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Species Differences in the Pharmacology and Toxicology of PEGylated Helper-Dependent Adenovirus

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Abstract: Clinically relevant doses of helper-dependent adenoviruses (HDAds) provoke the host response against capsid proteins in primates and rodents. To determine if PEGylation truly affects this, baboons and mice were given either HDAd or PEG-HDAd expressing beta-galactosidase at 5 $\times$ 10^{11} or 3 $\times$ 10^{12} virus particles per kilogram (vp/kg) by iv infusion. Serum cytokines and blood chemistries were assessed for 96 h. PEG-HDAd reduced IL-6 6-fold in mice and 3-fold in the primate. This vector reduced IL-12 by 50% in both animal models. PEGylation reduced serum transaminases by approximately 50% at each dose in the primate and the mouse. PEGylation did not alter hepatic transduction efficiency in the mouse but did reduce transduction efficiency in the liver and the spleen of primates. Unmodified and PEGylated virus suppressed hepatic CYP3A activity in both animal models. PEGylation doubled the half-life ($t_{1/2}$) of the virus in the mouse and cut plasma clearance (CL) in half without affecting the half-life in primates. These results suggest that there are notable species-specific differences in the biodistribution of and response to PEG-modified vectors which may be linked to differences in binding properties to coagulation factors, receptor density and tissue architecture in the liver.

Keywords: Adenovirus; PEGylation; non-human primate; pharmacokinetics; toxicology

Introduction

PEGylation, the modification of biological molecules by covalent attachment of polyethylene glycol,[1] was first conceptualized in the 1970s in the pioneering work by Davis and Abuchowski.[2,3] Today, this technology has evolved to produce highly sophisticated, extremely potent, target-specific biomolecules used clinically for many chronic diseases.[4,5] Conjugation of PEG to complex biological compounds significantly changes their physical and chemical properties and, in turn, improves their physical stability, solubility and pharmacokinetic and immunological/toxicological profiles without compromising bioactivity.[4−7] The latter property, reducing immunogenicity and toxicity, was the impending force that expanded the application of this technology to intact microorganisms used for gene transfer.

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Viruses were originally thought to be perfect "Trojan horses" for gene therapy due to their inherent ability to efficiently transduce cells to support their own replication.[8] Their use was limited by the fact that they are rapidly cleared from the circulation by the immune system preventing them from reaching their target and reducing transduction efficiency if a second dose is needed. This fostered much research devoted to genetic and molecular manipulation of virus capsid proteins to evade the immune response.[9,10] Of the methods tested, PEGylation has been applied to several different viruses like adenovirus,[11−16] adeno-associated virus (AAV)[17,18] and lentivirus.[19] PEGylation did not significantly compromise transduction efficiency of these vectors, reduced the production of inflammatory cytokines and cytotoxic T cells and protected vectors from inactivation by complement and neutralizing antibodies.[19−22] To date, most of the data generated using PEGylation technology for gene transfer has focused on recombinant adenoviruses in a mouse model.

Although the adenovirus is the most highly characterized virus currently used for gene transfer, it is afflicted with one of the most vicious toxicity profiles.[23,24] Use of helper-dependent adenoviruses has minimized toxicity, allowing long-term, high-level gene expression in several animal models.[25−27] However, the innate immune response against capsid proteins, characterized by the release of pro-inflammatory cytokines into the circulation hours after injection and widespread activation of neutrophils, macrophages and dendritic cells in the spleen and Kupffer cells in the liver, continues to induce unwanted side effects such as thrombocytopenia and elevated liver enzymes, which, at certain doses, can lead to tissue injury, multiorgan failure and death.[26,28−30]

It has been shown that PEGylation of helper-dependent adenoviruses significantly reduces adenovirus-induced toxicity in the mouse.[15,16] Preclinical studies required for the

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marketing approval of any medicinal agent generally include toxicological evaluation of single and repeat doses in rodent and non-rodent species.[31,32] In recent years, the non-human primate has been the non-rodent species of choice due to the need for a model with the pharmacology, immune system and pharmacokinetics phylogenetically similar to humans.[33] In this report, we assess the pharmacokinetics, biodistribution and toxicity of two doses (5×10^{11} and 3×10^{12} virus particles per kilogram (vp/kg)) of a PEGylated helper-dependent adenovirus in male baboons. Results were compared to animals given unmodified virus from the same production lot. The effect of PEGylation on the expression and function of the hepatic cytochrome P450 3A4 in the primate, an enzyme homologous to the human isoform,[34,35] capable of metabolizing over 50% of all medicines currently on the market,[36] is also discussed.

Experimental Section

HDΔ28E4LacZ Rescue, Amplification, and Large-Scale Production. HDΔ28E4-βGEO was rescued (serial passage P0) by transfecting a 60 mm dish of 293Cre cells at ~80% confluence with 20 μg of *Pme*I-digested pΔ28E4-βGEO followed, the next day, by infection with 1,000 vp/cell of the helper virus, AdLC8cLuc.[37] The virus (HDΔ28E4-LacZ) was further amplified by serial coinfection of 60 mm dishes (~90% confluence) of 293Cre cells with 10% of the total culture volume consisting of crude lysate from the previous passage and 200 vp/cell of AdLC8cLuc for three serial passages (P1−P3). At P4, four 100 mm dishes of 293Cre cells were coinfected with media containing 10% (total culture volume) of the P3 lysate and 200 vp/cell of helper virus. At P5, eight 150 mm dishes of 293Cre cells were coinfected with 10% of the P4 lysate and 200 vp/cell of helper virus. Cells were harvested 48 h postinfection for use as inoculum for subsequent passage after one freeze−thaw. Virus amplification was assessed during each passage by staining one dish 24 h after infection with X-Gal and noting the number of cells that contained the chromogenic precipitate produced by active beta-galactosidase. For large-scale production, 16 triple flasks (Corning Life Sciences, Lowell, MA) of 293Cre cells at ~90% confluence were coinfected with 5% (total culture volume) of the P5 crude lysate and 200 vp/cell of helper virus. Cells were harvested 48 h postinfection by centrifugation. The cell pellet was resuspended in buffer (10 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$ and 4% sucrose) and stored at −80 °C prior to purification.

Virus Purification. Cells were lysed by three freeze−thaw cycles, and debris was eliminated by centrifugation at 3,500 rpm for 10 min. Virus was purified by banding twice on cesium chloride gradients and desalted on an Econo-Pac 10DG disposable chromatography column (Bio-Rad, Hercules, CA) equilibrated with sterile 100 mM potassium phosphate buffered saline (pH 7.4). Vector concentration was determined by UV spectrophotometric analysis at 260 nm and a standard limiting dilution assay.[38] All experiments were performed with freshly purified virus. The infectious virus to particle ratio for unmodified virus was 1:21 while that of the PEGylated preparations was 1:30. Helper virus contamination was not detectable by Southern blot analysis (<0.2% contamination).[39]

Southern Analysis for Helper Virus Contamination. Purified vector (50 μL) was incubated in Proteinase K (0.5 mg/mL in 10 mM Tris HCl, pH 7.5/10 mM EDTA/0.5% SDS) at 37 °C overnight. DNA was prepared by phenol/chloroform extraction and ethanol precipitation. The vector HDΔ28E4-βGEO, the helper virus AdLC8cLuc and the plasmid pΔ28E4-βGEO were digested with *Pst*I and *Pme*I and fractionated on a 1.2% agarose gel and transferred to a nylon membrane (GeneScreen Plus; Dupont NEN, Boston, MA) using a slot blot apparatus (Minifold II; Schleicher & Schuell BioScience, Keene, NH). The membrane was hybridized using a probe designed against the ITRs of pΔ28E βGEO at 60 °C in buffer composed of 5× SSC, 0.1% SDS, 5% dextran sulfate and 100 μg/mL salmon sperm DNA. The membrane was washed at 55 °C with a solution of 0.2×

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Table 1. Summary of Primate Characteristics, Treatment and Early Response to Helper-Dependent Adenoviruses[a]

animal no.	treatment	dose (vp/kg)	age (years)	wt (kg)	infusion time (min)	status 1 h postinfusion
17988	unmodified HDAd	5×10^{11}	3	8.48	3	lethargic
18167	PEGylated HDAd	5×10^{11}	3	9.14	3	active
27506	unmodified HDAd	3×10^{12}	1.9	6.76	4	lethargic, labored breathing; active by 3 h
27846	PEGylated HDAd	3×10^{12}	1.7	6.4	5	active

[a] Animals given the same dose of either PEGylated or unmodified virus were treated on the same study day. Studies with the low dose of either virus were conducted on different days than those involving the high dose.

