Polyinosinic acid enhances delivery of adenovirus vectors in vivo by preventing sequestration in liver macrophages

Hidde J. Haisma,1 Jan A. A. M. Kamps,2 Gera K. Kamps,1 Josee A. Plantinga,1 Marianne G. Rots1 and Anna Rita Bellu1

1Department of Therapeutic Gene Modulation, Groningen University Institute for Drug Exploration, University of Groningen, The Netherlands
2Department of Pathology and Laboratory Medicine, Medical Biology Section, University Medical Center Groningen (UMCG), University of Groningen, The Netherlands

Adenovirus is among the preferred vectors for gene therapy because of its superior in vivo gene-transfer efficiency. However, upon systemic administration, adenovirus is preferentially sequestered by the liver, resulting in reduced adenovirus-mediated transgene expression in targeted tissues. In the liver, Kupffer cells are responsible for adenovirus degradation and contribute to the inflammatory response. As scavenger receptors present on Kupffer cells are responsible for the elimination of blood-borne pathogens, we investigated the possible implication of these receptors in the clearance of the adenovirus vector. Polyinosinic acid [poly(I)], a scavenger receptor A ligand, was analysed for its capability to inhibit adenovirus uptake specifically in macrophages. In vitro studies, the addition of poly(I) before virus infection resulted in a specific inhibition of adenovirus-induced gene expression in a J774 macrophage cell line and in primary Kupffer cells. In vivo experiments, pre-administration of poly(I) caused a 10-fold transient increase in the number of adenovirus particles circulating in the blood. As a consequence, transgene expression levels measured in different tissues were enhanced (by 5- to 15-fold) compared with those in animals that did not receive poly(I). Finally, necrosis of Kupffer cells, which normally occurs as a consequence of systemic adenovirus administration, was prevented by the use of poly(I). No toxicity, as measured by liver-enzyme levels, was observed after poly(I) treatment. From our data, we conclude that poly(I) can prevent adenovirus sequestration by liver macrophages. These results imply that, by inhibiting adenovirus uptake by Kupffer cells, it is possible to reduce the dose of the viral vector to diminish the liver-toxicity effect and to improve the level of transgene expression in target tissues. In systemic gene-therapy applications, this will have great impact on the development of targeted adenoviral vectors.

INTRODUCTION

One of the main challenges in gene therapy is to engineer vectors that are targeted systemically to specific receptors. Among the different gene-therapy vectors available, adenovirus is preferred because of its superior in vivo gene-transfer efficiency to a wide spectrum of both dividing and non-dividing cell types. However, its broad tropism also represents an important limitation for its use in therapeutic applications where specific gene transfer is required. Upon systemic administration, the majority of the adenovirus accumulates rapidly in the liver. This is accompanied by the induction of acute-phase responses, including elevation of cytokines (tumour necrosis factor alpha, interleukin-6, etc.) and chemokines (Mip-2, Ip-10, etc.) and expression of genes involved in leukocyte trafficking (Cotter & Muruve, 2005; Muruve, 2004; Muruve et al., 1999; Zhang et al., 2001). Hepatic Kupffer cells (KCs) have been shown to contribute to the inflammatory response, and to be responsible for the short half-life of the circulating viral particles and indirectly for the lower transgene expression in other cell types (Alemany et al., 2000; Lieber et al., 1997; Smith et al., 2008; Wolff et al., 1997). Elimination of viruses by KCs results in a non-linear correlation between low doses of injected viruses and transgene expression (Fechner et al., 1999; Tao et al., 2001; Ziegler et al., 2002). Administration of sufficient numbers of viral particles to exceed a threshold level necessary to saturate the uptake by KCs can produce a linear correlation with transgene expression (Fechner et al., 1999; Tao et al., 2001; Ziegler et al., 2002).
The development of systemically deliverable adenoviral vectors requires two main conditions: targeting of the virus to selected cells and detargeting of the virus from native receptors, especially those present in the liver, which sequesters the majority of intravenously injected virus via KCs, sinusoidal liver endothelial cells and hepatocytes. From in vitro studies, it was found that adenovirus infection starts by a high-affinity interaction of the viral fiber knob with the coxsackievirus–adenovirus receptor (CAR) (Bergelson et al., 1997; Tomko et al., 1997). Subsequently, internalization of the virus occurs via binding of the RGD motif in the penton base protein to cellular vβ3 and vβ5 integrins (Wickham et al., 1993). Furthermore, heparan sulfate glycosaminoglycans have been shown to provide a third cell-surface interaction with adenovirus particles (Dechecchi et al., 2000; Smith et al., 2003a).

