, AdG.LTail-T(ii)-MH, which contains an intact integrin-binding site, exhibited only a modest reduction in transduction of those tissues. Thus, interaction of the capsid with integrins is essential for AdV transduction of kidney and lung, consistent with previous observations (2, 4). Since the kidney and lung sequester only a minor fraction of systemically administered AdV, very effective de-targeting of those tissues might not increase target-cell transduction or substantially prolong circulation half-life. In absolute terms, the modest reduction in liver transduction is likely to have a larger impact on bioavailability than the complete de-targeting of kidney and lung. However, effective de-targeting of kidney and lung establishes a framework for the development of highly selective target-cell transduction of those tissues by isolated organ perfusion. This approach might be an attractive option for AdV oncolytic treatment of primary malignancies originating from kidney or lung or metastatic lesions involving those organs. Such approaches could be considered as alternatives for systemic gene delivery if liver and spleen transduction cannot be entirely abolished.

Reduced uptake of AdG.Lp*Tail-T(ii)-MH in tissues was associated with increased vector levels in the circulation. We think it is important that the assay used to quantify virus in the circulation, measured particles capable of transducing target cells. Therefore, AdVs detected in the circulation represented bioavailable AdVs. For the first 30 min following intravenous inoculation, AdG.Lp*Tail-T(ii)-MH exhibited slower clearance from the blood than did native AdG.L and AdG.LTail-
Targeted adenovirus lacking native tropism

T(ii)-MH. This delay in clearance resulted in approximately 10-fold higher levels of circulating AdV during the majority of the observation interval. While replacing fiber with the Tail-T(ii)-MH chimeric attachment molecule in AdG.LTail-T(ii)-MH removed most known native adenovirus binding sites, this modification did not increase circulation half-life. Instead, additional deletion of the integrin-binding motif from AdG.LTail-T(ii)-MH to generate AdG.Lp*Tail-T(ii)-MH was essential for prolonged AdV maintenance in the circulation. Sustained bloodstream persistence of comparable doses of intravenously administered AdVs was previously achieved by coating AdVs with polyethylene glycol or polymer to inhibit cellular uptake or by depleting Kupffer cells via GdCl₃ treatment prior to AdV infusion (33-36). It is possible that the combined effects of genetic modification of the vector and previously employed coating strategies might result in even more prolonged circulation half-life.

Interactions of AdV with human erythrocytes poses a significant limitation to the utility of AdVs for systemic administration. Following intravenous inoculation of humans, greater than 98% of the AdV dose in the blood is associated with erythrocytes, which significantly hampers infection of target cells (7). Moreover, this phenomenon prevents extrapolation of AdV bioavailability in mouse studies to humans, since human AdVs do not bind murine erythrocytes. Replacement of fiber with Tail-T(ii)-MH in the targeted AdVs used in this study abrogated interactions with human red blood cells. Both AdG.LTail-T(ii)-MH and AdG.Lp*Tail-T(ii)-MH exhibited a 90% reduction in erythrocyte binding and loss of detectable hemagglutination. These findings indicate that the integrin-binding site does not mediate erythrocyte interactions, confirming a previous observation of Lyons et al. (7). Rather, our findings localize the erythrocyte-binding site to the fiber shaft or knob domain, which is in agreement with a previous report that mapped hemagglutination capacity to the CAR-binding site (18). Thus, targeted AdVs encoding Tail-T(ii)-MH attachment molecules evade potential sequestration by human erythrocytes, suggesting that AdVs based on this platform would have prolonged bioavailability in the circulation of humans.

In this study, we developed a prototype targeted AdV, AdG.Lp*Tail-T(ii)-MH, which incorporates many desirable features for systemic gene delivery. Although caution should be taken in extrapolating observations using mouse models to humans, the highly specific transduction profile, improved bioavailability, and absence of erythrocyte binding of AdG.Lp*Tail-T(ii)-MH bring systemic
application of AdV-based gene delivery in closer proximity and warrant further investigation.

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References
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