SSC and 0.1% SDS. Autoradiography was performed with an ECL Plus system (GE Healthcare Bio-Sciences, Piscataway, NJ).

PEGylation of Helper-Dependent Adenovirus. Protein content of freshly purified virus was determined using BioRad DC Protein Assay reagents (BioRad, Hercules, CA) and bovine serum albumin (Sigma Aldrich, St. Louis, MO) in a standard in a microplate format. PEGylation was performed according to established protocols.[12] In brief, 10 μg of monomethoxypoly(ethylene) glycol, activated by tresyl chloride (Sigma Aldrich), was added for each microgram of protein present. The coupling reaction was performed for 2 h at 25 °C with gentle agitation and the reaction stopped by the addition of L-lysine, in a 10-fold excess with respect to the amount of PEG added. Unreacted PEG, excess L-lysine, and reaction byproducts were removed by buffer exchange over an Econo-Pac 10DG disposable chromatography column equilibrated with sterile 100 mM potassium phosphate buffered saline (pH 7.4). An aliquot of virus treated and processed in the absence of PEG in the same manner as the conjugated virus served as the unPEGylated control for each production lot. Characterization of these preparations revealed significant changes in biophysical properties of the virus such as the PEG−dextran partition coefficient and peak elution times during capillary electrophoresis.[12] Approximately 16,175 PEG molecules were associated with each virus particle in the studies outlined here as determined by a PEG−biotin assay.[15]

Animal Model. Animals were obtained from a large pedigreed colony of baboons maintained by the Southwest National Primate Research Center (SNPRC, San Antonio, TX) specifically for biomedical and behavioral research. At the SNPRC, baboons are generally housed in 6-acre, open-air corrals, capable of housing several hundred animals or large outdoor cages (300−1000 ft^2) where 10 to 30 animals reside in compatible social groups. These areas are equipped with climbing structures, perches, and swings for enrichment. Primates were given a stable, nutritionally complete diet as well as additional fruits and vegetables when appropriate. Primates used in the studies outlined in this manuscript were transferred from these facilities to single animal cages located inside the biomedical research buildings. Because this is a significant transition, approximately six animals for every two used in our studies were brought inside for a period of 14 days prior to the initiation of the study. During this time, animals were assessed for their ability to adapt in the inside environment as well as their tolerance for the backpack tethering system (see below). Animals not selected were promptly returned to the outdoor facilities prior to the initiation of the study. Specific details about the baboons used in our studies are summarized in Table 1.

Neutralizing Antibody Assay. Baboon serum was screened prior to the initiation of the study for the presence of anti-adenovirus serotype 5 antibodies. In short, serum was heated to 56 °C for 30 min to inactivate complement and then diluted in DMEM in 2-fold increments starting from a 1:20 dilution. Each dilution was mixed with adenovirus expressing beta-galactosidase (1×10^6 pfu), incubated for 1 h at 37 °C, and applied to HeLa cells in 96-well plates (1×10^4 cells/well). After this time, 100 μL of DMEM supplemented with 20% FBS was added to each well. Cells were incubated for 24 h and expression of beta-galactosidase measured by histochemical staining. NAB titers were calculated as the highest dilution at which 50% of the cells were positive for the transgene. The absence of neutralization in samples containing medium only (negative control) and FBS (serum control) and 1,280 ± 210 reciprocal dilution with an internal primate positive control stock serum were the criteria for qualification of each assay. This assay can detect the amount of antibody sufficient to neutralize 100 virus particles in as little as 10 μL of serum.

Administration of Virus. All procedures were performed in accordance with the guidelines established by the National Institutes of Heath for humane treatment of animals and approved by the Institutional Animal Care and Use Committees of the SNPRC and The University of Texas at Austin. Prior to initiation of the study, baboons (*Papio* sp.) were placed in individual specialized cages equipped with a tethering system consisting of a lightweight fiberglass backpack and a flexible stainless steel cable connecting the pack to a cannular slip-ring in the top of the cage, allowing for frequent blood sampling without sedation.[40] Blood was collected from each animal prior to treatment to establish baseline blood chemistries and hematological parameters via a 22 gauge catheter placed in the left cephalic vein. Virus was infused at a rate of approximately 0.5 mL/min for 3−5 min. Each dose was followed by 5 mL of sterile saline to ensure that the entire preparation was flushed from the iv line. Animals were housed under BSL 2 conditions. Five milliliters of blood was collected for assessment of virus concentration, cytokines, blood chemistries and hematological profiles 1, 3, 6, 24, 48, 72, and 96 h after vector

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administration. These time points were selected based upon documented clinical sequelae associated with the use of recombinant adenoviral vectors in non-human primates.[28,30,41] Urine and feces were collected at the same time as blood withdrawal and all samples screened for infectious virus by an *in vitro* limiting dilution assay and for viral genomes by real-time PCR (see below). Animals were euthanized and underwent full necropsy 96 h after treatment. The following tissues were collected: liver (4 lobes), lung (5 lobes), kidney, heart, spleen, lymph nodes (multiple locations), pancreas, stomach, duodenum, jejunum, ileum, cecum, colon, esophagus, trachea, bronchi, eye, bladder, prostate, testis, adrenal gland, thyroid/parathyroid, tongue, salivary glands (parotid and sublingual) thymus, skeletal muscle, tonsil and brain. Portions of each tissue were rinsed with saline and rapidly immersed in disposable peel-away molds containing Tissue-Tek O.C.T compound (Sakura Finetek, Torrance, CA) and stored at −80 °C for histochemical analysis of transgene expression. Samples of each tissue were also snap frozen in liquid nitrogen, and stored at −80 °C for microsome preparation (liver only) and DNA extraction for PCR.

Limiting Dilution Assay for Detection of Infectious Virus in Biological Samples. The term lac-forming unit (lfu) defines the number of infectious virus particles present in a preparation of recombinant adenovirus containing the beta-galactosidase transgene. In this assay, it is assumed that one lac-forming unit is equivalent to one active virus particle capable of inciting beta-galacotsidase expression in a single cell in a population as determined by histochemical staining. Prior to analysis, urine was concentrated by centrifugation with a Centricon Plus-20 Filter Device according to the manufacturer's instructions (Millipore, Billerica, MA). Ten microliters of sample (blood, urine) was diluted in 5-fold increments in DMEM supplemented with 2% fetal bovine serum (FBS, Cambrex BioScience Inc., Walkersville, MD). One hundred microliters of each dilution was added in triplicate to HeLa cells (ATCC CCL-2.2) seeded in 24 well tissue culture dishes (BD Biosciences, Bedford, MA) and incubated at 37 °C for 2 h. One milliliter of DMEM, supplemented with 10% FBS, was then added to each well. Twenty-four hours later, cells were fixed with 0.5% glutaraldehyde and beta-galactosidase activity was determined by incubation with the substrate 5-bromo-4-chloro-3-indolyl-beta-galactoside (X-gal) for 4 h at 37 °C in the dark. Staining medium was removed and blue colored positive cells tallied from a minimum of 20 microscope fields (approximately 48,000 cells). Lac-forming units were calculated as described previously.[42] This assay can detect virus concentrations that fall within the range of 10 to 1×10^{12} lfu/mL.

