Improved Animal Models for Testing Gene Therapy for Atherosclerosis

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Abstract

Gene therapy delivered to the blood vessel wall could augment current therapies for atherosclerosis, including systemic drug therapy and stenting. However, identification of clinically useful vectors and effective therapeutic transgenes remains at the preclinical stage. Identification of effective vectors and transgenes would be accelerated by availability of animal models that allow practical and expeditious testing of vessel-wall-directed gene therapy. Such models would include humanlike lesions that develop rapidly in vessels that are amenable to efficient gene delivery. Moreover, because human atherosclerosis develops in normal vessels, gene therapy that prevents atherosclerosis is most logically tested in relatively normal arteries. Similarly, gene therapy that causes atherosclerosis regression requires gene delivery to an existing lesion. Here we report development of three new rabbit models for testing vessel-wall-directed gene therapy that either prevents or reverses atherosclerosis. Carotid artery intimal lesions in these new models develop within 2–7 months after initiation of a high-fat diet and are 20–80 times larger than lesions in a model we described previously. Individual models allow generation of lesions that are relatively rich in either macrophages or smooth muscle cells, permitting testing of gene therapy strategies targeted at either cell type. Two of the models include gene delivery to essentially normal arteries and will be useful for identifying strategies that prevent lesion development. The third model generates lesions rapidly in vector-naïve animals and can be used for testing gene therapy that promotes lesion regression. These models are optimized for testing helper-dependent adenovirus (HDAd)-mediated gene therapy; however, they could be easily adapted for testing of other vectors or of different types of molecular therapies, delivered directly to the blood vessel wall. Our data also supports the promise of HDAd to deliver long-term therapy from vascular endothelium without accelerating atherosclerotic disease.

Introduction

ATHEROSCLEROSIS IS A LEADING CAUSE of human morbidity and mortality (Gersh *et al.*, 2010; Go *et al.*, 2013). Gene therapy for atherosclerosis has shown promise in several preclinical studies (Rissanen and Yla-Herttuala, 2007; Williams *et al.*, 2010); however, other than a pilot study of low-density lipoprotein receptor gene therapy to prevent premature atherosclerosis in patients with familial hypercholesterolemia carried out nearly two decades ago (Grossman *et al.*, 1995), gene therapy for atherosclerosis remains at the preclinical stage (Williams *et al.*, 2010). Potential gene therapies for atherosclerosis include manipulation of plasma lipoproteins via gene transfer to the liver (Van Craeyveld *et al.*, 2010, 2011) and direct gene transfer to the blood vessel wall to prevent or reverse atherosclerotic lesion growth (Ris-

sanen and Yla-Herttuala, 2007; Bradshaw and Baker, 2013). The latter approach is appealing because of its potential to deliver therapy directly to sites of atherosclerotic lesion development while minimizing systemic vector exposure.

Preclinical development of vessel-wall-directed gene therapy for atherosclerosis requires animal models in which humanlike atherosclerotic lesions develop quickly and gene therapy vectors can be delivered efficiently and with minimal expense and morbidity. To this end, several years ago we developed a rabbit model of carotid atherosclerosis for testing vascular-wall-directed gene therapy (Schneider *et al.*, 2000). Atherosclerotic lesions in this model are produced within 4 weeks by a combination of diet-induced hyperlipidemia and local infusion of a “null” (i.e., no expression cassette) first-generation (FG) adenoviral (Ad) vector (FGAdNull). Key histologic features of the lesions, which strongly resemble

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early human atherosclerosis, include abundant lipid and macrophages, a significant smooth muscle cell presence, a prominent inflammatory component, and preservation of the endothelium (Schneider *et al.*, 2000). This animal model is useful for testing short-term vascular-wall-targeted gene therapy because the carotid arteries are easily accessible with only a minor surgical protocol (expensive catheters are not required); lesions develop quickly and reproducibly; and the side branches of the common carotid can be ligated without morbid consequences, facilitating vector dwell time and gene delivery. Moreover, vector infusion is inherent in the model: to test whether a transgene will prevent early atherosclerosis, one needs to only insert the transgene into an FGAd vector and compare lesions in arteries treated with the new vector to lesions in arteries treated with FGAdNull (Schneider *et al.*, 2000; Falkenberg *et al.*, 2002).

