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A Novel Strategy to Modify Adenovirus Tropism and Enhance Transgene Delivery to Activated Vascular Endothelial Cells *In Vitro* and *In Vivo*

KEN-ICHI OGAWARA,[1,2] MARIANNE G. ROTS,[2] ROBBERT J. KOK,[1] HENK E. MOORLAG,[3] ANNE-MIEK VAN LOENEN,[1] DIRK K. F. MEIJER,[1] HIDDE J. HAISMA,[2] and GRIETJE MOLEMA[3]

ABSTRACT

To assess the possibilities of retargeting adenovirus to activated endothelial cells, we conjugated bifunctional polyethylene glycol (PEG) onto the adenoviral capsid to inhibit the interaction between viral knob and coxsackie-adenovirus receptor (CAR). Subsequently, we introduced an αv integrin-specific RGD peptide or E-selectin–specific antibody to the other functional group of the PEG molecule for the retargeting of the adenovirus to activated endothelial cells. *In vitro* studies showed that this approach resulted in the elimination of transgene transfer into CAR-positive cells, while at the same time specific transgene transfer to activated endothelial cells was achieved. PEGylated, retargeted adenovirus showed longer persistence in the blood circulation with area under plasma concentration-time curve (AUC) values increasing 12-fold compared to unmodified virus. Anti-E-selectin antibody-PEG-adenovirus selectively homed to inflamed skin in mice with a delayed-type hypersensitivity (DTH) inflammation, resulting in local expression of the reporter transgene luciferase. This is the first study showing the benefits of PEGylation on adenovirus behavior upon systemic administration. The approach described here can form the basis for further development of adenoviral gene therapy vectors with improved pharmacokinetics and increased efficiency and specificity of therapeutic gene transfer into endothelial cells in disease.

OVERVIEW SUMMARY

One of the major limiting factors for gene therapy is the lack of efficiency of gene transfer to desired disease-associated cell types. We have used a novel method for enhancing gene delivery to vascular endothelial cells by coupling cell selective homing ligands (i.e., αv integrin-specific RGD peptide and E-selectin–specific antibody) to PEGylated adenovirus. *In vitro* studies showed that the resulting adenoviral vectors were devoid of transgene transfer into coxsackie-adenovirus receptor (CAR)-positive cells, while specific transgene transfer to activated endothelial cells was achieved. Furthermore, anti-E-selectin antibody-modified PEGylated adenovirus exhibited improved behavior *in vivo* after systemic administration compared to its unmodified counterpart. It demonstrated an improved blood circulation time, and selectively homed to activated endothelium in skin in mice with a delayed-type hypersensitivity (DTH) skin inflammation, resulting in local expression of the reporter transgene luciferase.

INTRODUCTION

HUMAN ADENOVIRUS has been widely used as a vector for delivery of foreign genes to mammalian cells. The virus can stably incorporate relatively large insertions of foreign DNA (< 7.5 kb) into the genome. It can be concentrated to high titers from infected cells, and it has a highly efficient mode of gene transfer that does not require host cell replication.

Infection of adenovirus is initiated by the high-affinity binding of the C-terminal "knob" part of the fiber protein to coxsackie-adenovirus receptor (CAR; Bergelson *et al*., 1997). Biodistribution studies of systemically administered adenovi-

[1]Department of Pharmacokinetics and Drug Delivery, [2]Department of Therapeutic Gene Modulation, and [3]Department of Pathology and Laboratory Medicine, Medical Biology Section, Groningen University Institute for Drug Exploration, Groningen, The Netherlands.

rus have revealed that in rodents and non-human primates, adenovirus preferentially accumulates in the liver and to a lesser extent in the spleen (Huard *et al*., 1995; Sullivan *et al*., 1997). Hepatic uptake of adenovirus presumably takes place mainly via CAR on the hepatocytes although detailed information about the mechanisms of entry to other cell types remains to be elucidated.

Endothelial cells in general are active participants in varieties of diseases including cancer (Carmeliet and Jain, 2000) and chronic inflammation such as rheumatoid arthritis (Paleolog, 2002). In inflammatory reactions, endothelial cells facilitate transmigration of leukocytes by expression of cell adhesion molecules such as E-selectin, vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1), as well as production of cytokines and chemokines (Ebnet and Vestweber, 1999). Inflammatory mediators can also, either directly or indirectly, promote angiogenesis. Moreover, there is considerable evidence to suggest that angiogenesis and chronic inflammation are codependent processes. Thus, their active roles in disease pathophysiology together with their easy accessibility from the blood makes endothelial cells attractive targets for therapy. For the potential benefits of targeting activated endothelial cells in chronic inflammation and angiogenesis, the discrimination between endothelial cells in these pathologic conditions and the normal quiescent vascular endothelium is critical. In the past a number of target epitopes overexpressed on activated (i.e., angiogenic or proinflammatory) endothelial cells has been identified, including $\alpha v\beta 3$ integrins (Brooks *et al*., 1994), E-selectin (Luo *et al*., 1999), and vascular endothelial growth factor receptors (Veikkola *et al*., 2000). Certain conformationally constrained peptides, characterized by an Arg-Gly-Asp (RGD) motif, specifically bind αv-integrins and have been shown to be suitable homing ligands for the delivery of drugs into proangiogenic endothelium (Pfaff *et al*., 1994; Pasqualini *et al*., 1997; Ellerby *et al*., 1999). With regard to E-selectin targeting, our previous study showed that anti-E-selectin antibody can be used to deliver drugs selectively into activated endothelial cells *in vitro* and *in vivo* (Everts *et al*., 2002, 2003; Asgeirsdottir *et al*., 2003).

Based on this, we hypothesized that blocking the normal knob-CAR interaction and introducing an adequate homing device onto the adenovirus would result in a change of its natural tropism favoring endothelial target epitopes of choice. To test this hypothesis we conjugated bifunctional polyethylene glycol (PEG) onto the viral capsid of adenovirus encoding luciferase as a reporter gene. For the retargeting of adenovirus, we introduced RGD peptide or antibody against E-selectin to the other functional group of the PEG molecule. Improvement in specificity and efficiency of gene transduction was evaluated *in vitro* for both retargeting strategies, whereas kinetics, homing selectivity and transgene expression after intravenous administration of E-selectin directed adenovirus were studied *in vivo*.

MATERIALS AND METHODS

Chemicals and proteins

RGD- and control-peptide. The cyclic RGD-peptide c(RGDf(ϵ-S-acetylthioacetyl)K) and the RAD analogue c(RADf(ϵ-S-acetylthioacetyl)K), hereafter referred as RGDpep and RADpep, respectively, were prepared by Ansynth (Roosendaal, The Netherlands). This RGDpep was previously conjugated to human anti-mouse antibody (RDG-modified protein) thereby providing the protein with $\alpha v\beta 3$ integrin specificity (Kok *et al*., 2002; Schraa *et al*., 2002).

