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Scavenger Receptor A: A New Route for Adenovirus 5

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Abstract: Adenoviruses are common pathogens associated with respiratory diseases, gastrointestinal illnesses and/or conjunctivitis. Currently, this virus is used as a vector in gene therapy trials. The promise of viral gene therapy applications is substantially reduced because the virus is cleared by liver macrophages upon systemic administration. The mechanism underlying adenoviral tropism to and degradation in macrophages is poorly understood. We identified a new adenoviral receptor, the scavenger receptor A (SR-A), responsible for uptake of the virus in macrophages. CHO cells expressing SR-A showed increased viral transgene expression when compared with wild type cells. Preincubation of J774 macrophage cells with SR-A ligands decreased significantly adenoviral uptake. Electron-microscopy analysis of infected J774 cells showed activation of a viral degradation pathway. Infection of mice with adenovirus resulted in a substantial decrease of the virus in liver macrophages when SR-A was blocked. Our data provide a basis for understanding of the adenoviral uptake and degradation mechanism in macrophages *in vitro* and *in vivo*. Inhibition of adenoviral SR-A uptake can be utilized in gene therapy applications to increase its efficiency and efficacy.

Keywords: Adenovirus; degradation; gene therapy; Kupffer cells; scavenger receptor A

Introduction

Adenovirus type 5 (Ad5), a nonenveloped double-stranded DNA virus, is considered one of the most important vectors for gene delivery and is used in about a quarter of the clinical trials in oncology and cardiology.

Different receptors have been described for Ad5 cell interaction, including the coxsackie B virus and adenovirus receptor (CAR),[1,2] the heparan sulfate glycosaminoglycans (HS-GAGs),[3,4] the class I major histocompatibility complex[5] and the vascular cell adhesion molecules.[6]

The CAR-dependent adenovirus infection is the best characterized mechanism thus far.[1,2] It has been shown *in vitro* that, after attachment of the viral knob to CAR, the

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virus is internalized by interaction of the RGD sequence at the viral penton base with cellular integrins.[7,8] Adenovirus is then transported into the endosome,[9] from where it escapes to the cytosol. After partial disassembly of the capsid, the virus translocates toward the nucleus, where replication occurs.[9]

In systemic administrations, the majority of the injected adenovirus is sequestered in the liver particularly by the liver macrophages called Kupffer cells (KC), which are responsible for their clearance.[10−17] This contributes not only to the induction of the inflammatory response and liver toxicity[10,18,19] but also to a substantial decrease of the efficiency and efficacy of therapeutic viral applications. In order to optimize adenoviral vectors for clinical applications, it is fundamental to understand the mechanisms governing adenoviral interaction with KCs and their subsequent clearance.

Until recently, it was believed that the CAR receptor was the major protagonist in liver uptake. Various attempts, largely unsuccessful, have been made to avoid Ad liver sequestration by preventing hepatocyte and/or Kupffer cell uptake, by ablating CAR- or integrin-binding motifs in the adenoviral capsid.[20−24] In more recent studies, it has been shown that the uptake of Ad5 by liver is CAR-independent[25,26] and rather involves either a direct[21,22] or indirect interaction of Ad with the cellular heparan sulfate proteoglycans (HSPG).[27−30] Shayakhmetov and coauthors[27] demonstrated

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that adenovirus interaction with coagulation factor IX (FIX) and complement component C4-binding protein (C4BP) results in a bridge by which the adenovirus can bind to HSPG and low-density lipoprotein receptor-related protein. Mutations in the fiber knob, that abolish interaction of Ad5 with FIX and C4BP, were able to prevent adenovirus uptake by KCs.[27] On the contrary, mutations in the fiber shaft, that have been shown to be essential for binding of Ad to HSPG,[22] did not influence Ad uptake by KCs.[31] Adenoviral particles have been shown *in vivo* to interact with platelets.[32] Stone and coauthors[33] reported that adenovirus−platelet interaction in blood caused virus sequestration to the reticuloendothelial system of the liver. They proposed that virus platelet aggregates are further taken up by Kupffer cells and degraded.