A number of strategies to alter adenovirus tropism have been used to eliminate adenovirus–receptor interactions (Bangari & Mittal, 2006; Glasgow et al., 2006; Mizuguchi & Hayakawa, 2004; Nicklin et al., 2005). Initial attempts to detarget the liver have been made by ablating the CAR- and integrin-based interactions (Akiyama et al., 2004; Koizumi et al., 2003; Martin et al., 2003; Nakamura et al., 2003). Also, fiber-shaft modifications have been introduced to eliminate virus interactions with native receptors (Bayo-Puxan et al., 2006; Breidenbach et al., 2004; Nicol et al., 2004; Shayakhmetov et al., 2004; Smith et al., 2003b; Vigne et al., 2003). However, the results are variable. Some animal studies showed reduced transduction of the liver, whilst in others, liver uptake was not affected by these modifications. Smith et al. (2003b) examined the role of heparan sulfate proteoglycans (HSPGs) in liver adenovirus uptake in vivo by replacing a putative HSPG-binding motif (KKTK) in the fiber shaft with an irrelevant peptide sequence, and observed a reduction in liver transgene expression of 90 %. However, Di Paolo et al. (2007) demonstrated recently that the KKTK motif plays only a minimal role in hepatic infectivity in vivo.

Chemical methods have also been used to detarget adenovirus. These methods include PEGylation (Croyle et al., 2000, 2002; Fisher et al., 2001; Lanciotti et al., 2003; Ogawara et al., 2004; O’Riordan et al., 1999) or polymer coating of the adenovirus (Green et al., 2004), which resulted in decreased toxicity and increased plasma half-life of adenovirus, allowing site-specific targeting.

Specific strategies aimed at preventing the interaction of adenovirus with KCs have been devised based on the selective depletion of KCs or pre-dosing of animals with transcriptionally inactive adenovirus (Alemany et al., 2000; Lieber et al., 1997; Schiedner et al., 2003b; Wolff et al., 1997). Scavenger receptors on KCs are responsible for the elimination of blood-borne pathogens and polyanionic macromolecules, such as modified lipoproteins and modified albumins (Gordon, 2002; Gough & Gordon, 2000; Krieger, 1997; Mukhopadhyay & Gordon, 2004; Peiser et al., 2002; Platt & Gordon, 2001; Platt et al., 2002; Shirai et al., 1999; Swart et al., 1999; Taylor et al., 2005; Yamada et al., 1998).

In this study, we hypothesized that the scavenger receptors present on KCs may serve as a receptor for adenovirus. To investigate this hypothesis, we isolated KCs and hepatocytes and infected them with an adenoviral vector expressing a reporter gene. Specific inhibition of gene expression was observed when a scavenger receptor A ligand, polyinosinic acid [poly(I)], was applied to the cells prior to adenovirus administration. In vivo, pre-administration of poly(I) in mice and rats resulted in a higher level of transgene expression with no effect on toxicity. Overall, these findings suggest that this approach will be useful for increasing the selectivity and efficiency of gene transfer in gene-therapy applications.

**METHODS**

**Cells.** The murine macrophage-like J774 cell line, the hepatocellular carcinoma HepG2 cell line and the human embryonic kidney 293 cell line were obtained from the ATCC. The mouse endothelioma H5V cell line was kindly provided by Dr A. Vecchi (Mario Negri Institute for Pharmacological Research, Milan, Italy). All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Invitrogen) containing l-glutamine (Gibco-BRL), 10 % fetal bovine serum (FBS; BioWhittaker) and penicillin (100 IU ml$^{-1}$)/streptomycin (100 μg ml$^{-1}$) (Gibco-BRL) at 37 °C in a humidified 5 % CO$_2$/95 % air atmosphere. The adenovirus-transformed human embryonic kidney 293 cell line was cultured in DMEM/F-12 (Gibco-BRL) containing 10 % FBS, 2 mM l-glutamine (Gibco-BRL) and penicillin (100 IU ml$^{-1}$)/streptomycin (100 μg ml$^{-1}$) (Gibco-BRL).