Antibodies. The H18/7 (mouse IgG2a anti-human E-selectin) monoclonal antibody-producing hybridoma was kindly provided by Dr. M. Gimbrone, Jr. (Brigham & Women's Hospital, Boston, MA) and used for the *in vitro* studies described here. H18/7 antibody was purified from the culture medium by protein A affinity chromatography (protein A sepharose fast flow, Pharmacia, Roosendaal, The Netherlands), followed by dialysis against phosphate-buffered saline (PBS). The rat IgG2a anti-mouse E-selectin antibody MES-1 was kindly provided by Dr. D. Brown (Cell Tech Group, Slough, UK) and was used for the *in vivo* studies described here. An irrelevant control antibody (323A3, mouse IgG1 anti-human EGP-2) was kindly provided by Centocor (Leiden, The Netherlands).

Production of knob5. The knob domains of adenovirus 5 fibers were expressed in *Escherichia coli* with N-terminal His6 tags, using the pQE30 expression vector (Qiagen, Hilden, Germany; Krasnykh *et al*., 1996). Knob5 was purified on Ni-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.

Chemical conjugation of adenovirus

The E1- and E3-deleted AdTL vector was produced and purified in HEPES/sucrose buffer, pH 8.0 according to conventional double CsCl gradient centrifugation method, and the number of viral particles was calculated from the optical density at 260 nm (OD_{260}). This vector contains green fluorescent protein (GFP) and luciferase gene as the reporter gene under control of a cytomegalovirus (CMV) promoter (Alamany and Curiel, 2001). Conjugation reactions were performed by a modification of established methods (Romanczuk *et al*., 1999). Figure 1 schematically represents the concept of adenoviral retargeting by combining PEGylation and endothelial specific homing ligand introduction, as well as the chemical approach chosen in this study. In brief, an aliquot of heterobifunctional PEG linker (3.4 kD) with N-hydroxysuccinimide ester (NHS) and vinyl sulfone (VS) group at each end of the molecule (NEKTAR Therapeutics, Huntsville, AL) dissolved in dimethylformamide (DMF; 100 mg/1 ml DMF) was added slowly to the virus (1.0×10^{12} viral particles) in a ratio of 10^5:1 moles PEG:viral particles. Reaction mixture was protected from light and gently mixed for 1.5 hr at 4°C. After the purification using a PD-10 column (Amersham Biotech, Uppsala, Sweden), PEGylated virus was directly used in the following coupling reaction with either RGDpep, RADpep, cysteine, or respective antibodies (see below). RGDpep, RADpep or cysteine dissolved in an acetonitrile-water mixture (1:4) at a concentration of 10

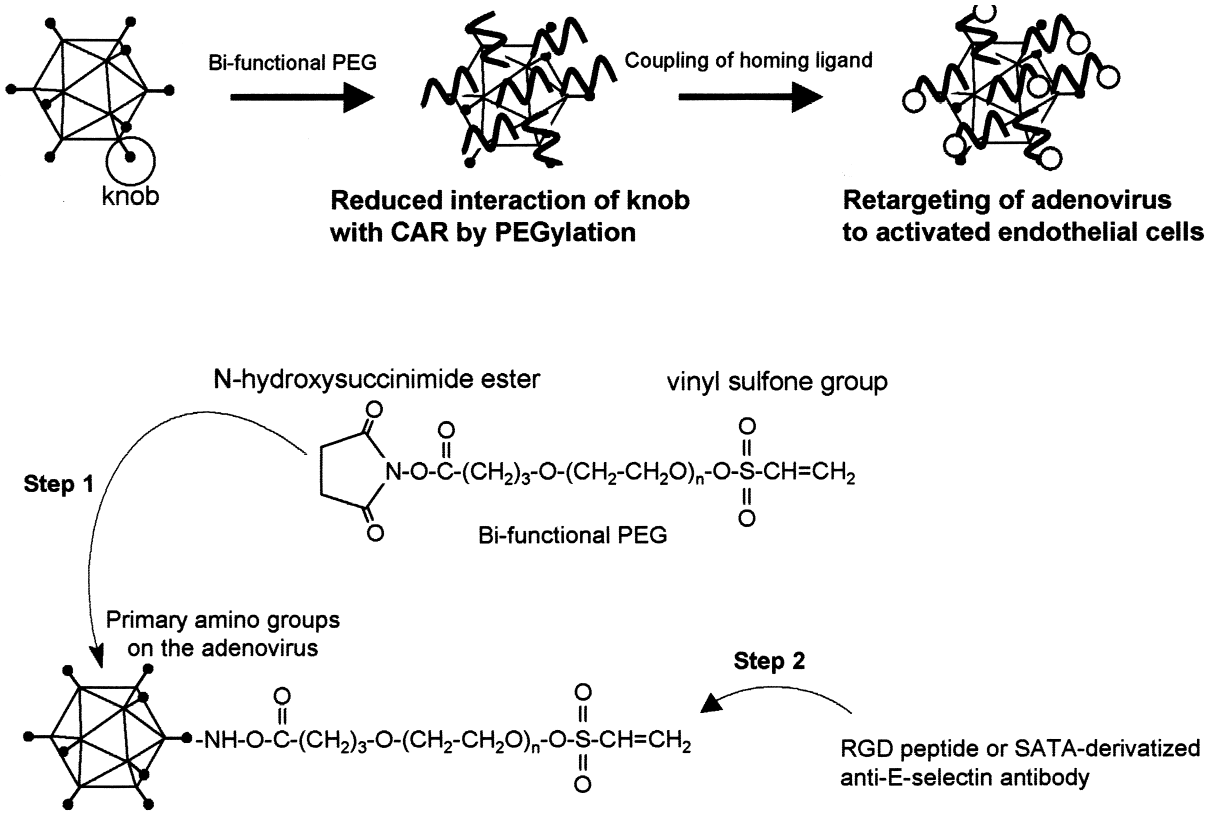

FIG. 1. Schematic representation of the concept of adenoviral retargeting by PEGylation and endothelial specific homing ligand introduction (**top**), and of the chemical approach chosen (**bottom**).

mg/ml was added dropwise to the PEGylated virus in the molar ratio of 10^5:1. After the addition of 25 μl of a freshly prepared 1 *M* hydroxylamine solution to unprotect the thiol group of the peptide, the mixture was reacted for 4 hr at 4°C under gentle mixing. Unreacted reagents were removed by dialysis (DispoDialyzers 300 kD MWCO, Spectrum Laboratories, Rancho Dominguez, CA) against HEPES/sucrose buffer (pH 8.0) at 4°C. The final virus preparation was collected and stored at −80°C in small aliquots until use. Initial studies showed that in the PD-10 column purification approximately 80% of the peak containing PEGylated virus was free from contamination with unconjugated PEG and that the dialysis procedure did not lead to loss of conjugated virus. Therefore, we collected the initial 80% of PEGylated virus that eluted from the PD-10 column and used the factor of 0.8 to calculate the final number of viral particles of each preparation. In the case of conjugation with antibodies, the protein was first reacted with N-succinimidyl S-acetylthioacetate (SATA) for 1 hr by an established method in the molar ratio of 1:10 (Koning *et al*., 1999). After the purification by dialysis against PBS, this SATA-modified antibody was directly used for the coupling with PEGylated adenovirus in the molar ratio of 10^3:1 and purified using the same procedure as described above.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from the Endothelial Cell Facility RuG/AZG (Groningen, The Netherlands). Primary isolates were cultured on 1% gelatin-precoated tissue culture flasks (Costar, The Netherlands) at 37°C under 5% CO_2/95% air. The culture medium consisted of RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine, 5 U/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml endothelial cell growth factor supplement extracted from bovine brain. The H5V mouse endothelioma cell line was kindly provided by Dr. A. Vecchi (Mario Negri Institute for Pharmacological Research, Milan, Italy). These cells were grown in tissue culture flasks at 37°C under 5% CO_2/95% air. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FCS, 2 m*M* L-glutamine, and 300 μg/ml gentamicin. Both cell types, upon confluency, were detached from the surface by trypsin/ethylenediaminetetraacetic acid (EDTA; 0.5/0.2 mg/ml in PBS) and split at a 1:3 ratio. For the experiments described, HUVEC were used up to passage three.