In our laboratory, we recently investigated the relevance of polyinosinic acid [poly(I)] in the adenoviral uptake by Kupffer cells.[34] We showed *in vitro* and *in vivo* that poly(I) can inhibit adenoviral uptake by macrophages and prevent Kupffer cell necrosis,[34,35] which normally occurs after adenoviral infection.[11] The reduced uptake of the adenovirus by KCs moreover resulted in an increase of circulating viral particles in the blood in the first few hours after injection and in an increase of gene expression in other tissues.[34]

Since poly(I) is a general scavenger receptor A (SR-A) ligand, in these studies, we investigated the role of this receptor in adenoviral uptake in macrophages. Scavenger receptors, originally defined by their ability to bind modified forms of low-density lipoprotein,[36] also function as pattern recognition receptors, which directly recognize conserved motifs on the surfaces of pathogens.[37] Alternative splice variants of SR-A (SR-AI, SR-AII and SR-AIII) have been identified. While SR-AIII is nonfunctional, SR-AI and SR-AII show similar ligand binding specificity and the collagenous region has been identified as the ligand binding domain.[38−41]

In the present studies, we show the relevance of SR-A in adenoviral uptake and subsequent degradation in macrophages. *In vitro* and *in vivo* analyses clearly demonstrate that Ad5 uptake and internalization occur in an SR-A-dependent manner. Infection of J774 cells by Ad5 results in the formation of autophagosome-like structures trapping adenovirus particles, indicating an Ad5 route of degradation. We also obtained evidence that the knob of the virus may be involved in Ad5 uptake by SR-A. The data presented will be relevant for fundamental studies because of the novel pathway for adenoviral entry and subsequent degradation in macrophages. By studying the interaction of Ad with Kupffer cells, it will be possible to improve systemic adenoviral gene therapy where it would be beneficial to prevent clearance of the virus.

Results

Scavenger Receptor A-Mediated Adenovirus Infection in CHO-SR-A Cells. The role of SR-A in Ad5 uptake and subsequent infection was investigated in CHO cells stably transfected with mouse scavenger receptor A type II (CHO-SR-A)[42] and compared with that of WT CHO-K1 cells (CHO). Ad5 poorly transduces CHO cells because of their lack of native receptors. Both cell lines were infected for one hour with AdTL, containing luciferase and GFP as reporter genes. 48 h post infection, cells were lysed and

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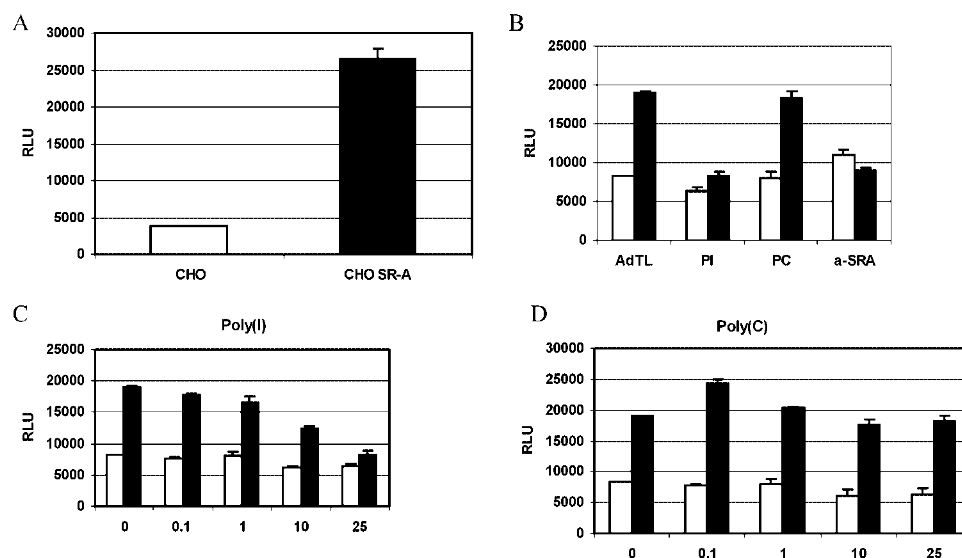

Figure 1. CHO-SR-A adenovirus infection. Relative luciferase gene expression in CHO (open bars) and CHO-SR-A cells (black bars), infected for 1 h with AdTL, containing the luciferase marker gene, at an MOI of 4000 viral particles/cell. (A) Difference in the luciferase expression in CHO and CHO-SR-A cells. $p < 0.05$. (B) Luciferase expression in cells infected respectively with AdTL alone or in the presence of 25 μg/mL of poly(I), 25 μg/mL of poly(C) or 15 μg/mL of the 2F8 antibody. $p < 0.05$ for AdTL compared to PI and a-SR-A; $p > 0.05$ for AdTL compared to PC. (C and D) Luciferase expression in cells infected with AdTL in the presence of increasing amounts of poly(I) or poly(C) (0; 0.1; 1; 10 or 25 μg/mL). To determine the gene expression, luciferase activity was measured at 48 h post infection. Results are expressed as the mean relative light units (RLU) $\pm$ standard deviation. Abbreviations: PI, poly(I); PC, poly(C); a-SR-A, 2F8 antibody.

analyzed for luciferase expression. As shown in Figure 1A, the luciferase level increases almost 7-fold in CHO-SR-A cells when compared to CHO cells. The capability of adenovirus to infect cells in a scavenger receptor A-dependent manner was further examined in CHO and CHO-SR-A cells by competition experiments. Before infection, cells were incubated with poly(I), a general SR-A ligand, or the mouse 2F8 scavenger receptor A antibody. Poly(C), an SR-A nonligand, was used as negative control. Both poly(I) and the 2F8 antibody were able to reduce transgene expression significantly in CHO-SR-A cells (Figure 1B). Poly(C) did not affect luciferase expression in both cell lines (Figure 1B).

