Materials and Methods

Animal Studies

All animal studies were approved by the University of Washington Office of Animal Welfare. Specific-pathogen-free male New Zealand white rabbits (3.0–3.5 kg; Western Oregon Rabbit Company, Philomath, OR) were fed either a high-fat diet including 0.25%, 0.5%, or 1% cholesterol (all with 3% soybean oil; Dyets Inc., Bethlehem, PA), normal rabbit chow (16% rabbit PLT; Albers Animal Feeds, Bellevue WA) or a mixture of high-fat and normal chow. After 1 week of adaptation to the animal facility, rabbits were fed 1% cholesterol for 2 weeks, followed by 0.5% cholesterol for 2 weeks. They then underwent neck surgery and bilateral common carotid infusions of Dulbecco's modified Eagle's medium (DMEM), as a means of atherosclerotic lesion initiation.[1] Postoperatively, rabbits were begun on sliding-scale diet (0%, 0.125%, or 0.25% cholesterol-containing chow), adjusted to maintain plasma cholesterol in the range of 200–800 mg/dL (Figure 1). Total plasma cholesterol was measured every two weeks with a colorimetric assay (Abbott Laboratories, Abbott Park, IL). Typically 24–25 weeks after the initial surgery, rabbits received bilateral intraluminal common carotid artery infusions of viral vector, as described below, and were placed on normal chow. 34 rabbits were enrolled: 5 rabbits were euthanized 3 days after vector infusion, 26 rabbits were euthanized 7 weeks after vector infusion, and 3 rabbits were euthanized at other times due either to surgical wound complications (2 rabbits) or severe anemia determined to be due to lymphoma (1 rabbit, not exposed to vector).

Adenoviral vectors

We used two adenoviral vectors: HDAdCMVNull (a “null” vector with an empty CMV-driven expression; referred to as HDAdNull)[2] and HDAdCMVgApoAI (contains a rabbit genomic apo A-I clone; referred to here as HDAdApoAI).[3] Both vectors are third-generation (also known as a “helper-dependent”) adenoviral (HDAd) vectors and accordingly lack all viral genes.[4] Vector stocks were propagated in human embryonic kidney 293 Cre4 cells (Microbix Biosystems, Toronto, Ontario, Canada)[5] and purified as described. Concentrations of vector stocks (measured by spectrophotometry) were 1–5 x 10^{12} viral particles (vp)/mL. Real-time polymerase chain reaction (PCR) revealed E1A-containing genomes were <1 in 10^6 total vector genomes (essentially undetectable) and helper virus contamination was <1% of total vector genomes.[2] All HDAdApoAI vectors were from a single preparation. HDAdNull vectors were from three independent preparations.

Surgical protocols, serum chemistries and blood counts

Rabbits were anesthetized with ketamine, xylazine, and isoflurane. After a midline neck incision, segments of common carotid arteries were temporarily isolated from the circulation with vascular clips (to arrest blood flow) and an arteriotomy was performed between the clips. The isolated carotid lumen was then rinsed with DMEM and filled either with DMEM or (24 weeks later) with HDAd (2×10^{11} vp/mL).[6] DMEM was incubated for 5 minutes; HDAd for 20 minutes. Each rabbit received HDAdNull on one side and HDAdApoAI on the other side, with the side for each vector randomized according a random number-generating program. After these incubations, the luminal contents were aspirated, the arteriotomy repaired, and blood flow re-established. This protocol yields gene transfer almost exclusively to luminal endothelium.[7-9] Blood was collected via the ear vein for plasma cholesterol measurements, plasma apo A-I measurements, complete blood count, and a metabolic panel (Phoenix Central Laboratory, test #1004).

Carotid arteries were harvested 3 days or 7 weeks after vector infusion, while rabbits were under general anesthesia. The neck was re-opened with a midline incision and silk ligatures were applied to isolate the transduced carotid segments. These segments

were excised, flushed gently with saline, and cut into smaller segments. Carotids harvested 3 days after vector infusion were cut into 5 equal segments designated A, B, C, D, and E from the cranial to the caudal end (Figure VIII in the online-only Data Supplement). Segments A, C, and E were snap-frozen in liquid nitrogen for later extraction of RNA. Segments B and D were placed into 1 mL DMEM, to be processed for protein and DNA analyses. Carotids harvested 7 weeks after vector infusion were cut into 8 equal segments designated 1–8 from the cranial to the caudal end (Figure VIII in the online-only Data Supplement). Segments 2 and 6 were snap-frozen in liquid nitrogen for later extraction of RNA. Segments 4 and 8 were placed into 1 mL DMEM to be processed for protein and DNA analyses. Segments 1, 3, 5, and 7 were embedded in OCT medium (1 block per artery) and frozen on dry ice.