Rat hepatocytes and KCs were isolated from male Wag/Rij rats (200–250 g; Harlan CBP) after collagenase perfusion of the liver, followed by centrifugation and counterflow centrifugal elutriation as described previously (Kamps et al., 1997). After isolation, hepatocytes were cultured in 24-well plates in Williams E medium (Gibco Invitrogen) supplemented with 10 % heat-inactivated FBS, 2 mM l-glutamine, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. KCs were grown in 24-well plates in RPMI 1640 medium (Gibco Invitrogen) supplemented with 20 % FBS, 2 mM l-glutamine, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. The purity of KCs was controlled by immunocytochemistry using the ED2 antibody and by staining for endogenous peroxidase activity, for which KCs are positive and endothelial cells are negative. The purity was >80 %.

**Inhibitors.** The following scavenger receptor-effective and -ineffective ligands were used: poly(I) and polycytidylic acid [poly(C)] potassium salts (both from Sigma) and plasmid DNA.

**Adenoviral vector.** AdTL is an E1- and E3-deleted recombinant serotype 5 adenovirus (He et al., 1998) that contains a green fluorescent protein (GFP) and luciferase gene-expression cassette, each under the control of a cytomegalovirus promoter (Alemany & Curiel, 2001). The virus was grown in 293 cells and purified in HEPES/sucrose buffer (pH 8.0) according to a conventional double CsCl gradient centrifugation method (Becker et al., 1994), and the number of viral particles (vp) was calculated from the OD$_{260}$. The number of p.f.u. was determined by plaque-forming assay. The ratio vp:p.f.u. ratio was determined to be 1:187.

**In vitro infection and reporter-gene expression analysis.** J774 and HepG2 cells were seeded into 96-well culture plates at a density of
10 000 cells per well for viral transduction experiments. After 24 h, cells were first incubated for 1 h at 37 °C in the presence or absence of scavenger-receptor inhibitors [poly(I), poly(C) or plasmid DNA] as indicated in the experiments. After 1 h, AdTL was added at a concentration of 1000 vp per cell in DMEM containing 2% FBS (infection medium). One hour after infection, the infection medium was replaced by normal culture medium and cells were incubated for 48 h before performing the luciferase assay. The cells were lysed with cell-culture lysis buffer (Promega) and the lysates were analysed with the luciferase assay system (Promega) on a LumiCount luminometer (Packard). All data are expressed as relative light units (RLU).

Adenovirus infection of freshly isolated hepatocytes and KCs was performed as described above in the presence or absence of poly(I). Forty-eight hours post-infection, cells were analysed for expression of the GFP reporter protein by using fluorescence microscopy.

**Animal infection and transgene-expression analysis.** The effect of scavenger receptor A blockade on liver transgene levels and adenovirus blood circulation times were determined in C57Bl mice or WagRij rats (Harlan CPB). Animals were anaesthetized (isoflurane/N₂O/O₂ inhalation) and the indicated amount of vp diluted in HEPES/sucrose buffer (pH 8.0) was injected intravenously via the orbital plexus (mice) or penis vein (rats). When indicated, 4.0 or 0.2 mg poly(I) in PBS was injected into rats or mice, respectively, 5 min prior to injection of the virus. An aliquot of blood was collected by orbital puncture 5, 30, 60 and 120 min after intravenous administration. The blood samples were then centrifuged to collect plasma and the numbers of vp in the plasma samples were subsequently measured by serial dilution and infection of 293 cells based on a conventional plaque-forming assay. In brief, each plasma sample was diluted serially in DMEM/F-12 supplemented with 2% FBS and 10 000 cells per well for viral transduction experiments. After 24 h, cells were first incubated for 1 h at 37 °C in the presence or absence of scavenger-receptor inhibitors [poly(I), poly(C) or plasmid DNA] as indicated in the experiments. After 1 h, AdTL was added at a concentration of 1000 vp per cell in DMEM containing 2% FBS (infection medium). One hour after infection, the infection medium was replaced by normal culture medium and cells were incubated for 48 h before performing the luciferase assay. The cells were lysed with cell-culture lysis buffer (Promega) and the lysates were analysed with the luciferase assay system (Promega) on a LumiCount luminometer (Packard). All data are expressed as relative light units (RLU).