SR-A-adenoviral infection was almost completely abolished at the dose of 25 μg/mL poly(I), indicating a saturation of the SR-A by its ligand (Figure 1C). In contrast, poly(C) did not affect adenoviral infection in both cell lines even at the highest dose (Figure 1D).

These data demonstrate that SR-A can mediate adenovirus infection in CHO cells and that it can be inhibited by the use of SR-A ligands or antibodies.

SR-A-Mediated Adenovirus Uptake and Internalization in the J774 Macrophage-like Cell Line. Recently we showed[34] that Ad transgene expression in the murine macrophage J774 cell line (SR-A positive and CAR negative)[43] could be prevented by almost 80% when cells were incubated with poly(I) or dextran (scavenger receptor A ligand). To determine if the SR-A ligands specifically affected the virus uptake, FACS analyses were performed. For this purpose we infected cells with Ad-PIX-GFP, obtained by the fusion of EGFP with the PIX viral capsid protein,[44] which is present 240 times on the capsid.

Adenovirus uptake in J774 cells was dependent on the length of the incubation time (Figure 2: A1, A2 and A3). Similarly, cells infected with increasing amount of viral particles demonstrated a dose response (Figure 2B). Uptake of adenovirus in J774 cells preincubated with poly(I), dextran or 2F8 was dramatically reduced when compared to cells preincubated with poly(C) or PBS (Figure 2C). Uptake of Ad-PIX-GFP in the A549 cell line (CAR positive and SR-A negative,[45,46] was not influenced by preincubation of the cells with SR-A ligands (Figure 2C). Cells infected in the presence of increasing amounts of poly(I) resulted in a reduction Ad5 internalization in a dose dependent manner in J774 cells but

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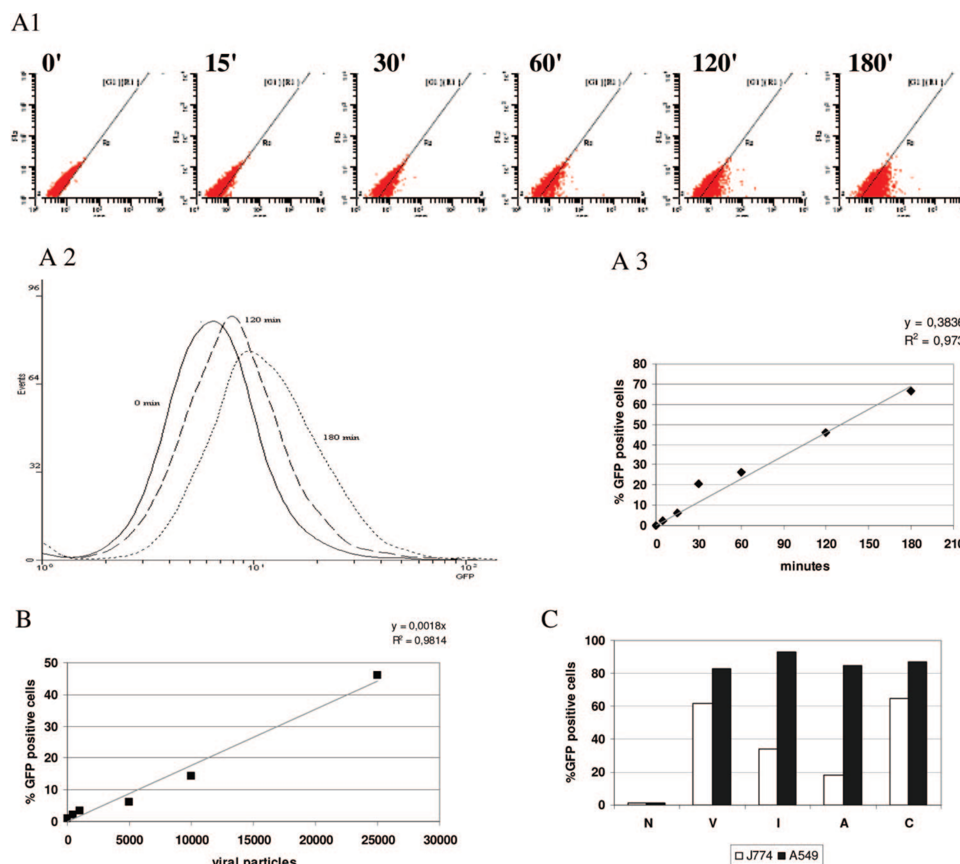