Human cervical carcinoma cells, HeLa cells, were cultured in DMEM containing 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) at 37°C in a humidified 5% CO_2/95% air. The adenoviral transformed human embryonic kidney cell line 293 was cultured in DMEM-F12 (Gibco BRL) containing 10% FBS, 2 m*M* L-glutamine (Gibco BRL) and penicillin (100 IU/ml)/streptomycin (100 μg/ml) (Gibco BRL) at 37°C in humidified 5% CO_2/95% air.

Expression levels of target epitopes and CAR in cell types used

Of the cell types used for *in vitro* transduction studies, the expression levels of $\alpha v\beta 3$ integrin, E-selectin, and CAR were determined by flow cytometry and/or real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. For this, resting and 4-hr tumor necrosis factor (TNF)-α (100 ng/ml for HUVEC and HeLa cells, 250 ng/ml for H5V cells) activated cells plated at 30,000 cells/cm^2 on the day before use were stained for E-selectin with monoclonal antibody H18/7 (HUVEC and HeLa cells) and MES-1 (H5V cells); for $\alpha v\beta 3$ with monoclonal antibody LM609 (Chemicon, HUVEC and HeLa cells), and anti-$\beta 3$ (Pharmingen, H5V cells), and respective isotype-matched control antibodies that do not recognize human or mouse epitopes, respectively. Detection was performed with fluorescein isothiocyanate (FITC)-labeled secondary antibodies specific for the primary antibodies used, and samples were analyzed using a Coulter Epics-Elite flow cytometer (Coulter Electronics, Hialeah, FL).

RNA was isolated from the cells and cDNA was synthesized using standard laboratory protocols. For quantitative real-time RT-PCR, unlabeled mRNA-specific primer sets were used: for HeLa and HUVEC: Hs99999905_m1 for GAPDH, Hs00174057_m1 for E-selectin, and Hs00154661_m1 for CAR; for H5V: mm0043-7762_m1 for β_2-microglobulin, mm00438361_m1 for CAR (all purchased from Applied Biosystems as Assay on Demand; Foster City, CA), and an in-house designed set for E-selectin, in combination with MGB probes labeled with 6-carboxyfluorescein (FAM) and nonfluorescent quencher. mRNA levels of the respective genes were established in triplicate on an ABI Prism 7900 HT sequence detection system.

Transduction protocols and reporter gene expression analysis

For the transduction experiments of AdTL, AdTL-PEG-Cys, AdTL-PEG-RAD, and AdTL-PEG-RGD, cells (HeLa, HUVEC, and H5V) were plated in 96-well tissue culture plates (Costar, Cambridge, MA) at a cell density of 10,000 cells per well. For the transduction experiments of AdTL-PEG-H18/7 or AdTL-PEG-323A3, HUVEC were cultured for 4 hr at 37°C in the presence or absence of TNF-α (Boehringer, Germany, 100 ng/ml). The various viral vectors diluted in DMEM without FCS were added at 1000 viral particles per cell and incubated for 60 min at 37°C. The incubation medium was replaced by normal culture medium and cells were further incubated for 48 hr before performing the luciferase assay. In the case of competition experiments, cells (HeLa, HUVEC, and H5V) were incubated with RGD-modified protein (25 RGDpep per protein backbone (Kok *et al*., 2002); final concentration of 50 μg/ml), recombinant knob5 (20 μg/ml), or both, for 30 min at 4°C prior to addition of viruses for the transduction experiments of AdTL, AdTL-PEG-Cys, AdTL-PEG-RAD, and AdTL-PEG-RGD. For the transduction experiments of AdTL-PEG-H18/7 or AdTL-PEG-323A3, HUVEC were incubated with H18/7 (20 μg/ml), 323A3 (20 μg/ml), recombinant knob5 (20 μg/ml), or both at room temperature for 10 min prior to addition of viruses.

Delayed-type hypersensitivity mouse model

Animals. Male BALB/c mice (20–25 g) were purchased from Harlan (Zeist, The Netherlands) and housed under standard laboratory conditions with free access to standard chow and acidified water. All experiments were approved by the Local Committee on Animal Experimentation.

Delayed-type hypersensitivity model. On day 1 and 2, mice were sensitized by painting on the shaved abdomen skin with 20 μl 0.5% (v/v) DNFB (2,4-dinitro-1-fluorobenzene, Sigma) in acetone:olive oil (4:1). On day 15, animals were challenged by application of 20 μl 0.2% (v/v) DNFB in acetone:olive oil (4:1) to shaved flank skin. Acetone:olive oil (4:1) without DNFB was applied to the other shaved flank and served as a noninflamed skin control.

In vivo *pharmacokinetics of adenovirus*

To determine the blood circulation time of the viral constructs, 10^{10} viral particles diluted in 200 μl HEPES/sucrose buffer (pH 8.0) were intravenously injected via the penis vein of mice. A small aliquot of blood (40 μl) was collected by orbital puncture at 3, 30, 60 and 180 min after intravenous administration. The blood samples were then centrifuged to collect plasma and the numbers of viral particles in the plasma samples were subsequently measured by serial dilution and infection of 293 cells based on a conventional limiting dilution assay. In brief, each plasma sample was serially diluted in DMEM/F12 supplemented with 2% heat-inactivated FCS and added to 293 cells plated in 96-well tissue culture plates (10,000 cells per well). After 14 days, cells were observed for cytopathic effect. To estimate the number of viral particles in the plasma sample, this limiting dilution assay was performed in parallel with the original solution of viral constructs injected into the animal.

In vivo *homing and transgene expression by E-selectin antibody-retargeted adenovirus*

Retargeted adenovirus homing studies. Homing of retargeted adenoviral constructs was studied immunohistochemically in DTH mice, 24 hr after DNFB challenge. Previous experiments demonstrated E-selectin protein expression in the inflamed skin, and not in the control skin, at this time point (Everts *et al*., 2003). Mice were anesthetized (isoflurane/N_2O/O_2 inhalation) and 10^{10} viral particles were administered via the penis vein. At 3 hr after administration, mice were sacrificed and organs were excised and frozen in isopentane (−80°C).