Forty-eight hours post-infection, cells were analysed for expression of the GFP reporter protein by using fluorescence microscopy.

**Quantitative real-time PCR.** DNA was isolated from liver tissue by using DNeasy Tissue kits (Qiagen) according to the manufacturer’s protocol. DNA was purified by using minicolumns and dissolved in elution buffer, and its concentration was determined spectrophotometrically (A₂₆₀/A₂₈₀). The concentration of adenovirus DNA was determined by real-time PCR using the Applied Biosystems Prism 7900HT sequence detection system with sso 2,1 software. Amplification was carried out in a total volume of 20 µl with SYBR Green PCR MasterMix (Applied Biosystems), forward (hexon: 5’-CCTCGATGTATGCGGGAGGTC-3’) and reverse (5’-GGGTCTCAG-GTACTCAGGAG-3’) primers and extracted DNA. The parameters used were one cycle of 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 56 °C for 15 s and 72 °C for 40 s. Adenovirus copy number was quantified by using a standard curve created from dilutions of adenovirus DNA from 1 500 000 copies to 15 copies in a background of mouse genomic DNA. Samples were amplified in duplicate and the mean total copy number was normalized to copies of viral DNA (µg DNA)⁻¹.

**Immunohistochemistry.** Immunohistochemistry was performed on frozen liver sections to locate KCs with the F4/80 antibody (Serotec). Livers were frozen in liquid N₂ and 7 µm sections were cut, fixed with acetone, air-dried and rehydrated in PBS. Primary antibody was used at a 1 : 50 dilution, followed by rabbit anti-rat–biotin at a 1 : 100 dilution. Colour development was performed with 3-amino-9-ethylcarbazole (AEC; Sigma) dissolved in N,N-dimethylformamide (Merck)/0.5 M acetate buffer, pH 4.9. Slides were counterstained with Mayer’s haematoxylin and mounted in Kaiser’s glycerin.

**Statistical analysis.** Differences in gene expression were analysed by using a one-sided paired Student’s t-test, assuming equal variance. Differences were considered to be significant when P<0.05.

**RESULTS**

**In vitro evaluation of scavenger-receptor inhibitors**

Macrophage scavenger-receptor ligands are polyanionic molecules or macromolecular complexes (Gordon, 2002). We used a panel of these ligands to determine the role of scavenger receptors in adenovirus transduction. First, we compared the effect of these ligands on virus transduction in the J774 cell line (scavenger receptor A-positive and CAR-negative) and a cancer cell line, HepG2 (scavenger receptor A-negative and CAR-positive) (Choi et al., 2004; Hamblin et al., 2000; Rhaïnds et al., 1999) (Fig. 1). For infection, a basic adenoviral vector (AdTL) containing reporter genes was used. In J774 cells, poly(I), a scavenger

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**Fig. 1.** Poly(I) prevents adenovirus infection of macrophages. Relative luciferase gene-expression levels of J774 (white bars) and HepG2 cells (black bars) infected with adenovirus particles in the presence of medium control (PBS), poly(C) (PC), plasmid DNA (PIDNA) or poly(I) (PI). To determine gene expression, luciferase activity was measured 48 h post-infection. Gene expression is presented as a percentage of that in cells infected with AdTL in the presence of medium control. Results are expressed as the mean ± SD relative light units (RLU) of at least three different experiments.
receptor-effective ligand (Krieger & Herz, 1994; Platt & Gordon, 2001), reduced the transgene levels by 83% when used at 25 μg ml⁻¹, whereas poly(C) (25 μg ml⁻¹) or plasmid DNA (10 μg ml⁻¹), scavenger receptor-ineffective ligands (Krieger & Herz, 1994; Platt & Gordon, 2001), did not. In HepG2 cells, no reduction in transgene levels was measured when the cells were infected in the presence of the ligands, including poly(I). Poly(I) did not reduce GFP expression in freshly isolated rat hepatocytes when infected with adenovirus (Fig. 2), whereas expression in KCs was blocked almost completely (Fig. 2). As poly(I) is a selective ligand of scavenger receptors, these data suggest that scavenger receptors are involved in adenovirus uptake in macrophages.