Figure 2. Adenovirus uptake by J774 cells. Flow cytometric analysis of Ad-PIX-GFP virus uptake in J774 and A549 cells. (A1) J774 cells were incubated with an MOI of 10,000 vp/cell at different time points. GFP negative cells (not infected) are visualized at the left of the oblique bar. GFP positive cells are shifted to the right of the oblique bar. (A2) Histogram of the results shown in A1 with selected time points (0, 120 and 180 min). (A3) Graph representing in percentage the data extrapolated from of A1. (B) J774 cell incubated with increasing amount of viral particles. The FACS results are presented as percentage of GFP positive cells. (C) Inhibition experiments. J774 and A549 cells were preincubated for 30 min with either 25 μg/mL poly(I), 25 μg/mL poly(C), 30 μg/mL SR-A antibodies or PBS before Ad-PIX-GFP infection at an MOI of 10,000 vp/cell. N represents not infected cells. Data extrapolated by the FACS analysis are presented as percentage of GFP positive cells. Abbreviations: I, poly(I); C, poly(C); A, SR-A antibody; V, PBS.

not in A549 cells (data not shown). Poly(C) on the other hand did not influence Ad uptake in both cell lines also at the highest dose (data not shown). J774 cells, analyzed by confocal laser scanning microscope 30 min post infection, showed GFP punctate structures corresponding to internalization of the PIX-GFP virus (Figure 3A). A similar profile was also observed in cells preincubated with 25 μg/mL of poly(C) (Figure 3B). In contrast, the amount of GFP-punctate structures diminished drastically when cells were preincubated with 25 μg/mL of poly(I) (Figure 3A).

Ultrastructural analysis of J774 cells, exposed for 30 min to AdTL, showed that after adenoviral uptake (Figure 4A) and internalization, the virus is clustered in vesicles (Figure 4B) or trapped in autophagosome-like structures (Figure 4C and 4E). The autophagosome-containing virus was also observed fused with cellular compartments resembling lysosomes (Figure 4E). These autophagosomes are most likely the first compartment were the cells trap the virus allocated for degradation. In A549 cells the virus was localized in the cytosol and not in degradation compartments (data not shown).

Primary Kupffer Cells Infected in an SR-A Dependent Manner. The ability to take up adenovirus via SR-A was also tested in primary Kupffer cells, preincubated or not with poly(I) or a soluble knob 5. As shown in Figure 5 preincubation with poly(I) prevents adenovirus uptake and subsequent infection by almost 50%. Preincubation of Kupffer cells with knob 5 resulted in strong inhibition of adenovirus infection suggesting a role of the knob in the uptake of the virus by scavenger receptor A.

***In Vivo* Adenoviral Uptake by Kupffer Cells via SR-A.** We previously showed[34] that the use of poly(I) prior to adenoviral injection results in an increment of circulating viral particles and an increase of other organ infection. In this study we show that poly(I) could partially prevent adenoviral uptake by KCs *in vivo*. Mice, preinjected with PBS or with poly(I) prior to Ad5 infection, were killed 10

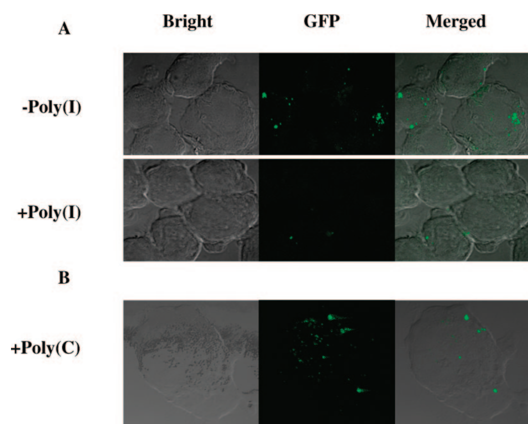

Figure 3. Adenovirus uptake by J774 cells. J774 cells were infected with Ad-PIX-GFP at an MOI of 20,000 vp/cell for 30 min and analyzed using a LEICA confocal laser scanning microscope. Panel A: before infection cells were preincubated respectively with plain medium (−Poly I) or with poly(I) (+Poly I). Panel B: pictures of a single J774 cell preincubated with poly(C) before adenoviral infection. Each picture derives from a 30 serial optical section of 0.3 μm.

min post infection. Immunohistochemistry was performed in liver cryosections stained with a viral hexon-specific antibody and the F4/80 macrophage-specific antibody. As shown in Figure 6, in liver sections of animals preinjected with PBS, the virus colocalized with Kupffer cells (dark brown-red color). In animals preinjected with poly(I), the staining of KCs is light brown, indicating lesser or no colocalization of the virus with KCs. Interestingly, similar results were obtained when animals were infected with AdTL preincubated with knob antibody. These data show that SR-A is involved in adenoviral uptake by KCs and indicate that the knob is implicated directly or indirectly in adenoviral recognition by KCs.