Immunohistochemical detection. Acetone-fixed 5-μm cryostat sections of inflamed and noninflamed skin were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-rat immunoglobulin (Ig; DAKO, ITK Diagnostics, The Netherlands) diluted in PBS containing 5% FCS for the detection of AdTL-PEG-MES-1. This was followed by HRP-conjugated goat anti-Rabbit Ig (DAKO, ITK Diagnostics), with peroxidase visualization with 3-amino-9-ethyl-carbazole (AEC; Sigma), and section counterstain with Mayer's hematoxylin (Merck, Darmstadt, Germany) according to standard laboratory protocols. To localize the endothelium in the skin, serial sections were parallel stained with rat antibodies recognizing the endothelial cell marker CD31 (Pharmingen, San Diego,

CA), followed by the same staining procedure as described above.

Immunofluorescence detection. Acetone-fixed 5-μm cryostat sections were blocked with 2% normal mouse serum and subsequently double-stained for the presence of AdTL-PEG-MES-1 using TRITC-conjugated goat anti-rat IgG (SBA, Birmingham, AL) and for endothelial cells using in house-labeled ALEXA488-MECA32 antibody. The sections were first incubated with TRITC-conjugated goat anti-rat IgG, and after extensive washing, with MECA32-ALEXA antibody. Nuclear counter staining was performed using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim, Mannheim, Germany). Sections were embedded in CITIFLUOR (CITIFLUOR Ltd., London, UK) and examined using a fluorescence microscope (DM RXA, Leica) equipped with a Kappa CF8/1 FMC camera (Kappa Opto-Electronics, Gleichen, Germany) and Leica Q600 Qwin software (Qwin V01.06, Leica, Cambridge, UK).

Measurement of transgene expression. For the detection of transgene in DTH mice, animals were sacrificed 48 hr after administration and organs were excised and frozen in isopentane (−80°C). Tissue samples were shock-frozen in liquid nitrogen and subsequently homogenized by using an Ultraturrax T25 homogenizer (IKA Labortechnik, Berlin, Germany) in 5× volume of cell lysis buffer (Promega, Madison, WI), then incubated for 20 min at room temperature. Cell debris was pelleted in a microfuge at 12,000*g* for 3 min. Fifty microliters of supernatant was transferred in a new tube and used for luciferase and protein assay. Luciferase activity was measured in LumiCount (Packard Bioscience, Meriden, CT) and protein assay was performed using the protein assay Dc kit (Bio-Rad, Mississauga, Ontario, Canada).

Statistics

Statistical significance was evaluated using *F*-test followed by Student's *t* test. Results are expressed as the mean ± standard deviation (SD) of three or more samples.

RESULTS

Expression levels of target epitopes and CAR in cell types used

As shown in Figure 2, all three cell types used expressed $\alpha v\beta 3$ integrin, albeit to a different extent, with HUVEC expressing approximately 4 times as much as HeLa cells. The expression of $\beta 3$ integrin expression by H5V was also prominently visible, yet direct comparison with staining intensity of HUVEC and HeLa cells cannot be performed because different antibody combinations were used. E-selectin expression was only detectable on the endothelial cells, with an increase in expression level on TNF-α activation (Fig. 2). This was confirmed by real-time RT-PCR analysis of E-selectin mRNA levels, which were undetectable in HeLa cells, while they increased in H5V and HUVEC upon TNF-α activation. H5V cells were completely devoid of CAR expression, whereas mouse liver as a positive control showed significant CAR mRNA levels. In HeLa cells, the ΔCt value of CAR expression (i.e., CAR Ct value corrected for housekeeping gene Ct value) was 9.8 ± 0.3 ($n = 3 \pm$ SD), whereas the ΔCt value of CAR in HUVEC was 14.3 ± 0.4 ($n = 3 \pm$ SD). From this, it was calculated that CAR mRNA levels in HeLa cells in the experimental condition used were approximately 22 times higher than the levels in HUVEC. Although mRNA levels do not directly reflect absolute protein levels, the observation that HeLa cells express higher levels of CAR compared to HUVEC is in line with data reported earlier (Carson *et al*., 1999).

PEGylation of adenovirus completely abrogates CAR-knob interaction

HeLa, HUVEC, and H5V cells, which exhibit differences in CAR and αv-integrin expression as described above, were infected with AdTL, AdTL-PEG-Cys, AdTL-PEG-RGD, and AdTL-PEG-RAD in the absence or presence of recombinant knob5 (20 μg/ml), RDG-modified protein (50 μg/ml), or both as competitive inhibitors (Fig. 3A–3C). In HeLa cells (Fig. 3A), infectivity of the unmodified AdTL was almost completely

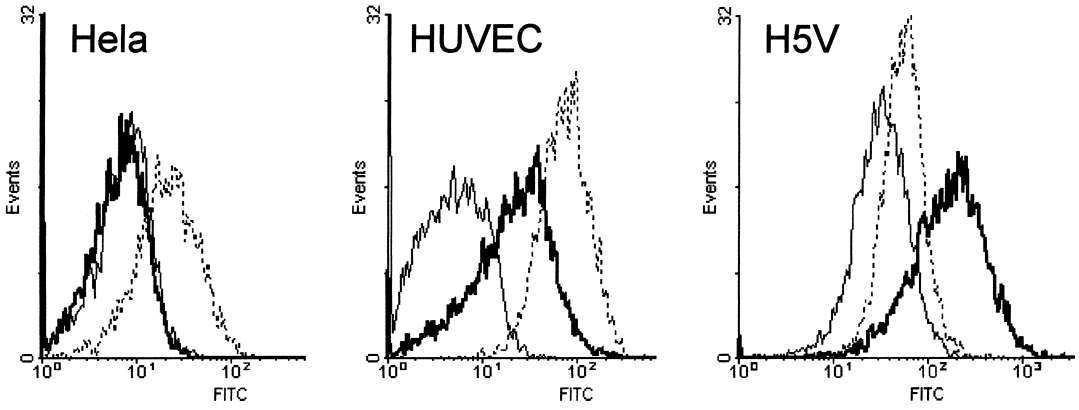

FIG. 2. Flow cytometric analysis of the expression levels of $\alpha v\beta 3$ integrin (dotted line) and E-selectin (resting conditions: thin solid line; tumor necrosis factor (TNF)-α–activated conditions: solid line) of the three cell types used for the *in vitro* transduction studies. For clarity, the histograms of the isotype matched negative controls, with mean fluorescence intensity values between 3 and 4, are not shown.