Pharmacokinetic Analysis. Pharmacokinetic parameters (plasma clearance (CL), the terminal elimination half-life ($t_{1/2}$), and the volume of distribution at steady state (V_{ss})) were calculated by a noncompartmental fit analysis using WinNonlin 4.0 (Pharsight Corporation, Mountain View, CA). The area under the plasma concentration−time curve (AUC) was calculated using the linear trapezoidal method with extrapolation of the terminal concentration to infinity.

Biochemical and Hematological Analysis of Blood. Hematological parameters (WBC, RBC, hemoglobin, hematocrit, platelets, neutrophils, lymphocytes, granulocytes, monocytes, eosinophils, basophils) and blood chemistries (glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, globulin, cholesterol, alanine transaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase, electrolytes, total bilirubin and lactate dehydrogenase (LDH)) were measured at the Southwest Foundation for Biomedical Research (San Antonio, TX). D-dimer concentrations were determined by the Scott & White Reference Laboratory (Temple, TX). Serum cytokine concentrations were determined using enzyme-linked immunosorbent assays (ELISA) for rhesus monkey IL-6, IL-12 p70 and TNF-α according to the manufacturer's instructions (Biosource, Camarillo, CA).

Histological and Biochemical Evaluation of Transgene Expression. Frozen sections (6 μm) were fixed in 0.5% glutaraldehyde in phosphate buffered saline (PBS) and washed twice in PBS containing 1 mM magnesium chloride. Slides were then incubated overnight in a solution containing X-gal and counterstained the following day with eosin (Fisher Diagnostics, Middletown, VA). Photomicrographs were captured using a Nikon Coolpix 4500 digital camera attached to a MicrosOptics IV900 microscope and Nikon View software. In order to quantitate the amount of beta-galactosidase in each tissue, separate samples were placed in 1 mL of lysis buffer (provided with the β-gal ELISA kit, Roche Applied Science, Indianapolis, IN) containing the Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL) and frozen at −80 °C until they were homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). Protein concentration of cleared supernatants obtained after centrifugation at 14,000 rpm for 10 min was determined by a microplate assay with Bio-Rad DC Protein assay reagents and bovine serum albumin as a standard. Beta-galactosidase concentrations were determined with the β-gal ELISA kit[43] according to the manufacturer's instructions.

Real-Time PCR. DNA was isolated from baboon blood, stool and tissue samples using a DNeasy Blood and Tissue extraction kit (Qiagen, Valencia, CA). Urine was concen-

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trated using a Centricon Plus-20 Centrifugal Filter Device (Millipore, Billerica, MA). DNA was then extracted from the concentrate using a QIAamp Viral RNA mini kit (Qiagen) and subsequently treated with DNase-free-RNase (Sigma Aldrich) according to the manufacturer's instructions. DNA was isolated from stool samples using a QIAamp DNA Stool Mini kit (Qiagen). Amplification of adenoviral ITR sequences in these samples was carried out using the Power SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) with the following primers: 5′-AGC CAA TAT GAT AAT GAG GGG GTG-3′ (forward) and 5′-TAC GCG CTA TGA GTA ACA AA-3′ (reverse).[44] Real-time reactions were run on the ABI Prism 7900HT sequence detector (Applied Biosystems) under the following conditions: 50 °C for 2 min, 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The viral genome copy number in unknown samples was calculated from standard curves generated from plasmid DNA containing the backbone for the helper-dependent adenovirus representing 1×10^2 to 1×10^7 viral genomes.

Microsome Isolation. Hepatic microsomes were prepared from 300−500 mg of tissue taken from each lobe at the time of necropsy according to established protocols.[45] Samples were stored at −80 °C prior to analysis.

Testosterone Hydroxylation Assay. In order to assess hepatic CYP3A activity, formation of the metabolite of testosterone, 6β-hydroxytestosterone, was characterized in microsomes isolated from each primate. In brief, 200 μg of liver microsomal protein was incubated with testosterone and glucose-6-phosphate dehydrogenase for 15 min at 37 °C with gentle agitation. The reaction was quenched with dichloromethane and 11α-hydroxyprogesterone added as an internal standard. The organic phase was then evaporated and samples redissolved in methanol. Testosterone and its metabolites were separated and quantified by high-performance liquid chromatography (HPLC) as described.[46] Peak areas of the hydroxylated metabolite were measured and compared to peak areas of an internal standard within the same run.

Western Blot Analysis. Twenty micrograms of hepatic microsomal proteins and CYP3A4 standard (human liver microsomes, Xenotech, Lenexa, KS) were separated on an 8% gel via sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS−PAGE).[47] Detection of CYP3A protein was achieved using a rabbit polyclonal antipeptide against human CYP3A4 antibody (1:4000, A4100, Xenotech) and a corresponding horseradish peroxidase conjugated secondary antibody (Cell Signaling Technology, Denver, MA). Immune complexes for CYP3A were detected by chemiluminescence (Western Lightning Detection Kit, PerkinElmer, Boston, MA). Protein band densities were analyzed using Kodak 1D image analysis software (Eastman Kodak, Rochester, NY).

Statistical Analysis. Statistical analysis of data was performed using Sigma-Stat (Systat Software Inc., San Jose, CA). Differences were evaluated by a one-way analysis of variances followed by a Bonferroni/Dunn post hoc test. Differences was determined to be significant when the probability of chance explaining the results was reduced to less than 5% ($P < 0.05$).

Results

Male baboons (*Papio* sp.) selected for this the study did not have circulating neutralizing antibodies to human adenovirus serotype 5. Specific details about each primate used in these studies are summarized in Table 1. Animals were given either a low (5×10^{11} vp/kg) or moderate dose (3×10^{12} vp/kg) of PEGylated HDAd by intravenous infusion. Animals receiving the corresponding dose of unmodified virus from the same production lot served as controls. Baboons were routinely monitored for abnormal signs and symptoms potentially related to vector administration such as significant changes in appetite, weight, stool consistency, heart rate, temperature and conjunctivitis throughout the study period. The low dose of each virus was well tolerated. The animal given 3×10^{12} vp/kg of unmodified HDAd was lethargic and inactive with moderate to labored breathing for the first 3 h while the baboon given the same dose of the PEGylated preparation continued to be active and alert throughout the entire study period.

Pharmacokinetics and Biodistribution of Viruses in the Baboon. One of the primary motives for PEGylating medicinal compounds is to strategically alter their pharmacokinetic profile and, in turn, improve their therapeutic potential.[6] Thus, in order to determine persistence of virus in the circulation, the amount of infectious virus present in the blood was quantified by an *in vitro* infectious titer assay. The maximum concentration of active circulating virus was found within 1 h after injection of 3×10^{12} vp/kg of each preparation (Figure 1). At this time, the concentration of PEGylated HDAd (1.65×10^7 BFU/mL) was approximately 1.6 times higher than the unmodified virus (1.01×10^7 BFU/mL). The systemic clearance rate of the PEGylated virus was also approximately half that of the unmodified virus while the area under the plasma concentration time curve (AUC) doubled (Table 2). Despite this, the terminal half-life ($t_{1/2}$) for each of the viruses was similar.