Discussion

In this paper we demonstrate *in vitro* and *in vivo* that the scavenger receptor A present on macrophages is an alternative route for adenovirus type 5 to enter cells. Uptake of adenovirus by macrophages results in activation of viral degradation responsible for the clearance of the virus.

Viral replication and/or viral gene expression has been the subject of studies not only to overcome the problems caused by adenoviral pathogenicity but also to improve the efficiency and efficacy in gene therapy applications when the virus is used as a gene transfer vector. The mechanism of adenoviral uptake and degradation upon systemic administration, mainly attributed to Kupffer cells,[10,11,13−17,47] has been highlighted only more recently. This issue, fundamental to understanding the defense mechanism of the body upon adenoviral infection, is also of primary importance in adenoviral gene therapy to avoid clearance of the vector upon systemic administration and liver toxicity.

Recent studies demonstrate that uptake of Ad in KCs is CAR independent[25,26] and can occur via the HSPG.[27]

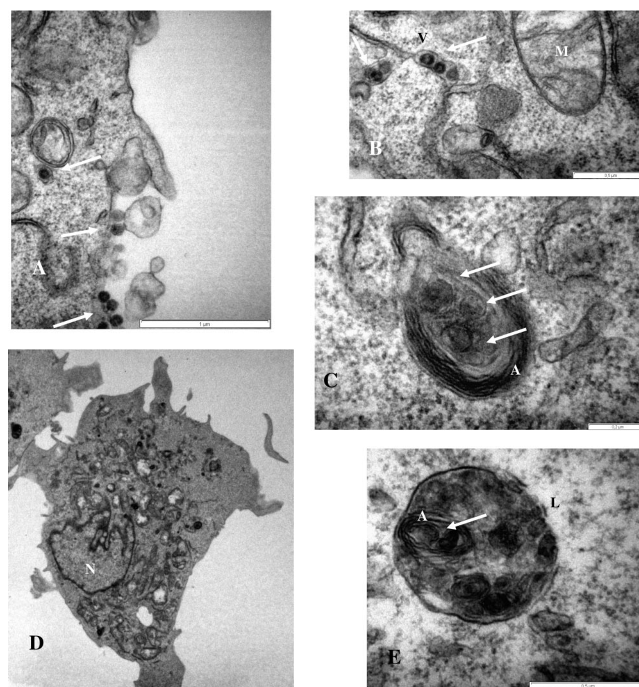

Figure 4. Intracellular localization of adenovirus in J774 cells. Representative electron micrographs of cells infected for 30 min with AdTL before glutaraldehyde/paraformaldehyde fixation. (A) Virus attachment to cellular membrane; bar: 1 μm. (B) Internalization of the virus into vesicles; bar: 0.5 μm. (C) Virus trapped in autophagosome like structures; bar: 0.2 μm. (D) Overview of the whole cell. (E) Cellular compartment containing autophagosomes; bar: 0.5 μm. White arrows indicate the adenoviral particle. A, autophagosome; L, lysosome; M, mitochondria; N, nucleus; V, vesicles.

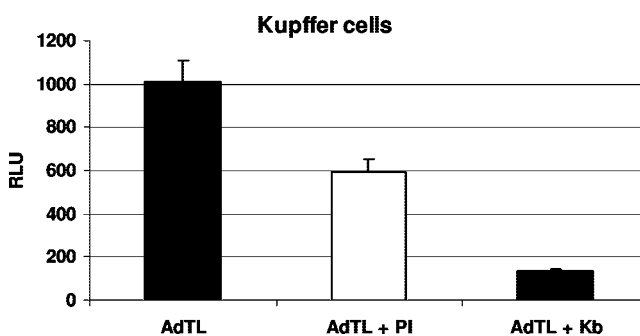

Figure 5. *In vitro* uptake of adenovirus by KCs in an SR-A-dependent manner. Relative luciferase gene expression on primary KCs isolated from rats. Cells were infected with AdTL at an MOI of 1000 for 1 h. To determine transgene expression, luciferase activity was measured at 48 h post infection. Before infection, the cells were preincubated with plain medium (AdTL), 25 μg/mL poly(I) (AdTL+PI) or 100 μg/mL knob 5 (AdTL+Kb). Results are expressed as the mean relative light units (RLU) ± standard deviation. $p < 0.05$ for AdTL compared to AdTL+PI and AdTL+Kb.