**Effects of poly(I) on gene expression in vivo**

Based on these results, we carried out in vivo experiments in rats to determine whether inhibition of the scavenger receptors would indeed result in a reduction of virus sequestration by KCs and thus an increment of virus transfer to tissues. Animals were injected with poly(I) or PBS prior to administration of AdTL. Transgene expression was analysed 48 h post-infection. In frozen liver sections of rats, GFP expression increased when poly(I) was administered (Fig. 3a) and distribution changed from a patchy pattern to a regular diffuse pattern. Together, these results can be attributed to diminished uptake of virus by liver macrophages and an increase in infection and transduction in surrounding liver tissue, through the CAR receptor and/or HSPGs on hepatocytes (Smith et al., 2003a).

Higher values for luciferase expression, measured in different organs, also resulted when poly(I) was injected prior to adenovirus administration (Fig. 3b). The higher luciferase activity, registered as a consequence of the use of poly(I), can be explained by a reduction of viral particles degraded by liver macrophages. This results in more viral particles being available to infect tissues expressing the virus native receptor.

**Effects of poly(I) on adenovirus clearance from blood**

To evaluate whether blockade of the scavenger receptors by poly(I) results in reduced clearance of adenovirus from the

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**Fig. 2.** Poly(I) blocks adenovirus infection of Kupffer cells. Hepatocytes and Kupffer cells were isolated from rats injected with PBS (−PI) or 4 mg poly(I) (+PI) per rat, 5 min prior to infection with 5×10¹¹ adenovirus particles per animal. Expression of GFP was analysed 48 h after adenovirus infection and representative images are shown.

**Fig. 3.** Poly(I) alters the distribution of adenovirus transgene expression in rats. Transgene expression in rats injected with PBS (−PI) or with PBS containing 4 mg poly(I) (+PI) per rat, 5 min prior to injection of 5×10¹¹ adenovirus particles per animal. (a) Rat liver sections were analysed by fluorescence microscopy 48 h after viral injection. (b) Luciferase measurements in tissue homogenates 48 h after injection of AdTL. White bars represent animals injected with PBS; black bars represent animals pre-injected with poly(I). Results are expressed as the mean±SD relative light units (RLU) of three rats. *P<0.05 compared with controls (animals injected with PBS).
circulation, blood samples from mice were analysed for adenovirus levels by plaque-forming assays. At the low dose of adenovirus, $5 \times 10^6$ vp per mouse, which was selected to be below the threshold of KC saturation (Tao et al., 2001; Ziegler et al., 2002), we observed a rapid clearance of virus from the blood, with a maximum level of virus present in the blood of approximately $5 \times 10^3$ p.f.u. ml$^{-1}$ at 5 min after virus administration. Pre-administration of poly(I) increased the maximum level by more than 10-fold. At the high dose of virus administration, $5 \times 10^{10}$ vp per mouse, a similar increase in the maximum level of circulating virus was measured 30 min after the virus injection (Fig. 4). These data demonstrate that pre-administration of poly(I) to animals increased blood levels of adenovirus.

Effects of poly(I) on adenovirus levels in the liver

In mice injected with the low and high doses of adenovirus, $5 \times 10^9$ or $5 \times 10^{10}$ particles, luciferase activity and adenovirus content in the liver were compared. At both doses, the viral copy number of liver tissue, as determined by RT-PCR 48 h after administration, was significantly higher when poly(I) was administered prior to AdTL, compared with AdTL alone (Fig. 5a). This resulted in an accompanying higher gene-expression level, as determined by luciferase measurements (Fig. 5b). The pre-virus injection of poly(I) results in a reduced clearance of adenovirus, with a consequent enhancement of viral particles and an increase in transgene levels in the liver.

Poly(I) toxicity in combination with adenovirus

Adenovirus administration can induce liver toxicity, reduction of KC numbers and elevated liver-enzyme levels (Liu et al., 2003; Manickan et al., 2006; Schiedner et al., 2003a; Shayakhmetov et al., 2004). Mouse liver sections were stained for KCs 48 h after administration of AdTL and KC numbers were quantified. With the low dose of adenovirus ($5 \times 10^9$ vp), KC numbers remained unchanged (data not shown). At the high dose of virus ($5 \times 10^{10}$ vp), a reduction in KC count was observed in animals injected with AdTL only, but not in mice pre-injected with poly(I) (Fig. 6a, b). The levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase in serum obtained 48 h after injection of AdTL only or AdTL in combination with poly(I) are low and very similar, indicating no toxicity from treatment with either adenovirus or virus combined with poly(I) (Fig. 7) at this time point. However, to study the direct effect of poly(I) on the
toxicity caused by uptake by KCs, a similar experiment should be performed within 24 h of adenovirus administration.