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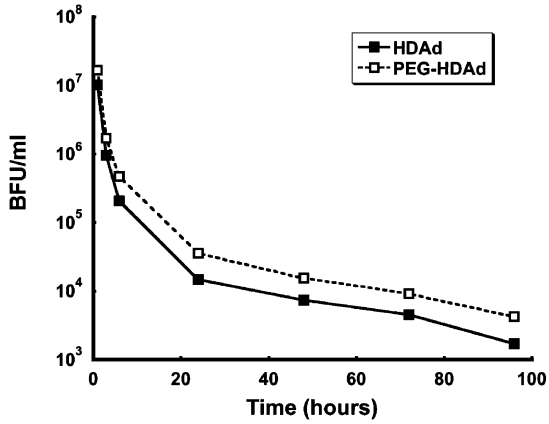

Figure 1. PEGylation improves the amount of active virus in the circulation and reduces clearance from the systemic circulation. Observed blood concentration vs time curve after intravenous administration of 3×10^{12} vp/kg of either unmodified (HDAd) or PEGylated (PEG-HDAd) virus. Whole blood was serially diluted in standard culture medium containing 2% fetal bovine serum. Aliquots were placed on confluent layers of HeLa cells for 24 h and assessed for beta-galactosidase expression by histochemical staining. The number of infectious virus particles was calculated as described previously.[42] Infectious virus could not be detected in urine and feces collected from the same primates and assayed in the same manner. Active virus particles could not be detected in any of the samples obtained from primates treated with 5×10^{11} vp/kg of either virus.

Table 2. Pharmacokinetic Parameters of Unmodified and PEGylated HDAd (3×10^{12} vp/kg) After Intravenous Administration in Non-Human Primates

parameter[a]	HDAd	PEG-HDAd
AUC (BFU/h)	3.68×10^7	6.13×10^7
CL (L/h/kg)	8.13×10^5	4.89×10^5
V_{ss} (L/kg)	1.51×10^5	1.13×10^5
$t_{1/2}$ (h)	23.89	24.07

[a] AUC: area under plasma concentration−time curve extrapolated to infinity. CL: systemic clearance. V_{ss}: volume of distribution at steady state. $t_{1/2}$: terminal elimination half-life.

Transduction Efficiency of PEGylated Virus. Preclinical studies have shown that covalent attachment of monomethoxy poly(ethylene) glycol derivatives to certain compounds can interfere with attachment and interaction with specific cellular receptors.[5] In order to determine if attenuation of virus-induced toxicity associated with systemic administration of PEGylated HDAd previously observed in rodents is due to changes in the affinity of the vector for the liver and the spleen, the primary sites for release of pro-inflammatory cytokines during the innate immune response,[26,48] beta-galactosidase expression was assessed in these and other tissues 96 h after treatment. A definite dose response with respect to transgene expression was observed with both viruses. Hepatic transgene expression in the animal given a dose of 3×10^{12} vp/kg of HDAd was approximately 400 times more than that of the baboon given 5×10^{11} vp/kg of the same virus (Table 3). Although hepatic transgene expression of the PEGylated virus was generally lower than that of the unmodified virus in the primate, transgene expression in the animal given the moderate dose of PEG-HDAd was approximately 84 times higher than that of the animal given a lower dose of the same virus. Histochemical staining patterns of hepatic tissue sections followed a similar trend (Figure 2A−D).

Table 3. Quantitative Assessment of Transgene Expression 96 h After Administration of Two Different Doses of Unmodified (HDAd) and PEGylated Virus (PEG-HDAd)[a]

	beta-galactosidase (pg/mg protein)			
	5×10^{11} vp/kg		3×10^{12} vp/kg	
tissue	HDAd	PEG-HDAd	HDAd	PEG-HDAd
liver	1.73	0.48	687.34	40.33
spleen	14.21	1.32	589.27	18.29
lung	nd[b]	nd	2.47	2.56
heart	nd	nd	nd	0.84
kidney	nd	nd	nd	nd

[a] Data were obtained from a beta-galactosidase ELISA of tissue homogenates. [b] None detected. Sample fell below the detection limit of the assay (6 pg/mL).

PEGylation also reduced transgene expression in the spleen by a factor of 11 and 32 in animals given the low and moderate doses respectively. Beta-galactosidase levels in the spleen were also much higher than that found in the liver for both viruses when a low dose was used (8-fold higher HDAd, 3-fold higher PEG-HDAd, Table 3). Samples obtained from animals given the moderate dose did not follow this trend as similar amounts of transgene were found in the liver and spleen of the animal given HDAd while the spleen of the animal given the PEGylated virus contained about half the beta-galactosidase found in the liver. Beta-galactosidase was not detected in samples obtained from the lung, heart and kidney of animals given the low dose of either virus. Similar levels of transgene expression (~2.5 pg/mg protein) were found in the lungs of animals given the moderate dose of either virus. Low but detectable levels of beta-galactosidase (0.84 pg/mg protein) were also found in samples from the heart of the baboon given the PEGylated virus at this dose while that from the animal given HDAd did not contain the transgene. Beta-galactosidase expression was not found in the kidneys of both animals given the moderate dose of either virus (Table 3).

Effect of PEGylation on the Distribution of Viral DNA. Tissues were screened for viral DNA by real-time PCR. Samples obtained from the liver and the spleen of the animal given 3×10^{12} vp/kg of unmodified virus contained the

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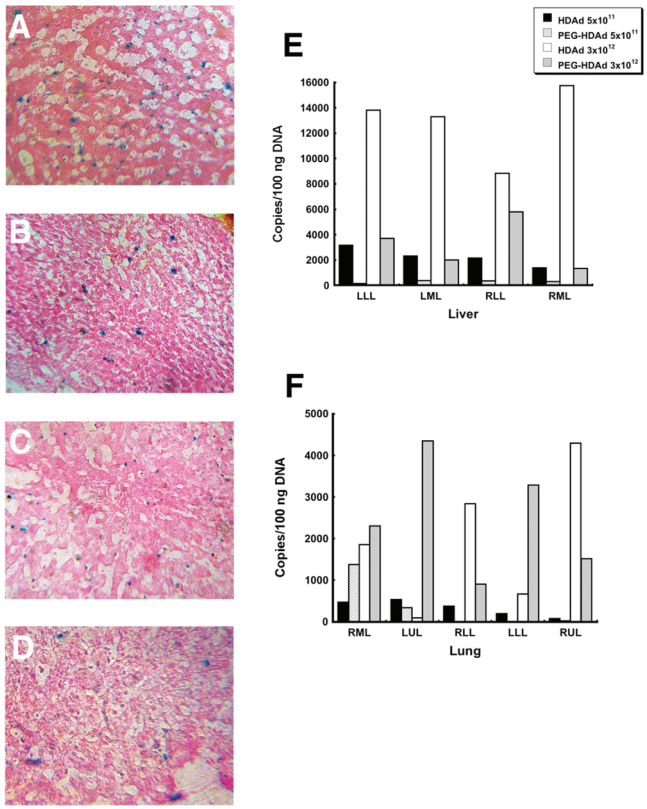

Figure 2. PEGylation reduces transduction efficiency in the liver. Representative sections of hepatic tissue harvested 96 h after systemic administration of unmodified (panels A and C) or PEGylated (panels B and D) HDAd expressing the beta-galactosidase transgene at a dose of either 3×10^{12} vp/kg (panels A and B) or 5×10^{11} (panels C and D). Magnification:100×. Biodistribution patterns in different lobes of the liver (E) and lung (F) of non-human primates 96 h after a single dose of unmodified and PEGylated HDAd. Animals were given vectors by intravenous infusion. Four days later, animals were sacrificed and DNA was extracted from samples obtained from specific lobes of each tissue. Virus genomes were measured by quantitative real-time PCR. LLL: left lateral lobe. LML: left middle lobe. LUL: left upper lobe. RLL: right lateral lobe. RML: right middle lobe. RUL: right upper lobe. Data represent values obtained from one primate per experimental condition.

highest amount of viral DNA (Figure 2E, Table 4). The number of virus genomes in samples from the same organs of the baboon given the same dose of PEGylated virus was reduced 4-fold. Samples obtained from the kidneys from these animals followed a similar trend (144 genomes/100 ng of DNA, HDAd vs 27 genomes/100 ng of DNA, PEG-HDAd). In contrast, samples from the heart of the baboon treated with the PEGylated virus contained approximately 8 times more viral DNA than those from the animal given unmodified virus. Samples obtained from the liver, spleen and kidneys of animals given 5×10^{11} vp/kg of the unmodified virus contained 8 times more viral DNA than those given the PEGylated virus (Table 4). Viral DNA was not detected in the heart of either animal at this dose.