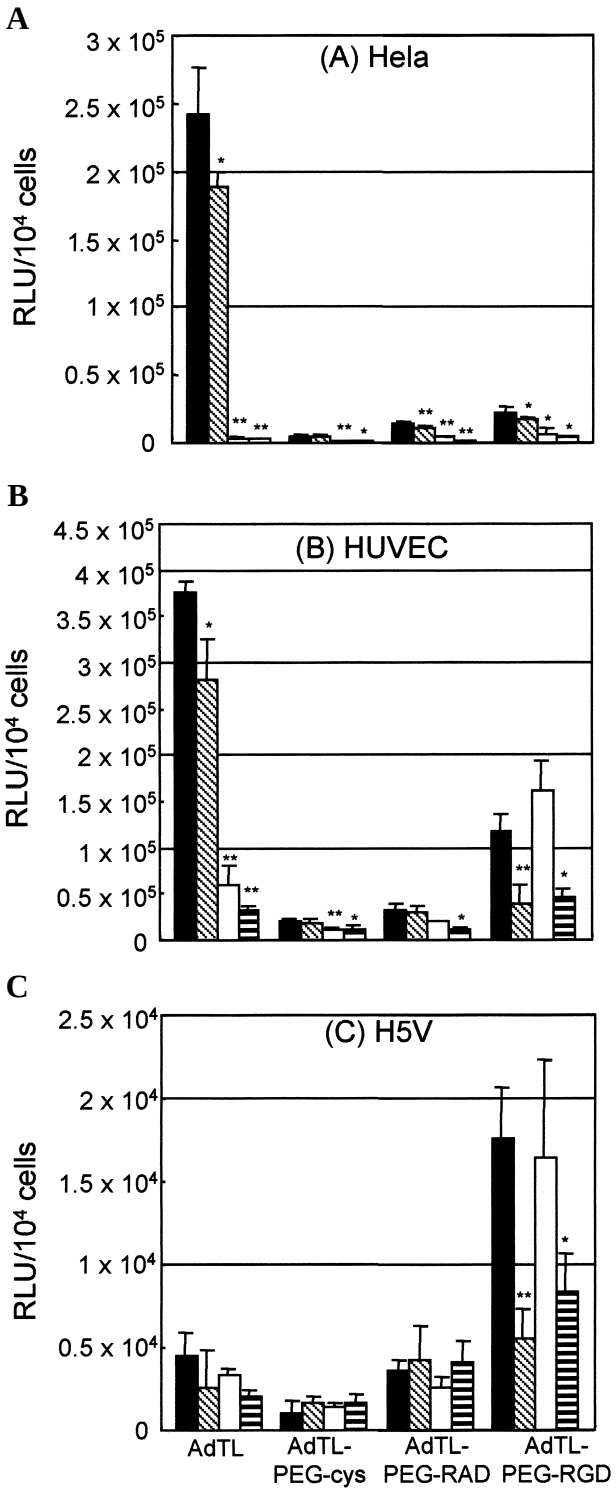

FIG. 3. Transduction of AdTL, AdTL-PEG-Cys, AdTL-PEG-RAD and AdTL-PEG-RGD in Hela (**A**), human umbilical vein endothelial cells (HUVEC) (**B**), and H5V cells (**C**) in the absence (■) or presence of RGD-modified protein (50 μg/ml, ▨), recombinant knob5 (20 μg/ml, □), or both (▤). Results are expressed as the mean relative light unit (RLU) ± standard deviation (SD) of at least three samples. $*p < 0.05$, $**p < 0.01$ compared to control (no block). The result shown here is the representative one among three independent experiments.

blocked by the presence of recombinant knob5 (< 5% of control). The infectivity of AdTL was also significantly inhibited in the presence of RGD-modified protein, although to a lesser extent (78% of control). These results show that the transduction of AdTL in HeLa cells is strongly dependent on CAR-knob interaction. In the case of PEGylated AdTL, viral infectivity was almost completely ablated, suggesting that the PEG molecule is shielding the viral knob from recognition by CAR.

In HUVEC (Fig. 3B), infectivity of AdTL could mainly be ascribed to CAR-knob interaction, similar to that in HeLa cells, because its infectivity was reduced to 16% of control in the presence of recombinant knob5. In the presence of both recombinant knob5 and RGD-modified protein, the inhibitory effect was even larger (9% of control), indicating that both CAR and αv integrin are involved in the transduction of AdTL into HUVEC. Also in these cells, PEGylated AdTL was barely able to infect and transfer the reporter gene.

RGD-PEG modification endows adenovirus with αv integrin-specific binding and infectivity

AdTL-PEG-RGD exhibited a relatively high transduction in HUVEC, compared to AdTL-PEG-Cys or AdTL-PEG-RAD. The transduction of AdTL-PEG-RGD was significantly inhibited by the presence of RGD-modified protein but not by recombinant knob5, suggesting that AdTL-PEG-RGD can efficiently infect in HUVEC mainly via the interaction of the introduced RGDpep with the endothelial αv integrin. In the case of AdTL-PEG-RAD, no such an increase in infectivity was observed, showing that the infectivity was RGDpep dependent, and not caused by peptide conjugation *per se*.

In H5V (Fig. 3C), the infectivity of AdTL was not affected by the presence of recombinant knob5, suggesting that the H5V mouse endothelial cell line does not express CAR on the cell surface. Direct measurement of CAR expression level supported this result. Of note is the observation that the infectivity of AdTL to H5V cells was 10-fold less compared to the infectivity of the two other cell types used, indicating that the existence of both CAR and αv integrin together likely facilitate the infectivity of adenovirus. A similar conclusion may be drawn from the AdTL infectivity levels of HUVEC and HeLa cells. While HUVEC express less CAR but more $\alpha v\beta 3$ than HeLa cells, AdTL infectivity is in the same range for both cell types (Fig. 3). AdTL-PEG-RGD showed highest transduction compared to the other adenoviral constructs tested. Also, the transduction of AdTL-PEG-RGD was significantly reduced by the presence of RGD-modified protein but not by recombinant knob5. These results, together with the fact that the transduction of AdTL-PEG-Cys and AdTL-PEG-RAD were only a fraction of that of AdTL-PEG-RGD, indicate that AdTL-PEG-RGD infected H5V via specific recognition of the RGDpep introduced.

These findings clearly demonstrate that chemically modified AdTL-PEG-RGD exhibit a change in specificity of cell entry from its intrinsic CAR-driven entry pathway to the αv integrin-mediated pathway via the RGDpep introduced.

Introduction of E-selectin specificity of infectivity by anti-E-selectin antibody modification of adenovirus

To evaluate the feasibility of specific delivery of PEG-modified AdTL to cytokine-activated HUVEC expressing E-selectin

on their surface, we compared the infectivity of AdTL, AdTL-PEG–anti-human E-selectin antibody (H18/7) and AdTL-PEG-control antibody (323A3) in resting and TNF-α–activated HUVEC (Fig. 4A and 4B). AdTL showed the highest transduction in resting HUVEC, which was almost completely blocked by the presence of recombinant knob5 (Fig. 4A). Furthermore, the two AdTL constructs modified with PEG and antibody exhibited approximately 10-fold less efficient transduction, presumably because of the PEG molecule on their surface preventing the interaction between CAR and viral knob, data corroborating those presented in Figure 3. On the other hand, in activated HUVEC, AdTL-PEG-anti-E-selectin antibody (H18/7) efficiently infected HUVEC to the same extent as AdTL, an enhancement in transduction efficiency that was not seen with AdTL-PEG-control antibody (323A3) (Fig. 4B). The transduction of AdTL-PEG-anti-E-selectin antibody was significantly inhibited by the presence of parental antibody (H18/7) but not by recombinant knob5 or by control antibody. These results clearly show that similar to RGD-PEG modification, anti-E-selectin antibody-PEG modification of AdTL abrogated the intrinsic viral knob-CAR interaction while gaining specificity for activated endothelium.