Processing of vessel segments for protein and DNA analysis
The artery segments (in DMEM) were transferred to a well in a 48-well plate in a tissue culture incubator and incubated 3 hours (from time of harvest). The segments were then rinsed 3 times with brief agitation in 500 µL DMEM, then placed in 1 mL DMEM for 3 hours. After another 3 rinses with brief agitation in 500 µL DMEM, the segments were placed in separate wells of a 96-well plate with 100 µL DMEM. After 20 hours incubation at 37 °C and 5% CO_2, the DMEM and vessel segments were placed in separate tubes and frozen at -80 °C for protein and DNA analyses, respectively. To measure HDAd vector copy number, DNA was isolated from segments B and D, as described below.

Measurement of vector DNA
Total DNA was extracted from artery segments with the DNeasy Blood and Tissue kit (Qiagen Sciences, Germantown, MD) and quantified by spectrophotometry (NanoDrop; Thermo Scientific, Wilmington, DE). Two segments from each vessel were pooled and extracted together. Copies of HDAdNull or HDAdApoAI were measured by quantitative real-time PCR amplification of 100 ng of total DNA, targeting a sequence of noncoding stuffer DNA in the HDAd backbone.[3] Vector copy number was measured with reference to a standard curve generated by serial dilution of the pC4HSU plasmid (Microbix) that contains the stuffer DNA sequence.[2] Vector copy number per vascular wall cell was calculated by dividing the number of vector genomes detected in a well by the number of diploid cells represented by 100 ng of DNA.

Measurement of apo A-I protein
To detect apo A-I in medium conditioned by artery explants, we used essentially the same western blot protocol reported previously,[2] but with a new primary anti-rabbit apo A-I antibody. Goat antisera to rabbit apo A-I were generated by Pacific Immunology (Ramona, CA) using 3 peptide immunogens within the rabbit apo A-I protein (near the N-terminus, near the C-terminus, and an internal sequence). Preliminary testing of the 3 affinity-purified antibodies showed that the N-terminal sequence-targeted antibody (amino acids 24-47: RDEPRSSWDKIKDFATVYVDTVKD-Cys) identified purified rabbit apo A-I[3] on a western blot and was the most sensitive of the 3 antibodies. We used this antibody for all subsequent western blots. Equal volumes of conditioned medium were resolved under reducing conditions by electrophoresis on 0.1% SDS 15% polyacrylamide gels. Proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Billerica, MA). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, protein was detected with the new N-terminal-directed goat anti-rabbit apo A-I primary antibody followed by incubation with HRP-conjugated Donkey anti-goat secondary antibodies (sc-2033; Santa Cruz Biotechnology, Dallas, TX). Bound antibody was detected with a chemiluminescence reagent kit (Amersham ECL Select; GE Healthcare Bio-Sciences, Pittsburgh, PA) and imaged on a Bio-Rad ChemiDoc MP system (Bio-Rad Laboratories, Hercules, CA). Western blots were evaluated for the presence or absence

of apo A-I protein by a blinded observer. Plasma apo A-I was detected with the same western blot protocol, and was quantified by densitometry using ImageJ (National Institutes of Health).