More recently, we found that third-generation or helper-dependent adenoviral vectors (HDA) (Parks *et al.*, 1996; Sandig *et al.*, 2000) are superior agents for atheroprotective gene therapy because they stimulate vascular inflammation only minimally and can express a transgene in the artery wall for at least 48 weeks (Flynn *et al.*, 2010, 2011). Accordingly, we began using HDA to develop atheroprotective gene therapy in our rabbit carotid model. However, we found that lesions in fat-fed rabbit carotids infused with HDANull are much smaller than lesions in fat-fed rabbit carotids infused with FGAdNull, and that—unlike FGAd-associated lesions—HDANull-associated lesions regress spontaneously between 4 and 8 weeks after vector infusion (Du *et al.*, 2011; Flynn *et al.*, 2011). The small and transient character of atherosclerotic lesions in HDANull-infused carotids is likely because of the less inflammatory nature of HDA (Wen *et al.*, 2004). This feature of HDA is useful for eventual clinical application; however, small transient lesions are a suboptimal substrate for testing gene therapy vectors and determining whether they have robust and durable atheroprotective effects.

Limited lesion development in HDANull-infused carotids may explain why we detected only a modest effect of HDA-expressed apolipoprotein (apo) A-I gene therapy on lesion size at 4 weeks (Flynn *et al.*, 2011) and failed to detect any effect of HDA-expressed interleukin-10 gene therapy on lesion size at either 4 or 8 weeks (Du *et al.*, 2011). Another limitation of this model is that exposure to Ad during lesion initiation immunizes the rabbit to Ad capsid proteins (Schulick *et al.*, 1997). Immunity to Ad might interfere with subsequent Ad-mediated gene transfer (Schulick *et al.*, 1997; Du *et al.*, 2011), precluding use of the model to test whether HDA-mediated gene therapy (delivered to established lesions) can promote lesion regression. To address these shortcomings, we developed three new rabbit models of carotid atherosclerosis. These models are well suited for testing HDA (or other) vectors for their ability either to prevent atherosclerosis or to promote atherosclerotic lesion regression.

Materials and Methods

Adenoviral vectors

We used two adenoviral vectors: FGAdNull and HDA-Null. FGAdNull is a first-generation E1/E3-deleted vector

with an “empty” expression cassette (Falkenberg *et al.*, 2002). HDANull is a third-generation (also known as a “helper-dependent”) vector that lacks all viral genes and contains the same empty expression cassette as FGAdNull (Flynn *et al.*, 2010). HDANull was made with commercially available reagents (Microbix Biosystems, Mississauga, Ontario, Canada). Vector stocks were propagated in either human embryonic kidney 293 cells (for FGAdNull) or 293 Cre4 cells (Chen *et al.*, 1996) (for HDANull). Vector preparations were characterized by measuring their concentration ($3\text{--}4 \times 10^{12}$ viral particles (vp)/ml for both vectors, measured by spectrophotometry) (Mittereder *et al.*, 1996) and the level of E1A-containing genomes (<1 in 10^6 vector genomes for both vectors, measured by real-time polymerase chain reaction [PCR]) (Flynn *et al.*, 2010). Helper virus contamination for HDANull was measured by real-time PCR (Flynn *et al.*, 2010) and was $<1\%$ of total vector genomes. Both vectors in this study were from single preparations.

Animals and surgeries

All animal studies were approved by the University of Washington Office of Animal Welfare. Twenty-six specific-pathogen-free male New Zealand white rabbits (3.0–3.5 kg; Western Oregon Rabbit Co, Philomath, OR) were enrolled. After 1 week of adaptation to the animal facility, rabbits were allocated into one of five experimental groups ($n=5\text{--}6$ in each group), with each group receiving a unique combination of diet, infusate, and surgical protocol (Fig. 1). We measured total plasma cholesterol in six of the rabbits before beginning the high-fat diet (47 ± 12 mg/dl). High-fat diets (Dyets Inc., Bethlehem, PA) included 0.3%, 0.5%, and 1% cholesterol, all with 3% soybean oil. These diets were fed to the rabbits either as purchased or after mixture with normal rabbit chow (Albers 16% rabbit PLT Pacific Northwest Milling, Bellevue, WA) as described below. Rabbits were fed ~ 230 g/day of chow. Total plasma cholesterol was measured with a colorimetric assay (Abbott Laboratories, Abbott Park, IL). Fifteen of the rabbits (groups I, II, and III) received bilateral intraluminal common carotid artery infusions of viral vector, using an open surgical protocol that includes infusion of vector solution into an arterial segment that is temporarily isolated from the circulation with vascular clips (Schneider *et al.*, 2000). After a 20 min incubation, the vector solution was aspirated, the arteriotomy repaired, and blood flow restored. This protocol results in gene transfer primarily to luminal endothelial cells (Schulick *et al.*, 1995; Jiang *et al.*, 2011).