In vivo *kinetics, homing, and transgene expression*

PEGylation increases blood circulation time of adenovirus. The above findings prompted us to proceed to *in vivo* experiments to evaluate how AdTL, AdTL modified with PEG, and AdTL modified with PEG-anti-E-selectin antibody behave after intravenous administration in a murine DTH skin inflammation model. For proof of principle on homing selectivity and gene delivery capacity of the chemically modified adenovirus, we focused on AdTL modified with PEG and anti-E-selectin antibody in this specific model. In this model, E-selectin expression kinetics have been well documented, whereas knowledge on neovascularization patterns and αv integrin expression is lacking at present.

The rationale behind the retargeting of adenovirus is to deliver therapeutic genes selectively into the cell type of interest, while simultaneously decreasing distribution to other sites in the body, thereby diminishing side effects. In this respect, the blood circulation time, the homing potential of retargeted adenovirus, and the transduction capacity to the target cell type of interest *in vivo* are important features to be studied. Blood circulation time of AdTL-PEG–anti-mouse E-selectin antibody (MES-1) was found to be significantly longer than that of AdTL after intravenous administration (Fig. 5), showing that the PEG molecules introduced are affecting the normal clearance pathway of the adenovirus *in vivo*. The area under the plasma concentration-time curve (AUC) value of AdTL-PEG-MES-1 (1018 $\pm$ 398% of injected viral particles per milliliter [vp/ml] plasma*min) was 12-fold increased compared to that of AdTL (85 $\pm$ 46% of injected vp/ml plasma*min; $p < 0.01$). In the

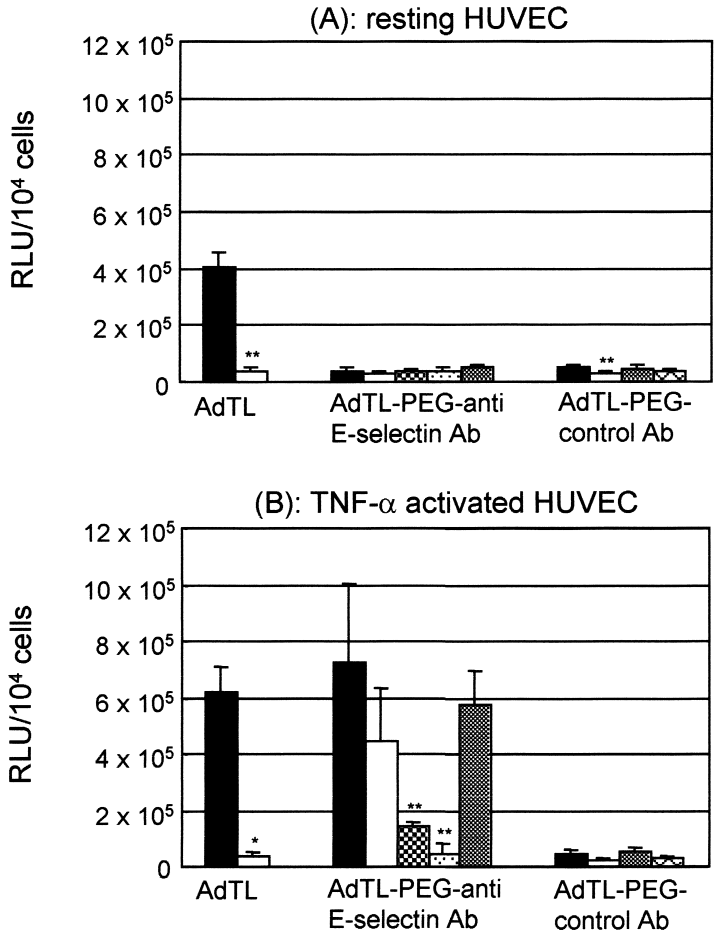

FIG. 4. Transduction of AdTL, AdTL-PEG anti-human E-selectin antibody (H18/7) and AdTL-PEG-control antibody (323A3) in resting (**A**) or tumor necrosis factor (TNF)-α–activated (4 hr) human umbilical vein endothelial cells (HUVEC) (**B**) in the absence (■) or presence of recombinant knob5 (20 μg/ml, □), parental H18/7 (20 μg/ml, ▩), both knob5 and H18/7 (⊡), parental 323A3 (20 μg/ml, ■) or both knob5 and 323A3 (▨), Results are expressed as the mean relative light unit (RLU) $\pm$ standard deviation (SD) of at least three samples. *$p < 0.05$; **$p < 0.01$ compared to control (no block). The result shown here is the representative among three independent experiments.

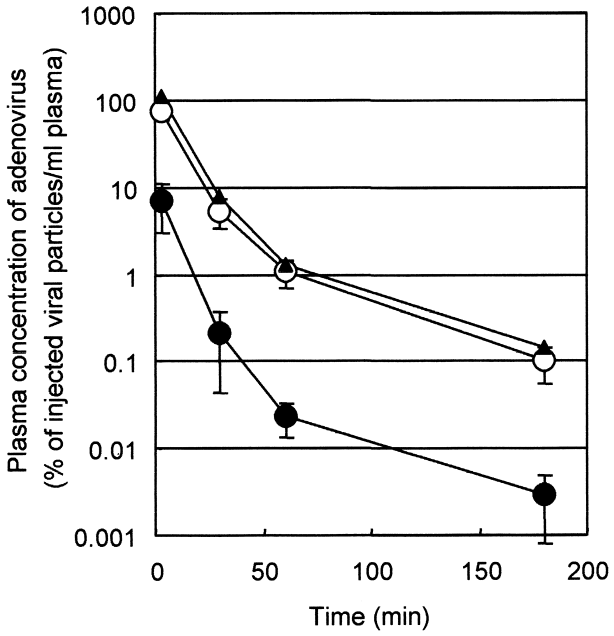

FIG. 5. Clearance of AdTL (●), AdTL-PEG-MES1 (○), or AdTL-PEG-Cys (▲) from the blood circulation after intravenous administration into mice with a local skin inflammation (delayed-type hypersensitivity [DTH] model). The number of injected viral particles (vp) was 10^{10}. Plasma was collected by orbital puncture at 3, 30, 60, and 180 min after injection. The number of viral particles in the plasma samples was evaluated based on a limiting dilution assay (see Materials and Methods section). Results are expressed as the mean ± standard deviation (SD) of three mice.

case of AdTL-PEG-Cys, AUC value (1479 ± 239% of injected vp/ml plasma*min) was 17-fold larger than that of AdTL ($p < 0.01$).