Previously, we showed that administration of poly(I) *in vivo* could enhance the lifetime of systemic injected adenoviral particles, resulting in an increase of organ transduction.

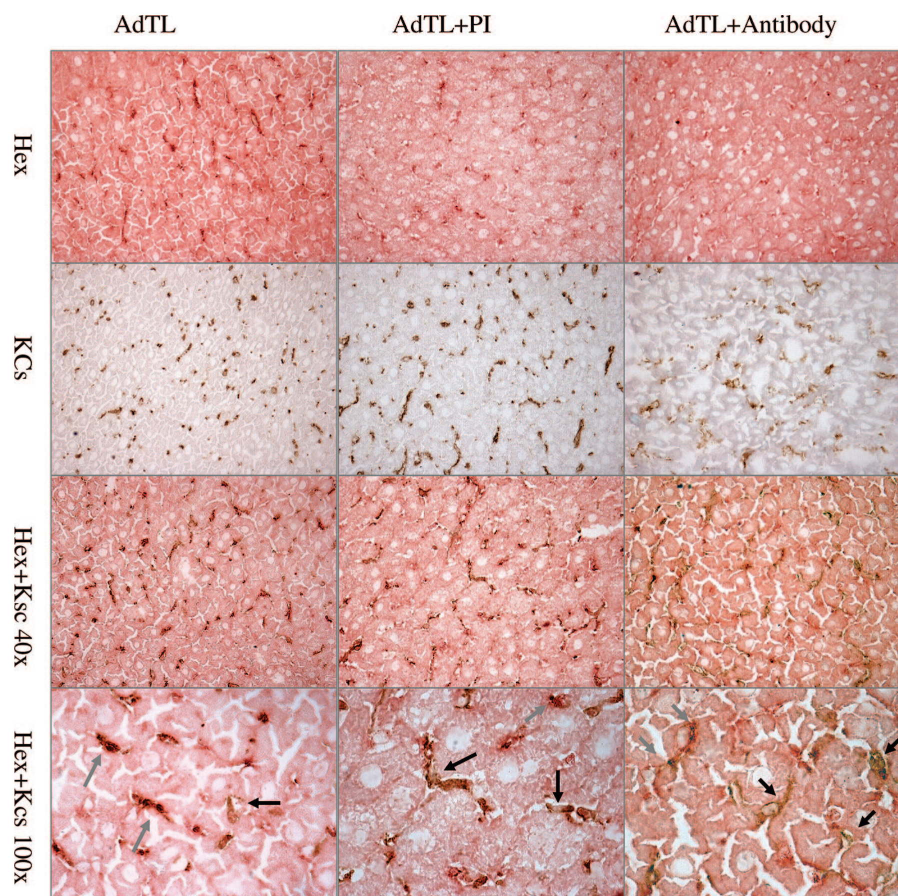

Figure 6. *In vivo* uptake of adenovirus by KCs in an SR-A-dependent manner. Immunohistochemistry on liver cryosections of mice injected with PBS (AdTL) or with PBS containing 0.2 mg of poly(I) (AdTL+PI) per animal 5 min prior to injection of 5×10^{10} adenoviral particles per animal. AdTL+antibody represents a group of animals injected with AdTL coupled to a viral knob single chain antibody. Viral localization was shown using an antihexon antibody, red color (Hex). KCs were detected with the F4/80 antibody, brown color (KCs). Colocalization studies were performed with hexon and KCs double-staining, dark brown/red color (Hex+KCs). Random fields at 40 and 100 times magnification are presented. In the AdTL group, most of the Kupffer cells are infected with virus and are double positive in the staining (gray arrows). Single positive (black arrow), noninfected cells are occasionally observed. In the AdTL+PI group, more uninfected Kupffer cells (black arrows) are observed, indicating that viral entry into Kupffer cells is abrogated/prevented. In the AdTL+antibody group, predominantly uninfected Kupffer cells (black arrows) are observed, indicating that viral entry was almost completely inhibited.

Therefore, we investigated the role of SR-A in viral uptake. Using CHO cells expressing the scavenger receptor A, we showed that adenovirus can be taken up and result in viral transgene expression. The luciferase expression could be partially blocked by inhibiting adenoviral uptake using SR-A ligands and/or an SR-A antibody. Most likely, adenovirus interacts directly with the scavenger receptor A. Interaction of adenovirus with blood factors has been shown to be necessary for the entry of adenovirus via HSPG.[27] However, preincubation of the virus with human serum before infection of CHO-SR-A cells did not result in an increase of adenoviral uptake, but rather in blocking of uptake (data not shown), indicating a direct interaction of the virus with SR-A. Electron microscopic analysis showed that within half an hour of uptake the majority of adenovirus in J774 macrophages was present in enclosure of the virus in multilayer cellular structures (autophagosome), fused or not with lysosome compartments, resembling a viral degradation pathway.