Measurement of RNA

Total RNA was extracted from transduced artery segments by grinding two (7-week time point) or three (3-day time point) frozen segments from each artery with a liquid nitrogen-cooled mortar and pestle. The combined tissue (i.e., from all of the 2 or 3 segments from each artery) was then homogenized with a Polytron PT3100 (Polytron Devices, Paterson, NJ) in RLT buffer from the RNeasy mini kit (Qiagen Sciences, Germantown, MD) with 1% added β-mercaptoethanol. RNA extraction was completed as described in the RNeasy mini kit instructions, and total RNA quantified by spectrophotometry (NanoDrop; Thermo Scientific, Wilmington, DE). Apo A-I, MRC1, and IL-1 β mRNA in the extracts was measured by quantitative reverse transcriptase-mediated-PCR performed on 100 ng total RNA. Results were normalized to GAPDH mRNA measured in the same extracts. Primers and probes for Apo A-I were as reported.[3] MRC1 and IL-1β were quantified using SYBR Green and the following primers: $MRC1_{forward}$, 5′-GAAGAAAGGCAGGATGTG-3′; $MRC1_{reverse}$, 5′-ATCTGGGTTCAGGAGTTG-3′; $IL\text{-}1\beta_{forward}$, 5′-TCCAGACGAGGGCATCCA-3′; $IL\text{-}1\beta_{reverse}$, 5′-CTGCCGGAAGCTCTTGTTG-3′.

Histochemical and immunohistochemical staining

The OCT blocks (each containing four artery segments) were sectioned (6-μm-thick) at each of three 150-μm steps (Figure IX in the online-only Data Supplement). Ten serial sections were cut at each of the 3 steps and were placed on 10 separate slides, allowing serial sections to be stained with different reagents. Slides were stained with hematoxylin and eosin (H&E) and Oil Red O as well as with antibodies that detect a macrophage-associated antigen (RAM-11; 1:200 dilution; Dako, Carpinteria, CA), smooth muscle actin (HHF-35; 1:200 dilution; Thermo Scientific), T cell-associated antigen (KEN-5; 1:50 dilution; sc-59373; Santa Cruz Biotechnology),[10] ICAM-1 (1:200 dilution; from Dr. Myron Cybulsky, University of Toronto, Toronto, ON, Canada) and VCAM-1 (1:50 dilution; also from Dr. Myron Cybulsky).[11] Omission of the primary antibodies was used as a negative control. Stained sections were evaluated by an observer blinded to the treatment group. For H&E, RAM-11, and HHF-35 all intact sections (8–12 sections from each artery) were analyzed, using digital images were obtained with a Leica D4000B microscope and DFC295 camera (Leica Microsystems, Buffalo Grove, IL). Analysis of these data revealed that use of data from only 4 sections per artery (1 section per segment) provided results nearly indistinguishable from those obtained by using data from all 12 sections of the same artery. Therefore, only a single ORO-, KEN-5-, ICAM- and VCAM-stained section (in all cases the most caudal intact section) per artery segment (i.e., 4 sections per artery) was analyzed.

Intimal and medial areas were measured by planimetry performed on H&E-stained sections (ImageJ software). Measurements were made on 12 sections per artery (as described above). Intimal area was calculated by subtracting the lumen area from the area within the internal elastic lamina. Medial area was calculated by subtracting the area within the internal elastic lamina from the area within the external elastic lamina. The percentage of intima staining positively with Oil Red O, RAM-11, HHF-35, KEN-5, VCAM-1, and ICAM-1 was calculated by dividing the stained area (obtained with color thresholding; Image Pro-Premier 9) by the total intimal area measured on the nearest H&E-stained section. When multiple step sections were analyzed within a vessel segment, the step section measurements were averaged to determine the mean values for each segment. Mean values per artery were then calculated from the segment

means, and these individual artery values were used both to compare the groups and to compare paired HDAdApoAI- and HDAdNull-treated arteries in the same rabbits.