Table 4. Biodistribution of Viral DNA in Select Tissues of Non-Human Primates 96 h after a Single Dose of Unmodified or PEGylated HDAd[a]

	viral genomes/100 ng of DNA			
	5×10^{11} vp/kg		3×10^{12} vp/kg	
organ	HDAd	PEG-HDAd	HDAd	PEG-HDAd
spleen	2626.42	310.19	7062.45	1719.91
heart	nd[b]	nd	17.29	140.60
kidney	52.27	nd	144.32	26.92

[a] Data were obtained by real-time Taq Man PCR on genomic DNA isolated from baboon tissues. [b] None detected. Sample fell below the detection limit of the assay (10 viral genomes/100 ng of DNA).

Careful analysis of specific regions of the liver and lungs of animals given 3×10^{12} vp/kg of virus revealed that the unmodified virus was preferentially distributed to the right upper lobe of the lung while the PEGylated vector was retained primarily in the left upper lobe (Figure 2F). In addition, DNA from the unmodified virus was present in slightly larger amounts in the right middle lobe of the liver while that from the PEGylated vector was found primarily in the right lateral lobe (Figure 2E). In contrast, the lower dose of both vectors was distributed evenly throughout the liver. This was also the case in the lung for the unmodified virus, however, DNA from the PEGylated vector was only found in the right middle and left upper lobes (Figure 2F).

Toxicology of PEGylated HDAd in the Non-Human Primate. *Pro-Inflammatory Cytokines.* A sharp rise in systemic cytokines is one of the hallmarks of the acute innate immune response associated with systemic administration of recombinant adenoviruses.[26,48] To evaluate the effect of PEGylation on this response in the baboon, serum interleukin-6 (IL-6), IL-12, and tumor necrosis factor-α (TNF-α) were measured 6 h after treatment since notable levels of cytokines were not detected in samples collected at any other time points. Although IL-6 (62 U/mL) was found in serum from the baboon given 5×10^{11} vp/kg of the unmodified virus (Figure 3A), it could not be detected in the animal given the same dose of the PEGylated virus. Serum from the animal given the 3×10^{12} vp/kg dose of PEGylated HDAd contained 49 U/mL IL-6, approximately 70% lower than that found in the animal given the same dose of the unmodified virus (160 U/mL). A trace amount of TNF-α (8 pg/mL) was noted after treatment with HDAd at a dose of 5×10^{11} vp/kg (Figure 3B). This increased by a factor of 10 in the serum of the animal given the higher dose of the same virus. TNF-α was not detected in the serum of animals given the PEGylated virus at either dose. IL-12 as found in the serum of each animal (Figure 3C) and increased in a dose dependent manner. PEGylation reduced IL-12 secretion by a factor of 3 and 2 with the low and moderate doses, respectively.

Platelets and Coagulation Characteristics. Virus-induced thrombocytopenia and aberrant activation of the coagulation cascade contribute to the toxicity of recombinant adenovi-

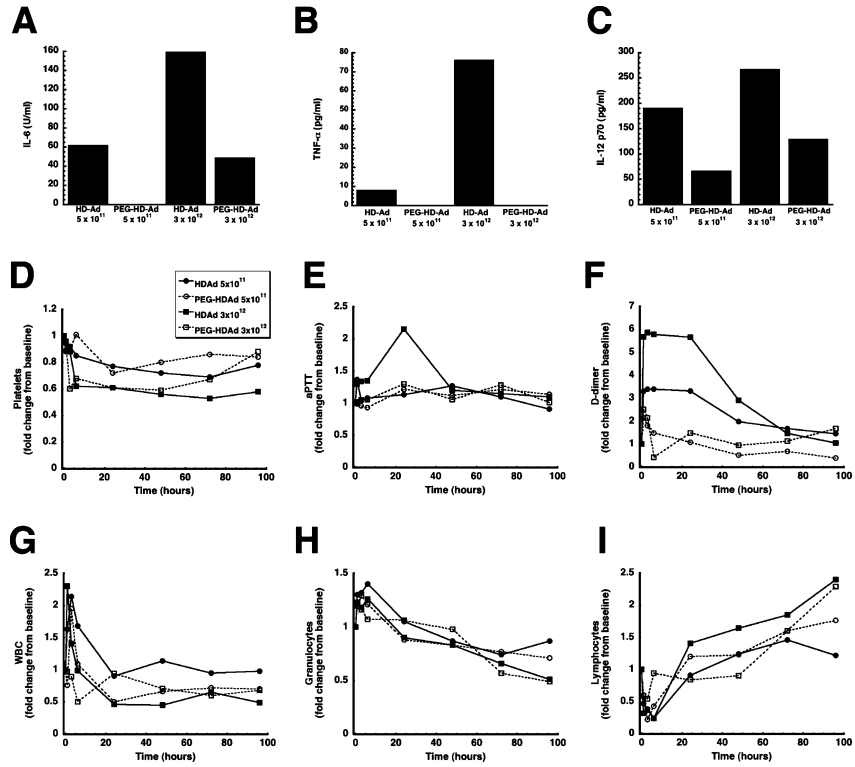

Figure 3. PEGylation reduces cytokine production after vector administration. IL-6 (A), TNF-α (B) and IL-12 p70 (C) were measured in serum samples obtained from animals 6 h after administration of 5×10^{11} or 3×10^{12} vp/kg of either unmodified or PEGylated helper-dependent adenovirus in the non-human primate. Clinical parameters such as platelet counts (D), activated partial thromboplastin time (aPTT) (E), D-dimer (F), white blood cell (WBC) counts (F), granulocytes (G) and lymphocytes (H) were measured over time in baboons given either modified or PEGylated helper-dependent adenovirus expressing beta-galactosidase. Data represent values obtained from one primate per experimental condition.

ruses when administered systemically at high doses.[49] Platelet counts fell to approximately 30% of what was measured prior to initiation of the study in animals given 5×10^{11} vp/kg of either virus within 24 h after treatment (Figure 3D). Platelets fell to 40% of baseline levels in animals given 3×10^{12} vp/kg of either virus. This effect was seen as early as 6 h in the animal given the unmodified virus and did not resolve throughout the study. The baboon given PEGylated HDAd experienced a similar reduction in platelet count 6 h after treatment, but values returned to baseline within 72 h.

Increases in the activated partial thrombin time (aPTT) were also noted in animals given unmodified HDAd. This value peaked at approximately 1.4 times baseline in the animal given the 5×10^{11} vp/kg dose at the 1 h time point (Figure 3E). Similar results were obtained from the animal given 3×10^{12} vp/kg of HDAd, however, levels continued to increase to 2.2 times that of baseline at 24 h. aPTT did not return to baseline in this animal until the 96 h time point. In contrast, changes in aPTT values were not detected in any samples obtained from animals given the PEGylated virus at the early time points although a slight increase above the normal upper limit was noted 24 and 72 h after treatment with 5×10^{11} vp/kg and 3×10^{12} vp/kg of the virus respectively. Although changes in prothrombin time[50] followed a similar trend, an increase above the normal upper limit was found only in the animal given 3×10^{12} vp/kg of HDAd at 1 and 6 h (data not shown).