Rabbits in group I ($n=5$; the “higher cholesterol” group) were fed chow containing 1% cholesterol/3% soybean oil for 2 weeks, and then 0.5% cholesterol/3% soybean oil for 2 weeks. Their carotids were then infused with HDANull (2×10^{11} vp/ml). We initially planned to continue the 0.5% cholesterol/3% soybean oil diet for 24 weeks postoperatively (Kaul *et al.*, 1992); however, the rabbits did not tolerate this diet, becoming anorectic and anemic, with one rabbit dying 8 weeks postoperatively. The four surviving rabbits were therefore placed on a “sliding scale” of cholesterol-containing chow: 0%, 0.15%, or 0.3% cholesterol, with adjustments of their diet made typically at 7–14-day intervals, after measuring plasma cholesterol. Adjustments were made

FIG. 1. Protocols for accelerating development of rabbit carotid atherosclerosis. Rabbits were fed high-fat diets beginning in week 0. The percentage of dietary cholesterol during each block of time is indicated. “Sliding scale” indicates adjustment of dietary cholesterol within the range of 0–0.3% to target plasma cholesterol to 200–800 mg/dl. The ends of the bars correspond to the time of artery harvest. The “Historical Control” protocol was reported previously (Du *et al.*, 2011; Flynn *et al.*, 2011). Triangles indicate the time of carotid surgery; the infusate for each group is indicated here. Group II also underwent periarterial Silastic collar placement at the time of vector infusion.

with a goal of achieving plasma cholesterol levels of 200–800 mg/dl. The four rabbits were fed this sliding-scale diet from 8 to 24 weeks postoperatively, at which time they were euthanized and their carotid arteries removed.

Rabbits in group II ($n=5$; the “collar placement” group) were fed 0.3% cholesterol for 4 weeks. Their carotids were then infused with HDAdNull (2×10^{11} vp/ml), and Silastic periarterial collars (Fig. 2A; fabricated by G.R.Y.D.M.) placed around the transduced segments (Booth *et al.*, 1989; Kockx *et al.*, 1992). The collars were sealed by placing a small amount of silicone glue (Dow Corning 732; Dow Corning Corporation, Midland, MI) along the open edges of the collar. The 0.3% cholesterol diet was continued for 4 weeks postoperatively at which time the rabbits were euthanized and their carotid arteries removed. Plasma cholesterol levels in this group were 591 ± 274 mg/dl at the time of vector infusion and 726 ± 322 mg/dl at the time of artery harvest.

Rabbits in group III ($n=5$; the “coinfusion with FGAd” group) were fed 0.3% cholesterol for 4 weeks. Their carotid arteries were then infused with a mixture of HDAdNull (2×10^{11} vp/ml) and FGAdNull (7×10^{11} vp/ml). The 0.3% cholesterol diet was continued for 8 weeks postoperatively, at which time they were euthanized and their carotid arteries removed. Plasma cholesterol levels in this group were 142 ± 61 mg/dl at the time of vector infusion and 562 ± 249 mg/dl at the time of artery harvest.

Group IV rabbits ($n=5$; the “longer-time only” group) were fed 0.3% cholesterol for 4 weeks and then a sliding-scale (0–0.5%) cholesterol diet for 24 weeks with adjustment of the diet to target a plasma cholesterol of 200–800 mg/dl. They were not operated on and received no vector infusions.

Group V rabbits ($n=6$; the “surgery without vector” group) were fed 1% cholesterol for 2 weeks and then 0.5% cholesterol for 2 weeks. They then underwent a “mock”

FIG. 2. Protocols for processing rabbit carotid arteries for histologic and molecular analyses. (A) Arteries that were wrapped in Silastic collars (shaded area) were cut into four segments. Segments A, B, and D were processed for histology; segment C was used for molecular analyses. (B) All other arteries were cut into seven segments. Segments a–d were processed for histology; segments 1–3 were used for molecular analyses.

gene transfer surgery with bilateral common carotid infusions of Dulbecco's modified Eagle's medium (DMEM) instead of viral vector. Postoperatively, they were begun on a 0.3% cholesterol diet with sliding-scale adjustments as described above to target plasma cholesterol to 200–800 mg/dl. Their arteries were harvested 24 weeks after the surgical procedure. Cholesterol levels for groups I, IV, and V (all of which received 7 months of high-fat diet) are presented below.