AdTL-PEG-MES-1 homed to inflamed endothelium. Three hours after intravenous injection of AdTL-PEG-MES-1, the virus could be clearly detected in the vasculature in the inflamed skin upon staining for its MES-1 fragment (Fig. 6A). This localization did not occur in unaffected skin upon performing the same staining protocol. In addition, staining with anti-rat IgG conjugated with TRITC colocalized with MECA32-ALEXA staining of endothelial cells, while being absent in unaffected skin (Fig. 6B and 6C). In contrast, AdTL-PEG–control antibody (323A3) could not be detected in the inflamed skin (data not shown). From these results, we concluded that introduction of E-selectin specificity by conjugating both PEG and MES-1 to the adenovirus endowed the virus with homing specificity for endothelial cells in the inflamed skin.

The AdTL-PEG-MES-1 delivered reporter transgene is expressed in inflamed skin, not control skin. Transgene expression in the skin samples 48 hr after intravenous injection was measured by the luciferase assay (Fig. 7). Significantly higher luciferase activity was detected in the inflamed skin (1717 ± 220 relative light unit [RLU]/mg of protein) than in the normal skin (316 ± 41 RLU/mg of protein) in the mice injected with AdTL-PEG-MES-1 (Fig. 7). Neither in those mice injected with AdTL-PEG-cys (Fig. 7), nor in those mice injected with AdTL-PEG–control antibody (323A3) (data not shown), could an increase in luciferase activity in inflamed skin be detected, implying that MES-1–PEG conjugation to AdTL harnessed the virus with specificity of infection for inflamed tissue. Using antiluciferase antibodies, we tried to determine the cellular expression of the transgene. Whereas the staining protocol was sufficiently robust to detect luciferase expression in HUVEC infected with AdTL-PEG-anti-E-selectin antibody (H18/7), no signal was found in the inflamed skins of the mice treated with AdTL-PEG-MES-1 (data not shown). However, taking into account that the homing of the AdTL-PEG-MES-1 was specific for activated endothelium in inflamed skin as assessed by immunohistochemistry (Fig. 6), and that transgene expression requires active internalization of the retargeted virus by a target cell, the data presented imply that the AdTL-PEG-MES-1 specifically infected E-selectin–expressing activated endothelial cells.

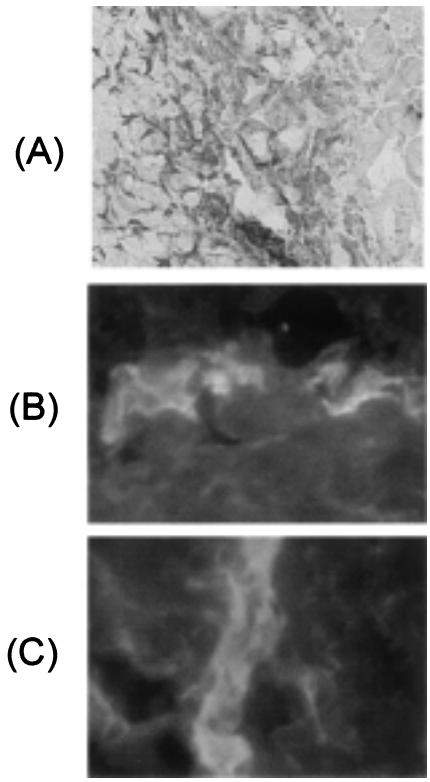

FIG. 6. Homing of AdTL-PEG-MES-1 to endothelial cells in the inflamed skin 3 hr after injection into mice with a local skin inflammation (delayed-type hypersensitivity [DTH] model). As described in the Materials and Methods section, 3-amino-9-ethyl-carbazole (AEC) staining was performed for MES-1 using anti-rat-HRP/AEC (**A**), whereas fluorescence double-staining was performed for MES-1 (using TRITC-labeled anti-rat immunoglobulin G) and endothelial cells (using MECA32-ALEXA) (**B** and **C**). Inflamed skin, (A) and (B); normal skin, (C). In (B) and (C) nuclear counter staining was performed using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Original magnification, 400×. (The color image of Fig. 6 can be accessed at http://www.rug.nl/med/faculteit/disciplinegroepen/plg/medbiol/research/ec/ec_pub)

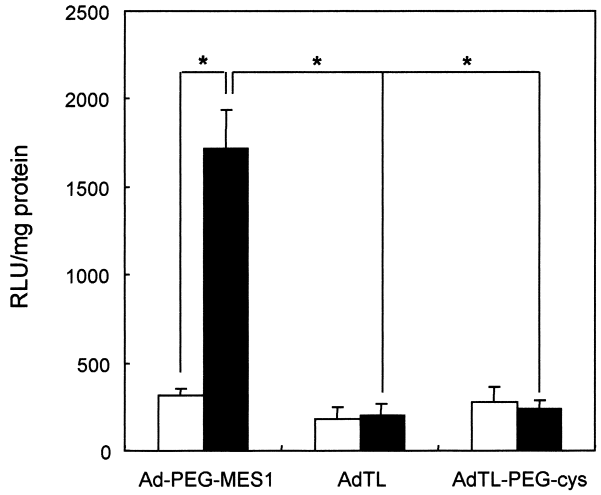

FIG. 7. Reporter transgene expression in the inflamed and control skins of delayed-type hypersensitivity (DTH) mice at 48 hr after intravenous administration of AdTL-PEG-MES-1, AdTL, and AdTL-PEG-Cys. □, noninflamed skin; ■, inflamed skin. Results are expressed as the mean relative light unit (RLU) ± standard deviation (SD) of three mice. $*p < 0.05$ compared to the respective group.

Expression of luciferase in the liver and spleen was also evaluated 48 hr after intravenous administration to investigate whether the PEG molecule affected the level of transduction into these two organs. Figure 8 shows that luciferase activity in the liver was significantly lower in the mice injected with AdTL-PEG-MES-1. In contrast, luciferase activity in the spleen was not significantly lower in the mice injected with AdTL-PEG-MES-1. In the case of the mice injected with AdTL-PEG-Cys, luciferase activity in both liver and spleen paralleled that observed for AdTL-PEG-MES-1 (i.e., levels were significantly lower than that of the mice injected with AdTL).

DISCUSSION

One of the major limiting factors for gene therapy is the lack of efficient gene transfer to desired disease-associated cell types. Viruses have their natural tropism for certain cell/tissue types but are relatively inefficient in infecting others. To overcome this problem, direct genetic modification of the capsid proteins was studied. In this approach, one of the drawbacks encountered was that genetic modification of the virion capsid and/or fiber knob did not eliminate the endogenous tropism of the virus to the desired extent (Wickham *et al*., 1996, 1997; Bouri *et al*., 1999; Hidaka *et al*., 1999; Reynolds *et al*., 1999).