D-dimer, derived from the degradation of cross-linked fibrin polymers, is a specific marker for increased procoagulatory activity and fibrinolysis.[51] Elevated D-dimer levels, often observed in patients with severe sepsis,[52] were noted in all animals in this study. D-dimer was 3.3 and 2.2 times baseline values 1 h after treatment in animals receiving 5×10^{11} vp/kg of unmodified and PEGylated virus,

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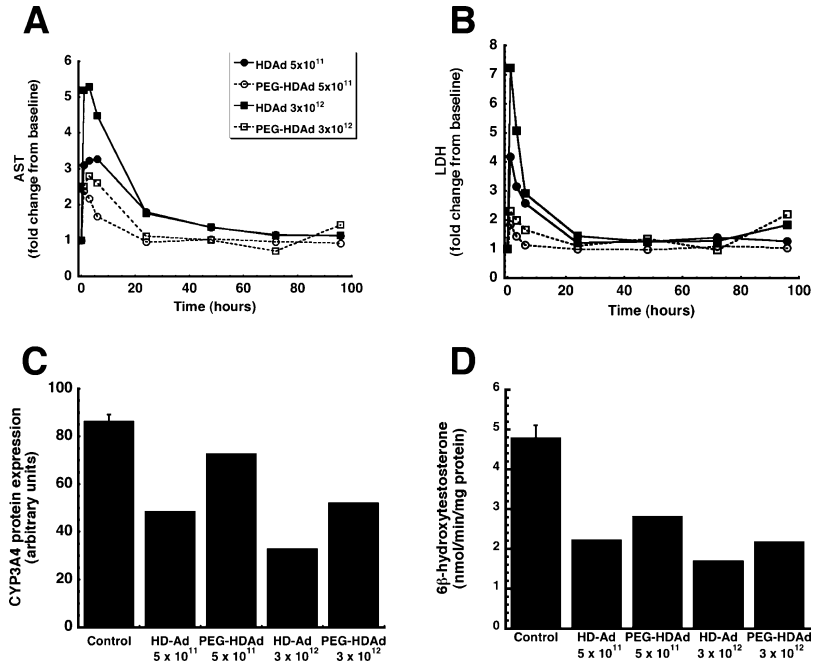

Figure 4. PEGylation alters the expression and function of hepatic cytochrome P450 3A (CYP3A) in the baboon. Full blood chemistry panels were run on baboons given either unmodified or PEGylated helper-dependent adenovirus expressing beta-galactosidase at a dose of 5×10^{11} or 3×10^{12} vp/kg. Changes in serum aspartate aminotransaminase (AST) (A) and serum lactate dehydrogenase (LDH) (B) profiles were noted. All other parameters measured (see Experimental Section under Biochemical and Hematological Analysis of Blood) fell within normal limits for each primate (data not shown). (C) Immunoblot analysis of hepatic CYP3A in male baboons 96 h after a single systemic dose of 5×10^{11} or 3×10^{12} vp/kg of either unmodified or PEGylated helper-dependent adenovirus expressing beta-galactosidase. Protein levels are reported as arbitrary units of relative density with respect to a known protein standard. (D) *In vitro* catalytic activity of CYP3A microsomal proteins 96 h after vector administration as measured by the production of the isoform-specific metabolite, 6β-hydroxytestosterone. Data represent values obtained from one primate per experimental condition.

respectively (Figure 3F). D-dimer declined thereafter in the baboon given PEGylated HDAd. In the animal treated with unmodified virus, D-dimer remained high at 3 times above baseline until the 24 h time point, after which it began to decline. A rapid increase in D-dimer to six times that of baseline was observed within 1 h after treatment with 3×10^{12} vp/kg unmodified HDAd. Values remained at that level for the first 24 h, when they then began to decline. Conversely, D-dimer doubled during the first 3 h following administration of PEGylated virus at the same dose and then returned to baseline. Hemoglobin, hematocrit and red blood cell counts were within the normal range in all animals throughout the course of the study (data not shown).

Other Hematological Parameters: White Blood Cell Count and Differentials. The number of circulating white blood cells (WBCs) peaked to approximately twice that of baseline 1 h after treatment with either dose of unmodified virus (Figure 3G). WBCs in the animal given the low dose of virus returned to baseline levels within 24 h, while that of the animal treated with 3×10^{12} vp/kg of HDAd returned to baseline within 6 h. WBCs in this animal then fell to less than half of baseline throughout the remainder of the study. WBCs generally fell slightly below baseline in animals given the PEGylated virus throughout the study. A small increase in granulocytes to no more than 1.4 times baseline was observed in response to vector administration in all animals within the first 6 h after which it began to decline (Figure 3H). Granulocytes in the animal given unmodified virus dropped to 13% of control while those in the animal given PEGylated virus dropped by 30%. In contrast, granulocytes were approximately 50% of baseline in animals given 3×10^{12} vp/kg of either virus. Lymphocytes initially dropped in each animal soon after treatment then rose to ~1.2 and 1.8 times that of baseline in animals given 5×10^{11} vp/kg unmodified and PEGylated virus respectively (Figure 3I). A similar trend was noted in animals given 3×10^{12} vp/kg of either virus.

Serum Chemistry. Full blood chemistry panels were run on each of the baboons included in this study. Sharp changes in serum aspartate aminotransaminase (AST) levels were seen as early as 1 h after treatment with each virus at each of the doses tested (Figure 4A). A dose response was seen with AST levels at 3.1 and 5.2 times that of baseline in animals receiving 5×10^{11} vp/kg and 3×10^{12} vp/kg of HDAd at this time point. A similar trend was observed in animals given the PEGylated virus, however, serum AST was much lower than that seen in animals given similar doses of HDAd. AST rose to 2.4 and 2.5 times that of baseline 1 h after treatment

with 5×10^{11} vp/kg and 3×10^{12} vp/kg of PEG-HDAd. Significant differences were also noted in the time at which serum AST peaked in animals given 5×10^{11} vp/kg of virus (6 h HDAd, 3.3 times baseline vs 1 h PEG-HDAd) while levels in primates given 3×10^{12} vp/kg of either virus peaked 3 h after treatment (5.3 and 2.8 times baseline, HD-Ad and PEG-HDAd, respectively).

Serum alanine aminotransaminase, bilirubin, and albumin for each primate fell within normal limits throughout the entire study period (data not shown). Serum lactate dehydrogenase (LDH) followed a similar trend (Figure 4B). Although this parameter plateaued in all animals within 1 h, differences were noted in the degree of LDH release. Serum LDH rose to 4.1 and 1.8 times baseline after treatment with 5×10^{11} vp/kg of unmodified and PEGylated virus, respectively. LDH returned to baseline 18 h earlier in the animal treated with PEGylated HD-Ad with respect to the animal given unmodified virus. An increase of LDH to 7.4 times that of baseline was observed in animals given 3×10^{12} vp/kg of unmodified virus while the animal given the same dose of PEGylated virus experienced a 2-fold increase at the same time point.

Effect of Systemic Administration of PEGylated and Unmodified HD-Ad on Hepatic Drug Metabolism. A single dose of a recombinant adenovirus can significantly suppresses hepatic cytochrome P450 (CYP) 3A2 in the rat for 14 days.[47,53,54] In order to determine if this occurs in the primate, changes in CYP3A were evaluated. Unmodified and PEGylated virus at a dose of 5×10^{11} vp/kg reduced CYP3A protein levels by 44% and 15%, respectively (Figure 4C). Increasing the dose further suppressed protein levels by 60% and 40%. Both viruses suppressed hepatic CYP3A activity as determined by an *in vitro* assay measuring the production of 6β-hydroxytesterone, the primary isoform-specific metabolite of testosterone, 96 h after treatment. PEGylated virus at a dose of 5×10^{11} vp/kg reduced catalytic activity by 40% with respect to an untreated control while 3×10^{12} vp/kg reduced activity by 55% (Figure 4D). At each of the doses tested, CYP3A activity in animals given the unmodified virus was approximately 10% lower than that observed in baboons given PEGylated virus.

Toxicology, Biodistribution and Kinetics of Unmodified and PEGylated HDAd in Mice. Some of the findings reported here, most notably the fact that PEGylation reduces the ability of HDAd to effectively transduce the liver, were quite different from that previously reported in the mouse. Thus, in order to determine if these differences were due to the particular PEGylated virus used in this study, mice were given 3×10^{12} vp/kg of either unmodified or PEGylated HDAd left over from the same production lots used in primates by tail-vein injection. As noted previously, PEGylation reduced production of IL-6 and IL-12 in these animals by a factor of 6 and 2 with respect to mice given the unmodified virus ($p < 0.05$) while significant elevations were noted in mice given unmodified virus (Figures 5A and 5B, $p < 0.01$). In a similar manner, ALT and AST levels of mice given the unmodified virus were significantly elevated (9 and 4 times baseline respectively, $p < 0.05$) while those in mice given the PEGylated virus were not statistically different from mice given saline (Figures 5C and 5D).