Because these models were developed for the purpose of increasing lesion size above what we had obtained in HDAdNull-infused arteries harvested from rabbits enrolled in earlier studies (Du *et al.*, 2011; Flynn *et al.*, 2011), we include data from these “historical control” rabbits ($n = 14$ arteries from 7 rabbits) in one of the figures (Fig. 3).

Carotid artery harvests

Rabbits were given general anesthesia, and their common carotid arteries dissected free via a midline neck incision. Silk ligatures were applied to isolate the transduced/mock-transduced artery segments (or to isolate a similar segment of untransduced carotid artery in group IV rabbits). The isolated segments were flushed gently with DMEM and excised. In all groups except the collar group, the carotids were then cut into seven segments (Fig. 2B). Four evenly spaced segments (a–d) were embedded in OCT medium for frozen sectioning, and the three intervening segments (1–3) were snap-frozen in liquid nitrogen for molecular analyses. Because the collar size limited the length of artery that was exposed to vector, collared carotids were cut into only four segments (Fig. 2A). Three of these pieces (A, B, and D) were embedded in OCT for

frozen sectioning and one segment (C) was frozen for molecular analyses.

Measurement of vector DNA

Total DNA was extracted from artery segments with the DNeasy kit (Qiagen Sciences, Germantown, MD) and quantified by spectrophotometry (NanoDrop; Thermo Scientific, Wilmington, DE). In the collar group, DNA was extracted from segment C. In the other two groups that received vector (groups I and III), two segments (1 and 3) were pooled and extracted together. Copies of HDAdNull were measured by quantitative real-time PCR amplification of 100 ng of total DNA, targeting both a sequence of noncoding stuffer DNA (present only in HDAdNull) and a sequence in the CMV promoter (present in both FGAdNull and HDAdNull) (Flynn *et al.*, 2011). The stuffer DNA amplification specifically detects HDAdNull, whereas the CMV promoter amplification both provides an independent measurement of HDAdNull genomes and permits detection of FGAdNull genomes. Vector copy number was measured with reference to a standard curve generated by serial dilution of the pC4HSU plasmid (Microbix) (Sandig *et al.*, 2000) that contains the stuffer DNA sequence (Flynn *et al.*, 2010). 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.

Histochemical and immunohistochemical staining

The OCT blocks (containing either three or four artery segments) were cut into 6- μ m-thick sections. Two step sections of each of the segments (at steps of $\geq 180 \mu$ m) were

FIG. 3. Intimal lesion size in models of accelerated atherosclerosis. (A) Intimal area. (B) Medial area. (C) Intimal/medial area ratios. Data points are individual rabbit carotid arteries; bars are group means. Protocols for generating lesions are depicted in Fig. 1. HC, historical control group. * $p < 0.001$.

placed on a single slide to ensure the presence of at least 3–4 intact sections per slide for later analysis. Serial sections cut at each of the two steps were placed on separate slides, allowing serial sections to be stained with different reagents. Slides from all five groups were stained with hematoxylin and eosin (H&E) and with either Verhoeff–Van Gieson (VVG) or Movat stains. Slides from groups I, II, and V were stained with oil red O and with antibodies that detect macrophages (RAM-11; 1:200 dilution; Dako, Carpinteria, CA) or smooth muscle actin (HHF-35; 1:200 dilution; Thermo Scientific). The specificity of the primary antibodies was tested by substituting an isotype-matched antibody (for RAM-11; #14-4714; eBiosciences) or by omitting the primary antibody (for HHF-35). Evaluations of stained sections were carried out by an observer blinded to the treatment group.

Digital images of sections were obtained with a Leica DFC295 image system (Leica Microsystems, Buffalo Grove, IL). Intimal and medial areas were measured by planimetry performed on either VVG- or Movat-stained sections (ImagePro 5.0 software; Bethesda, MD). Measurements were made on three evenly spaced step sections from collared arteries and on four evenly spaced step sections from the other four groups. 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 RAM-11, HHF-35, and oil red O was calculated by dividing the stained area (obtained with color thresholding) by the total intimal area measured on the same section. Mean values were computed from areas measured on three or four step sections per artery and were then used as individual artery values to compare the groups.