In the current study, a strategy to achieve both reduction of CAR interaction reflected by diminished hepatic uptake of adenovirus (its natural tropism) and selective delivery of the adenovirus to the activated endothelial cells in chronic inflammatory lesions is presented. In this strategy, we conjugated bifunctional PEG onto the viral capsid of adenovirus. Subsequently, we introduced RGD peptide or antibody against E-selectin to the other functional group of PEG molecules for the retargeting of the adenovirus. Although the general concept of the modification of adenovirus with bifunctional PEG and homing device was postulated earlier (Romanczuk *et al*., 1999), we show for the first time that this retargeting strategy leads to selective gene transfer *in vivo* into the diseased, inflammation-associated endothelial cells after systemic administration. Chemical modification of adenovirus with PEG efficiently blocked the normal knob-CAR interaction. We additionally showed that PEGylation leads to an advantageous pharmacokinetic profile of the resulting retargeted adenoviral construct. As a result, the target cells are exposed to the construct for a prolonged period of time. This improvement in vector behavior is mainly caused by introduction of the PEG molecule because AdTL-PEG-Cys showed similar kinetics in the blood. PEGylation can have the additional advantage that it partially masks the immunogenic proteins of the capsid and likely represents a vector for *in vivo* gene transfer that evokes a limited immunologic reaction (the stealth approach) (Croyle *et al*., 2001). Recently it was reported that, in addition to neutralizing antibodies, other specific and nonspecific factors (e.g., soluble CAR, immunoglobulin, and serum albumin) exist in the milieu of diseased tissues, which can interact with adenovirus (Bernal *et al*., 2002). This interaction could possibly impair gene transfer by adenovirus. Moreover, introduction of PEG molecules onto the viral capsid seems to be an appropriate approach, because this molecule is universal in inhibiting the interaction between adenovirus and blood components and other components in the tissues and/or organs.

In the *in vivo* skin inflammation model, we unequivocally showed specificity for inflammation-associated endothelium. At present we cannot elaborate on the extent to which the PEGylation contributed to the selective homing potential of AdTL-PEG-MES-1, because we did not perform experiments with MES-1 directly conjugated to AdTL. No literature data are available on adenoviral retargeting to endothelium in inflamed tissue *in vivo*. We can, however, conclude that the modification with MES-1 for E-selectin targeting and PEG for the abovementioned purposes resulted in an adenoviral vector that se-

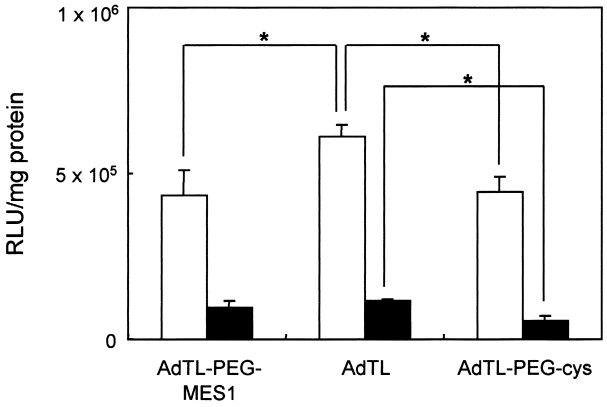

FIG. 8. Reporter transgene expression in the liver and spleen at 48 hr after intravenous administration of AdTL-PEG-MES-1, AdTL, and AdTL-PEG-Cys. □, liver; ■, spleen. Results are expressed as the mean relative light unit (RLU) ± standard deviation (SD) of three mice. $*p < 0.05$ compared to the respective group.

lectively localized in the endothelium in the inflamed skin and was capable of luciferase transgene delivery in the diseased endothelium.

Ideally, the present retargeting strategy should also lead to liver and spleen untargeting. Although AdTL-PEG-MES-1 or AdTL-PEG-Cys showed significantly lower luciferase expression in the liver in spite of their higher AUC values, the eventual levels of transgene of these PEGylated AdTL in the liver or spleen 48 hr after intravenous administration did not differ greatly from that of AdTL (Fig. 8). Moreover, the transduction of AdTL-PEG-MES-1 in the liver and spleen was 250 and 55 times larger than that of inflamed skin, respectively. One explanation for this observation is that activated endothelium in the skin represents only a small fraction of the cells in the tissue. Furthermore, the blood flow in the skin is low. Therefore, the chance for the retargeted adenovirus to be taken up by the endothelium in the skin is much smaller compared to the chance of uptake by other tissues like liver and spleen.

The choice of target molecules ($\alpha v\beta 3$ integrin versus E-selectin) defines the type of endothelium aimed at. E-selectin is expressed by activated endothelium that upon clearance of the inciting stimulus likely goes back into a resting state without going into apoptosis. $\alpha v\beta 3$ integrin is mainly expressed by endothelium in a proliferative (i.e., angiogenic) stage. Depending on the balance of growth factors at the site of inflammation, a newly formed vessel may regress in time. Whether this corrupts the therapeutic effectiveness of the targeted gene depends on the time frame in which the gene is being expressed in relation to the time frame in which the vessel regresses. A follow-up study including a therapeutic gene in the retargeted vector will reveal whether absolute amounts of gene product will be sufficient to interfere with endothelial cell activation and subsequently inhibit the inflammatory process while exerting no effect on the function of the eliminating cells in the liver and spleen. In this respect, the optimum number of viral particles to be injected to give the best therapeutic outcome will also have to be studied carefully. Knowledge regarding the number of viral particles being targeted to the inflamed endothelium and the level of gene product subsequently being produced is of importance to appreciate the potential of the adenoviral retargeting strategy proposed here for future therapeutic application. At present, these studies are, however, hindered by the fact that delivery of vectors selectively targeting the endothelial compartment cannot be accurately quantified *in vivo*, because the target compartment is minimal in size compared to the local blood and extracellular fluid compartment (Schraa *et al*., 2002; Cooke *et al*., 2001).

To improve retargeting adenoviral vectors with regard to their specificity, a combination of transductional and transcriptional retargeting may be used. By using a retroviral system, better specificity for endothelial cells was gained with the use of heterologous hypoxic and cytokine-inducible enhancers (Modlich *et al*., 2000). Reynolds *et al*. (2001) furthermore showed a tremendous improvement in specificity and efficiency of retargeting of adenovirus to endothelial cells in the lung by the combination of transductional targeting to a pulmonary endothelial marker angiotensin-converting enzyme, and an endothelial-specific promoter of vascular endothelial growth factor receptor type 1. Based on these accomplishments and the data presented here, a rational strategy to obtain higher specificity for and efficacy of gene transfer into endothelium in inflamed tissues would be a combination of our chemical homing approach with an adenovirus that encodes a therapeutic gene under the control of a disease-induced endothelial specific promoter. This would prevent liver and spleen associated toxicity while preserving the capacity of these organs to clear the therapeutic vectors from the body. Therapeutic genes of interest include dominant negative IκB, to block NF-κB–mediated cell activation (Iimuro *et al*., 1998), and heme oxygenase-1, one of the genes that can protect endothelium from excessive activation (Kushida *et al*., 2002).

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Address reprint requests to:
Grietje Molema, Ph.D.
Department of Pathology and Laboratory Medicine
Medical Biology Section
Hanzeplein 1
9713 GZ Groningen
The Netherlands

E-mail: g.molema@med.rug.nl

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