Four days after vector administration, mice were necropsied and biodistribution and transgene expression assessed. We found no difference in the number of viral genomes distributed to liver, lung, kidney and heart of animals receiving either virus (data not shown). A similar result was seen with respect to the transgene expression. Hepatic tissues of animals treated with unmodified and PEGylated virus contained 2.2×10^4 and 2.0×10^4 pg of beta-galactosidase/mg of protein, respectively. Histochemical staining of hepatic tissue sections showed a similar trend (Figures 5E and 5F). In contrast, transgene expression in the spleen of animals given PEG-HDAd (258 pg of beta-galactosidase/mg of protein) was approximately half that found in the spleen of mice given HDAd (485 pg/mg of protein, $p < 0.05$). The number of virus genomes found in the spleen followed a similar trend with 1.4×10^4 and 8.9×10^3 vg/100 ng of genomic DNA found in mice given HDAd and PEGylated HDAd, respectively.

To further evaluate the kinetic profile of PEGylated HDAd, mice were injected via the tail vein with 3×10^{12} vp/kg of either virus. The amounts of infectious virus and viral DNA present in the blood were analyzed soon after administration (5, 10, 20, 30, 40, and 60 min) by an *in vitro* infectious titer assay and real-time PCR. The number of virus genomes of either virus found in the circulation was approximately one log higher than the amount of infectious virus obtained from the same sample at each time point (Figures 5G and 5F). Despite this, the rate at which each declined was similar (5.6 min distribution half-life infectious HDAd vs 5.5 min distribution half-life HDAd genomes). In both cases, the distribution half-life of PEGylated HDAd was twice as long as that of the unmodified virus (11.2 min infectious, 11.0 min genomic DNA).

Discussion

Development and characterization of helper-dependent adenoviruses have revealed that the innate immune response against vector capsid proteins is, at this moment, the last and most significant hurdle to be overcome for clinical application of these vectors for gene transfer.[25,26] Significant strides have been made in reducing this effect in rodents through PEGylation, a technology with over three decades of research supporting its compatibility and use in medicinal products.[5,7,10,22] Human infectious diseases with diverse etiologies such as viruses, bacteria, fungi, parasites, and prions have been modeled in non-human primates over the

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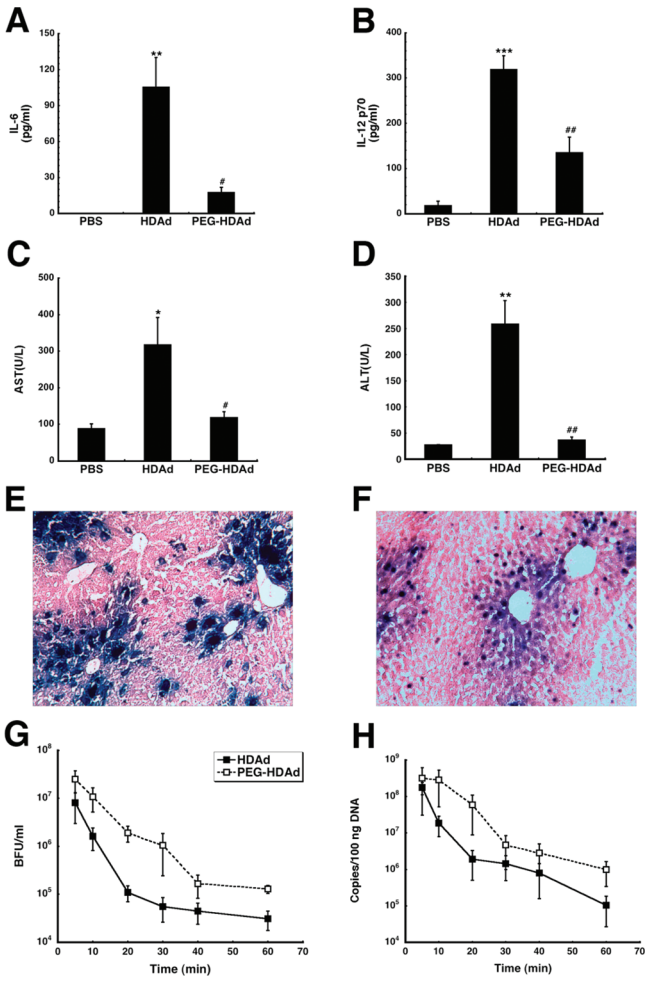

Figure 5. Toxicology, transduction efficiency and kinetics of unmodified and PEGylated HDAd in mice. Serum IL-6 (A), IL-12 p70 (B), aspartate aminotransferase (AST) (C) and alanine aminotransferase (D) levels were assessed 6 h after administration of 3×10^{12} vp/kg of either unmodified or PEGylated helper-dependent adenovirus. Results are reported as the mean ± SEM ($n = 4-5$ animals per treatment group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ with respect to vehicle control (phosphate-buffered saline, PBS). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ with respect to unmodified virus. Representative sections of hepatic tissue harvested 96 h after systemic administration of unmodified (E) or PEGylated (F) HDAd expressing the beta-galactosidase transgene at a dose of 3×10^{12} vp/kg. Magnification:100×. Observed blood concentration vs time curve after intravenous administration of 3×10^{12} vp/kg of either unmodified or PEGylated helper-dependent adenovirus. Numbers of infectious virus (G) were determined by an *in vitro* infectious titer assay. Viral genomes (H) were obtained by real-time PCR of genomic DNA isolated from whole blood.

past century.[55] Baboons are an excellent model for infectious disease and genetic studies because they exhibit the same physiological characteristics that are critical to common diseases in humans,[56] they are amenable to strict control of breeding and environmental factors and detailed genetic linkage maps are available that illustrate direct relationships to human disease.[55,57] In this report, we describe for the first time the pharmacological, toxicological and pharmacokinetic properties of a recombinant, PEGylated helper-dependent adenovirus in this animal model.

The most surprising aspect of this study was that few of our findings correlated to what has been previously observed in rodents with similar modified viruses. As expected, pro-inflammatory cytokines were elevated 6 h after systemic administration of unmodified virus in a dose-dependent manner (Figures 3A−3C). As previously described in the mouse[15,16] and observed in the pilot study outlined in this manuscript (Figures 5A and 5B), PEGylation reduced cytokine levels at this time point, further suggesting that conjugation of monomethoxypoly(ethylene) glycol to adenovirus capsid proteins reduces the innate immune response against the virus in both the primate and the mouse. Aspartate aminotransaminase (AST) and lactate dehydrogenase (LDH) were elevated shortly after systemic administration of the unmodified virus in a manner similar to that described previously in the non-human primate (Figures 4A and 4B).[28,30,58] Covalent conjugation of PEG to the virus capsid reduced circulating concentrations of both enzymes, indicating that PEGylation can also alleviate virus-induced toxicity in primates. We also found that both the modified and PEGylated vectors altered the activity of hepatic CYP3A, which, like the human isoform, plays a key role in drug metabolism and elimination,[34,35,59] in a dose-dependent manner similar to that previously reported in the rat[47,53,54] (Figures 4C and 4D). Although the mechanism by which adenoviral infection alters CYP remains to be elucidated, this finding is important for several reasons. First, it suggests that adenovirus infection in humans may significantly alter the expression and function of hepatic CYP which is important in the context that recombinant adenoviruses are currently used in the clinic for gene therapy and vaccine protocols and that this ubiquitous virus is capable of causing

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significant illness in specialized patient populations.[60−62] Pioneering studies with bacterial systems suggest that cytokines and reactive oxygen species generated during the early stage of infection interfere with transcription factors needed for expression of many CYP isoforms.[63,64] While we do not discount that these components play a role in controlling drug metabolism, similar findings with helper-dependent viruses in the rat long after these things have been cleared suggest that other aspects of the infection may activate a series of cell signaling cascades in the hepatocyte that could have long-term effects on cytochrome P450 expression.[54] Finding correlations between these observations and those in non-human primates is currently underway.