Statistical analysis

Results are reported as mean $\pm$ standard deviation. Normally distributed data with equal variances were compared with the unpaired *t*-test. Data not normally distributed or with unequal variances were compared with the Mann–Whitney rank-sum test. For comparing more than two groups, we used one-way ANOVA, with correction for *post-hoc* pairwise comparisons with the Holm–Sidak method. Statistical tests were performed with the SigmaStat program (Systat, San Jose, CA).

Results

Addition of a second “insult” increases intimal lesions in HDAdNull-infused arteries of hyperlipidemic rabbits

We and others have shown that development of atherosclerosis is accelerated in hyperlipidemic animals by addition of a second vascular insult [e.g., balloon injury (Weidinger *et al.*, 1991), cryodamage (Aliev *et al.*, 1998), electrical injury (Chiesa *et al.*, 2001), periarterial collar placement (Baetta *et al.*, 2002), or infusion of first-generation Ad (Schneider *et al.*, 2000)]. We therefore tested three interventions (Fig. 1) for their ability to generate large and/or persistent intimal lesions in HDAdNull-infused carotids of hyperlipidemic rabbits. These interventions included a diet of higher cholesterol content administered over a longer period of time (group I); placement of a periarterial collar at the time of HDAdNull infusion (group II); and coinfection of

FGAdNull at the time of HDAdNull infusion (group III). All arteries in these three groups had a neointima (Fig. 3A and Supplementary Fig. S1A–C [Supplementary Data are available online at www.liebertpub.com/hgtb]; $p < 0.001$ for each group vs. historical control arteries from rabbits fed a lower concentration of cholesterol, infused with HDAd only, and harvested at 8 weeks). Neointimal area was greatest in group I ($6.2 \pm 5.0 \times 10^5 \mu\text{m}^2$), followed by group II ($3.1 \pm 1.7 \times 10^5 \mu\text{m}^2$) and group III ($1.4 \pm 0.6 \times 10^5 \mu\text{m}^2$). Compared with the historical control group ($7.3 \pm 13 \times 10^3 \mu\text{m}^2$), these are increases of 20–80-fold. Medial areas of groups I–III were similar to the medial areas of the historical control arteries, although the mean medial area of group I arteries was slightly higher (20% increase vs. historical controls; $p < 0.05$ by ANOVA; Fig. 3B) and the mean medial area of group III arteries was nominally lower ($p = \text{not significant [NS]}$). The medial areas of groups I and II increased far less, proportionately, than their intimal areas. As a result, the intimal:medial area ratios of all three groups were significantly increased compared with the historical control group ($p < 0.001$ for all; Fig. 3C).

Coinfusion of FGAd causes loss of HDAd genomes

For an animal model to be useful for testing whether a locally delivered gene therapy vector can prevent atherosclerosis, the vector genome must persist in the blood vessel wall during lesion growth. To assess vector persistence, we extracted DNA from artery segments of groups I, II, and III at the time of euthanasia and measured HDAdNull and FGAdNull genomes by quantitative PCR. HDAdNull DNA was present at a mean of 0.5 vector genomes per vascular wall cell in arteries from groups I and II. However, neither HDAdNull nor FGAdNull genomes were detected in any of the arteries from group III (Fig. 4).

Lesion composition in two models of atherosclerotic lesion development

Groups I and II combine robust lesion development with HDAd vector persistence and therefore would allow testing the atheroprotective activity of therapeutic genes expressed from HDAd. Intimal lesions in these groups appeared different on H&E stain, with group I lesions relatively eccentric

FIG. 4. HDAdNull vector genomes detected in rabbit carotid artery extracts. DNA was extracted from arteries harvested at 6 months (group I), 1 month (group II), or 2 months (group III) after vector infusion. Protocols for generating lesions are depicted in Fig. 1. Data points are individual arteries; bars are group means.