Since some viral transgene expression has been detected upon AdTL infection in the macrophage cell line[34] and in primary KCs, it is possible that some of the virus is able to escape degradation. It has to be established if the transgene expression results from adenoviral saturation of the degradation pathway or from a proportion of the virus that enters the cells via another receptor.

Adenoviral infection in primary KCs was partially blocked either by preincubation of the cells with poly(I) or with

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soluble knob 5 before viral infection. This result suggests that the knob of the virus may be necessary for adenoviral uptake in macrophages via the scavenger receptor A. However it cannot be excluded that the adenoviral knob may be responsible for adenoviral uptake via other receptors. To confirm these data, similar experiments need to be performed in primary KCs deleted for the SR-A and/or other adenoviral receptors such as HSPG. IHC performed in animals preinjected with poly(I) shows a reduced colocalization of the virus with KCs when compared with animals directly injected with Ad. Very little colocalization of the virus was also observed when the virus was conjugated with a single chain antibody directed against the viral knob before injection. This result shows that SR-A is involved in adenoviral uptake *in vivo* and it is possible that blocking the viral knob can prevent entry of adenovirus in an SR-A-dependent manner. Although the role of the knob in adenoviral uptake via SR-A remains to be investigated in more detail, these results already show its potential involvement. In gene therapy applications, the single chain antibody directed against the viral knob can be used first to prevent adenoviral uptake by KCs, and moreover to direct the virus to a specific target tissue by attaching to the single chain antibody a targeting moiety, that can be another antibody or a peptide.[13,48,49]

Although this paper gives the first proof of adenoviral uptake via SR-A, very intriguing questions still need to be answered. Is the interaction of the virus with SR-A crucial in activation of a degradation pathway? Which cellular components in macrophages result in virus degradation when compared to other cell types? Since adenoviral uptake and subsequent clearance can be partially prevented by the use of poly(I) or by covering the adenoviral knob by the use of a single chain antibody, we believe that these studies signify the possibility to improve systemic gene therapy applications.

Material and Methods

Cells. CHO and CHO-SR-A obtained from Prof. S. Gordon[42] were cultured in Ham's F12 medium (Gibco BRL, Paisley, Scotland); the human epithelial lung carcinoma A549 cells and the adenoviral transformed human embryonic kidney cell line 293, derived from ATCC, were cultured in DMEM-F12 (Gibco BRL); and the murine macrophage-like J774 cell line derived from ATCC was cultured in DMEM. The growth medium contained L-glutamine (Gibco BRL, Paisley, Scotland), 10% fetal bovine serum (FBS; BioWhittaker Europe, Verviers, Belgium), and penicillin (100 IU/mL)/streptomycin (100 μg/mL) (Gibco BRL). Cells were grown at 37 °C in a humidified 5% CO_2/95% incubator.

Rat Kupffer cells were isolated from male Wag/Rij rats (200−250 g) after collagenase perfusion of the organ, followed by centrifugation and counterflow centrifugal elutriation as described previously.[50] Kupffer cells were grown in 24-well plates in RPMI-1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. The purity of Kupffer cells was controlled by immunocytochemistry using the ED2 antibody and by staining for the endogenous peroxidase activity for which KCs are positive and endothelial cells are negative. The purity was >80%.

Inhibitors. The following scavenger receptor A ligands were used: polyinosinic acid potassium salt [poly(I)] (Sigma), dextran (Sigma), antimouse SR-A antibody 2F8 (CD204, SR-A, mouse, mAb 2F8) (Hycult biotechnology). Polycytidylic acid potassium salt [poly(C)] was used as a cognitive nonligand for SR-A.

Adenoviral Vector. AdTL is an E1- and E3-deleted recombinant serotype 5 adenovirus.[51] It contains a green fluorescent protein (GFP) and luciferase gene expression cassette, each under control of a cytomegalovirus (CMV) promoter.[20] The vector was grown on 293 cells and purified in HEPES/sucrose buffer, pH 8.0 according to conventional double CsCl gradient centrifugation method,[52] and the number of viral particles was calculated from the optical density at 260 nm (OD 260). The number of plaque forming units (PFU) was determined by plaque forming assay. The ratio vp:PFU was determined to be 1:18.7. Ad-PIX-GFP[44] was produced and isolated as the AdTL virus.