Statistical analysis

Results are reported as mean ± standard deviation or median (25%–75% range), as indicated. Intimal lipid and macrophage accumulation were prospectively designated as the primary end points of this study. Because lesion severity in the left and right carotids of the same rabbit are highly correlated and lesion size is highly variable among rabbits (Figure II in the online-only Data Supplement,[1] and our unpublished data), we prospectively selected an analytic approach that compares the results of experimental (HDAdApoAI) and control (HDAdNull) treatments in the same rabbits. This approach tests the null hypothesis that the median ratio between a measurement made of a HDAdApoAI-treated lesion and the same measurement made of the HDAdNull-treated lesion in the same rabbit is 1 (i.e., that the median difference between the paired lesions is 0). Accordingly, we used a ratio-paired test to analyze all of the histologic data. To do this, the logarithms (base 10) of all data points were taken, and the transformed data were analyzed with a Wilcoxon signed-rank test. For data sets that contained a zero value, log (1+x) was used to transform the data. Vector genomes at 7 weeks were compared by unpaired t test, after confirming normality and equal variance of the 2 groups. The presence of apo A-I protein in explant culture conditioned media was assessed with a chi-square test. Plasma apo A-I levels before gene transfer and at harvest were compared by a Wilcoxon signed rank test. We assessed the relationship between left and right carotid artery intimal areas in the same rabbit by calculating the Pearson product-moment correlation coefficient. To test for a difference in the effect of apo A-I gene therapy between subgroups with either large or small-to-moderate-sized lesions, we used a mixed-design analysis of variance to detect interaction between treatment and subgroup. Treatment (HDAdApoAI or HDAdNull) was the matched within-subjects factor, and lesion size (small-to-moderate or large) was the between-subjects factor. As with the Wilcoxon signed-rank test, analysis was performed on the log-transformed data. Data for all endpoints met assumptions of homoscedasticity and sphericity, while all endpoints except RAM-11 passed normality (by Lilliefors corrected Kolmogorov-Smirnov test). For RAM-11, the non-log-transformed data met all assumptions and analysis of these non-log-transformed data provided further support for a significant interaction between lesion size and effect. All statistical tests were performed with the SigmaStat program (Systat, San Jose, CA).

References for Materials and Methods

1. Du L, Zhang J, De Meyer GR, Flynn R, Dichek DA. Improved animal models for testing gene therapy for atherosclerosis. *Hum Gene Ther Methods*. 2014;**25**:106–114
2. Flynn R, Buckler JM, Tang C, Kim F, Dichek D. Helper-dependent adenoviral vectors are superior in vitro to first-generation vectors for endothelial cell-targeted gene therapy. *Mol Ther*. 2010;**18**:2121–2129
3. Flynn R, Qian K, Tang C, Dronadula N, Buckler J, Jiang B, Wen S, Dichek H, Dichek D. Expression of apolipoprotein a-i in rabbit carotid endothelium protects against atherosclerosis. *Mol Ther*. 2011;**19**:1833–1841
4. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: Removal of helper virus by cre-mediated excision of the viral packaging signal. *Proc. Natl. Acad. Sci. U. S. A.* 1996;**93**:13565–13570

5. Chen L, Anton M, Graham FL. Production and characterization of human 293 cell lines expressing the site-specific recombinase cre. *Somat. Cell Mol. Genet.* 1996;**22**:477–488
6. Schneider DB, Vassalli G, Wen S, Driscoll RM, Sassani AB, DeYoung MB, Linnemann R, Virmani R, Dichek DA. Expression of fas ligand in arteries of hypercholesterolemic rabbits accelerates atherosclerotic lesion formation. *Arterioscler. Thromb. Vasc. Biol.* 2000;**20**:298–308
7. Jiang B, Qian K, Du L, Luttrell I, Chitaley K, Dichek DA. Helper-dependent adenovirus is superior to first-generation adenovirus for expressing transgenes in atherosclerosis-prone arteries. *Arterioscler. Thromb. Vasc. Biol.* 2011;**31**:1317–1325
8. Schulick AH, Dong G, Newman KD, Virmani R, Dichek DA. Endothelium-specific in vivo gene transfer. *Circ. Res.* 1995;**77**:475–485
9. Gruchala M, Bhardwaj S, Pajusola K, Roy H, Rissanen TT, Kokina I, Kholova I, Markkanen JE, Rutanen J, Heikura T, Alitalo K, Bueler H, Yla-Herttuala S. Gene transfer into rabbit arteries with adeno-associated virus and adenovirus vectors. *J Gene Med*. 2004;**6**:545-554
10. Pospisil R, Kabat J, Mage RG. Characterization of rabbit cd5 isoforms. *Mol. Immunol.* 2009;**46**:2456–2464
11. Newman KD, Dunn PF, Owens JW, Schulick AH, Virmani R, Sukhova G, Libby P, Dichek DA. Adenovirus-mediated gene transfer into normal rabbit arteries results in prolonged vascular cell activation, inflammation, and neointimal hyperplasia. *J. Clin. Invest.* 1995;**96**:2955–2965