One of the most touted benefits of PEGylation is that it can improve the pharmacokinetic profile of delicate biologicals by protecting them from enzymatic and immunological attack in the general circulation.[6] PEGylation has been shown to increase the half-life, reduce the clearance and increase the volume of distribution of recombinant adenoviral and lentiviral vectors in mice.[19−21] PEGylation did reduce the clearance of virus by 40% in the primate. This most likely facilitated the observed increase in transduction efficiency of this vector in the heart, an organ saturated with adenovirus receptors in which transgene expression is often limited after systemic administration of virus due to its rapid clearance from the circulation.[65,66] The fact that this modification did not seem to affect the circulation half-life of the virus in non-human primates was concerning. This may be due to interspecies differences in blood volume distributions and hydrolases, often responsible for degradation of PEGylated products in the circulation, as well as the particular assay chosen for the study.[7] A pilot study in the mouse using the same vector employed in primates revealed that similar pharmacokinetic parameters can be elicited from data generated by infectious titer and PCR assays for both viruses (Figures 5G and 5H). This data also suggested that, in the primate study, a significant amount of virus was cleared from the blood before the 1 h time point, limiting our ability to detect dramatic differences in the overall kinetics of each vector. It is also important to realize that PEGylation does not always substantially improve the pharmacokinetic parameters of biologicals.[6,7] For example, Graham found that PEGylation of L-asparaginase did not always improve the circulating half-life of the enzyme in humans while improvements from 2.9 to 56 h were found in mice.[67,68] Although very little work has been done with PEGylated compounds in non-human primates, compilations of data suggest that degree by which the pharmacokinetic profile of a PEGylated compound improves is related to the animal model used for testing. For example, increases in half-life are generally reported as 100−300-fold increments in small animals such as mice and rats, while improvements in primates and humans are often on the order of 2−3-fold.[6]

In an effort to identify the mechanism by which PEGylation attenuates the innate immune response, tissue biodistribution and transduction efficiency of both PEGylated and unmodified virus was evaluated in the non-human primate. The PEGylated virus had a reduced capacity for entering hepatocytes with respect to the unmodified virus. This was quite different from that previously reported in rodents in which PEGylation did not compromise hepatic gene transfer[14−16,69] and was confirmed in our pilot study in mice using the same virus preparation tested in baboons (Figures 5E and 5F). Although the mechanism underlying the differences in hepatic transduction efficiency of PEGylated virus between these species is not clear, the smaller size (90 nm (baboon) vs 100 nm (mouse)) and lower density (1.4/μm^2 (baboon) vs 14/μm^2 (mouse)) of fenestrae in the liver of non-human primates could pose as significant anatomical barriers for PEGylated viruses which generally have larger diameters than unmodified viruses.[70] Studies in non-human primates and rodents have shown that recombinant adenoviruses enter hepatocytes through heparan sulfate proteoglycans (HSPGs).[71−73] Additional studies in our laboratories and those of others *in vitro* and *in vivo* suggest that PEGylated virus relies on HSPGs for entry in cellular targets.[70] Interaction of the hexon protein with coagulation factors (VII, IX, X and protein C) facilitates subsequent binding to HSPGs and low-density

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lipoprotein receptor-related proteins and is crucial for efficient transduction of hepatocytes *in vivo*.[65,74,75] Hofherr et al. have recently reported that PEGylation prevents direct interaction with blood components, which can limit hepatic transduction efficiency.[69,76] Morral et al. have also suggested that the lower relative density of adenovirus receptors in the liver of non-human primates with respect to their rodent counterparts may redirect the virus to the endothelium, also responsible for severe adenovirus-induced toxicity.[30] Taken together, we believe that a reduction in binding properties to coagulation factors in combination with interspecies differences in receptor density and tissue architecture in the liver contributes to the reduced hepatic transduction observed in the non-human primate with PEGylated HDAd.

Despite the fact that hepatic transgene expression was significantly lower in primates given the PEGylated vectors with respect to the unmodified virus, a distinct nonlinear dose response, commonly reported in both mice[77] and non-human primates,[28,30,58,78] where a slight increase in dose incites a disproportionately higher level of transgene expression, was observed. Studies in mice have shown that depletion of reticuloendothelial cells, namely Kupffer cells, before vector administration improves hepatocyte transduction and, as a result, a nearly linear dose response is observed.[77] The doses selected for each vector in our studies, one below and one within the documented threshold for hepatic transduction, were chosen to determine if PEGylation would prevent interaction with these cells and the subsequent dose-dependent acute toxicity associated with high doses of adenovirus.[30,78] It is also interesting to note that, unlike the liver, a defined nonlinear dose response with respect to transgene expression was not observed in the spleen of animals given the PEGylated virus, despite the fact that the number of genome copies did follow the nonlinear trend in a manner similar to that described for PEGylated vectors in the mouse[16] (Tables 3 and 4). Microarray analysis of primary macrophages, Kupffer and dendritic cells isolated from mice treated with either native or PEGylated virus suggest that covalent attachment of PEG to adenovirus capsids does not alter the ability of the virus to enter these targets, but modifies the manner by which the virus is processed and presented (manuscript in preparation). Taken together, these findings suggest that PEGylation does not necessarily prevent interaction and uptake of these vectors by Kupffer and antigen presenting cells but does change their ability to respond to the virus in the mouse and possibly the non-human primate.

Despite the fact that PEGylation did significantly reduce the amount of inflammatory cytokines and serum transaminases produced in response to the adenoviral vector, this modification did not abrogate the virus-induced thrombocytopenia documented to occur in several animal models and humans shortly after administration of doses similar to those used in this study.[29,41,79,80] Although PEGylated virus has been shown to reduce thrombocytopenia in the mouse through steric hindrance, preventing the virus from interacting with platelets, disrupting the clotting cascade and limiting endothelial cell-mediated platelet activation and clearance,[15] in the study outlined here, platelet levels fell within 6 h after administration of the highest dose of PEGylated virus in a manner similar to that of the unmodified virus (Figure 3D). One may argue that this effect may be due to interspecies differences in hydrolases which may have been more efficient at uncoating the virus in the primate than the mouse.[7] It is also important to note that, in each case, platelet levels from each animal given the PEGylated virus quickly returned to baseline during the course of the study while those of animals given the unmodified virus remained low. Given that an intravenous catheter was surgically implanted in each animal approximately 7 days prior to initiation of the study and that each animal was given heparin to maintain the port, we believe that the transient drop in platelets seen with the low

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dose PEGylated virus and the mild increase in D-dimer noted at each dose of this vector is very similar to what is seen during heparin-induced thrombocytopenia,[81] making it hard to determine if this transient drop was due to the virus preparation, the maintenance therapy or a combination of both.

This manuscript summarizes our initial attempt to characterize PEGylated helper-dependent adenovirus in the nonhuman primate. Virus, prepared in the same manner as previously done for mice,[15] but on a much larger scale, retained transduction efficiency *in vitro* and, in some aspects, performed in a similar manner. PEGylation reduced cytokine secretion, serum transaminases and other markers associated with cytotoxicity and tissue damage most likely due to significant changes in transduction efficiency in the liver and spleen also observed in this animal model. Although our results were obtained from a limited number of animals, results in the mouse with the same set of vectors suggest that there are notable species-specific differences in the biodistribution of and response to PEG-modified vectors. Additional studies with larger cohorts of animals to further characterize interspecies variations in response to these vectors and those with different PEG densities are warranted and are planned in our laboratories.

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