Production of Knob 5. The knob domains of adenovirus 5 fibers were expressed in *E. coli* with N-terminal His6 tags, using the pQE30 expression vector (Qiagen, Hilden, Germany). Knob5 was purified on nitrilotriacetic acid agarose columns (Qiagen) and dialyzed against PBS. The ability of knob5 to form homotrimers was verified by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of boiled and unboiled samples. The concentration of the purified knob5 was determined by the Bradford protein assay (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard.

***In Vitro* Infection and Reporter Gene Expression Analysis.** The CHO and CHO-SR-A cells were seeded in 96-well culture plates for viral transduction experiments. After 24 h, cells were first incubated for 1 h at 37 °C as indicated in the experiments in the presence of SR-A ligands [poly(I), SR-A antibody, dextran] or SR-A nonligands [poly(C)]. After 1 h, AdTL was added in culture medium containing 2% FBS (infection medium). One hour after

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infection, the infection medium was replaced by normal culture medium and cells were incubated for 48 h before performing the luciferase assay and GFP observations. The cells were lysed with cell culture lysis buffer (Promega) and the lysates analyzed with the Luciferase Assay System (Promega) on a Lumicount luminometer (Packard, Groningen, The Netherlands). All data are expressed as relative light units (RLU). Adenoviral infection and luciferase expression on primary Kupffer cells were performed as described above in the presence of poly(I) or a soluble knob5.

FACS Analysis. To determine uptake and internalization of virus in cells, J774 and A549 cells were infected with the green fluorescent virus Ad-PIX-GFP. When indicated, cells were incubated with poly(I), 2F8 or poly(C) before infection. Prior to flow cytometry analysis, cells were fixed with 0.5% paraformaldehyde in PBS. Cells were analyzed for the presence of GFP derived by the uptake of the virus.

Confocal Microscopy. Uptake and internalization of the Ad-PIX-GFP virus in J774 cells was visualized using the Leica TCS SP2(AOBS) microscope (University Medical Hospital Groningen Microscopy and Image Center). Cells were grown on 8 well Permanox chamber coverglass (Laboratory-Tek). Infection was performed in growth-medium containing 2% FBS. When indicated, cells were preincubated with poly(I) or poly(C). Thirty minutes after infection, cells were fixed with 3.3% paraformaldehyde in PBS for 10 min at room temperature. Cells were than washed in 10 mM glycine in PBS and mounted with Citifluor (Agar scientific). Z-stacks of randomly selected areas were made, and representative sections showing virus internalization were selected.

Electron Microscopy. J774 cells infected for half an hour at an MOI of 10,000 viral particles per cell (Ad-PIX-GFP) were fixated in a 0.1% glutaraldehyde and 4% paraformaldehyde buffer and post-fixated for 4 h in a solution of 1% osmium tetroxide and 1.5% potassium ferrocyanide in a 0.1 M phosphate buffer pH 7.4. After dehydration in alcohol, the samples were embedded in Epon according to routine procedures. Semithin sections were screened with a light microscope, and areas of interest were selected for ultrathin sectioning. The ultrathin sections were examined in a transmission microscope CM100 (Philips, Eindhoven, The Netherlands) at 80 kV.

Animal Infection Studies. Anesthetized (isoflurane/ N_2O/O_2 inhalation) C57/Bl mice (Harlan CPB) were injected via the orbital plexus with adenoviral particles, diluted in HEPES/sucrose buffer (pH 8.0). The virus was injected alone or conjugated to knob antibodies. When indicated, animals were injected via the orbital plexus with 0.2 mg of poly(I) in a PBS solution 5 min prior to viral infection. For immunohistochemistry, animals were killed 10 min after viral injection and organs excised and frozen in liquid N_2. The experiments were performed according to the European ethical board statement. Approval and description of the experiments are contained in the D4314B documents at the University of Groningen.

Immunohistochemistry. Double immunohistochemistry was performed on frozen liver sections as follows: Livers were frozen in liquid N_2, and 7 μm sections were cut, fixed with acetone, air-dried and rehydrated in phosphate buffered saline (PBS). Kupffer cells were stained with the F4/80 antibody (Serotec) a macrophage specific antibody raised in goat (Abcam plc, Cambridge, U.K.), followed by rabbit antigoat IgG conjugated to biotin and a streptavidin−alkaline phosphatase complex followed by DAB staining. Adenovirus was detected using AB1056, an adenovirus type 5 hexon specific antibody raised in rat as primary reagent followed by rabbit antirat IgG conjugated to biotin. The signal was amplified by use of the AB Complex-HRP kit (Dako Denmark A/S), which provides a conjugated peroxidase. Staining was performed using fuchsin (Dako). Slides were mounted in Kaiser's glycerin.

Statistical Analysis. Differences in gene expression were analyzed using a two-tailed paired Student's t test. $P < 0.05$ was considered significant. $n \geq 3$.

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