**DISCUSSION**

We present a new method to detarget adenoviral vectors from KCs, leading to an increase in adenovirus-mediated transgene expression in tissues and reduced liver toxicity. In various studies, detargeting of the liver of systemic administrated adenoviral vectors has been addressed in different ways. Although the early steps of adenovirus infection have been studied extensively *in vitro*, much is still unknown about the mechanisms governing adenovirus infection *in vivo*. To date, attempts to modify adenovirus liver tropism by abolishing CAR and/or integrin interactions have not been successful in all animal models analysed (Brunetti-Pierri *et al.*, 2004; Hodges *et al.*, 2005; Lievens *et al.*, 2004; Nicol *et al.*, 2004; Park *et al.*, 2005; Smith *et al.*, 2002).

Shayakhmetov *et al.* (2004) demonstrated that coagulation factor IX and the complement component C4-binding protein can bind the adenovirus fiber-knob domain and provide a bridge for virus uptake through cell-surface HS PGs and low-density lipoprotein receptor-related protein. An adenoviral vector that contained mutations in the fiber-knob domain that ablated blood-factor binding demonstrated significantly reduced infection of liver cells and liver toxicity *in vivo* (Shayakhmetov *et al.*, 2005). Intravenous delivery of adenovirus results in interaction of the virus with circulating platelets (Stone *et al.*, 2007). Virus–platelet aggregates are then taken up by KCs and degraded. Depletion of platelets prior to virus infection could improve levels of target-cell transduction at a lower vector dose (Stone *et al.*, 2007).

The ability of KCs to take up large particles, such as adenovirus, is well established (Smith *et al.*, 2008). In these studies, we show that poly(I), a scavenger receptor A inhibitor, has a profound effect on the sequestration of adenovirus by KCs.

We demonstrated previously (Kamps *et al.*, 1997) that poly(I) inhibited the uptake of negatively charged liposomes by KCs efficiently, indicating that scavenger receptors are involved in the uptake of these liposomes. Scavenger-receptor binding is thought to result from the intrinsic negative charge of the binding ligand. Although the fiber of Ad5 is positively charged to promote binding to target cells, the rest of the capsid displays predominantly negatively charged residues (Mei & Wadell, 1995). Also, serum proteins associated on the surface may play an important role in hepatic uptake of negatively charged particles via scavenger receptors (Furumoto *et al.*, 2004). Here, we show that binding of adenovirus to KCs can be abolished by pre-treatment with poly(I). The reduced liver sequestration of adenovirus observed after pre-treatment with poly(I) allows lower doses to be used with similar transgene-expression levels.

In our study, we did not attempt to detarget the adenovirus from its natural receptor (CAR) and, thus, we observed increased hepatic expression after pre-treatment with poly(I). This may be a feasible approach for treating liver or metabolic diseases, but may not be desirable for other gene-therapy applications. Detargeting hepatocytes may be accomplished by genetic CAR ablation of adenovirus, with concomitant introduction of targeting ligands into the

![Fig. 6. Poly(I) reduces the Kupffer cell toxicity of adenovirus in mice.](image-url)

Mice were injected with PBS or PBS containing 0.2 mg poly(I) per mouse, 5 min prior to injection with $5 \times 10^{10}$ adenoviral particles. (a) Frozen liver sections were prepared 48 h after injection of AdTL and stained with the macrophage-specific antibody F4/80. (b) Quantification of Kupffer cell staining in mouse liver. Results are expressed as the mean ± SD of at least three different Kupffer cell counts. *P*<0.05.
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adeno virus capsid by chemical modification (Ogawara et al., 2004) or by using bifunctional ligands, such as bispecific antibodies (Haisma et al., 2000).

The results indicate that blocking of scavenger receptor A by administration of poly(I) prior to adenovirus injection can be used to reduce the clearance of virus by KCs and is a feasible approach to increase the availability of adenovirus without affecting toxicity. Higher availability of adeno vir al particles for specific targeting will increase the efficiency of gene therapy. Poly(I) combined with targeted viral vector is a powerful tool for gene-therapy applications.

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