FIG. 5. Intimal lesion composition analysis. Sections of carotid arteries harvested from rabbits in groups I, II, and V were stained with (A) the RAM-11 antibody to detect macrophages, (B) the HHF-35 antibody to detect smooth muscle cells, and (C) Oil red O (ORO) to detect lipid. The percentages of total intimal area staining positively are indicated. Protocols for generating lesions are depicted in Fig. 1. Data points are means of individual arteries; bars are group means. $*p < 0.001$.

and foamy and group II lesions more concentric and dense (Supplementary Fig. S1A vs. S1B). To more fully assess the suitability of these lesions to serve as substrates for testing specific gene therapy strategies (e.g., lipid depletion, macrophage depletion, inhibition of smooth muscle proliferation/migration), we measured intimal content of lipid, macrophages, and smooth muscle cells. Macrophages were far more abundant in group I lesions than in group II lesions ($47\% \pm 13\%$ intimal RAM-11-stained area in group I vs. $5\% \pm 4\%$ in group II; $p < 0.001$; Fig. 5A and Supplementary Fig. S2A vs. S2B). Group II lesions had a higher content of smooth muscle cells than group I lesions ($40\% \pm 8\%$ vs. $6\% \pm 4\%$ HHF-35-stained intimal area; $p < 0.001$; Fig. 5B and Supplementary Fig. S2D vs. S2E). The percentage of oil red O-stained area did not differ significantly between the two groups ($10\% \pm 4\%$ vs. $6\% \pm 5\%$, $p = 0.1$; Fig. 5C).

Prolonged hypercholesterolemia alone is insufficient to generate carotid artery intimal lesions

We next attempted to generate a rabbit model for testing whether transgenes expressed from HDAd vectors could mediate atherosclerosis regression. Lesions in groups I and

II are potential substrates for regression studies that would be performed by infusion of HDAd into arteries in which lesions had already formed. However, both of these models include exposure of the animal to Ad at the time of lesion initiation (Fig. 1). Exposure of an immune-competent animal to Ad—even HDAd, because of its capsid protein content—generates a systemic antigen-specific immune response to viral capsid proteins (Du *et al.*, 2011) and could interfere with HDAd-mediated vascular gene transfer attempted at a later time point (Newman *et al.*, 1995). Accordingly, we sought to develop a regression model in which lesions are generated without exposure to Ad.

The large lesions in group I suggested that lipid- and macrophage-rich carotid lesions might develop by 6 months with diet alone. Carotid atherosclerosis develops relatively slowly in cholesterol-fed rabbits and is minimal after 3 months on diet (Kamimura *et al.*, 1999). We therefore fed rabbits a high-fat diet for 7 months, performing neither carotid surgery nor virus infusion (group IV; Fig. 1). Cholesterol levels in these rabbits rose rapidly and remained elevated (~ 400 – $1,000$ mg/dl; $n = 5$; Fig. 6). Despite equivalence of plasma cholesterol between group IV and group I ($p = \text{NS}$), we found no intimal lesions in any of the carotid

FIG. 6. Plasma cholesterol in rabbits fed a high-fat diet for 7 months. Data points are group means; variances are standard deviations. The last measurement in Group IV was performed 4 weeks before harvest. Protocols for generating lesions are depicted in Fig. 1.

arteries from group IV ($n=10$; Fig. 3A and Supplementary Fig. S1D). Therefore, generation of large lesions similar to those in group I within 6 months requires the surgical infusion procedure, and possibly HDAd infusion as well.

Infusion of medium is sufficient to generate carotid intimal lesions in hyperlipidemic rabbits

We next tested whether surgical manipulation of carotid arteries with infusion of medium alone (no virus) could substitute for infusion of HDAd as a second “insult” that accelerates lesion formation. Group V rabbits ($n=6$) were fed cholesterol for 4 weeks then subjected to bilateral infusion of DMEM alone, with harvest of carotids 6 months later (Fig. 1). Cholesterol levels in group V were approximately 50% higher than those in groups I and IV ($p<0.001$; Fig. 6). Carotids harvested from group V rabbits had large lesions, with intimal area and I:M ratios similar to those of group I rabbits (Fig. 3A and C, and Supplementary Fig. S1E; $p=0.3$ for both intimal area and I:M ratio). Lesion analysis revealed similar composition of lesions from group I and V rabbits (Fig. 5; $p\geq 0.1$ for macrophage, smooth muscle, and lipid content; Supplementary Fig. S2C and S2F vs. S2A and S2D, respectively). Therefore, diet-induced hyperlipidemia and infusion of medium alone yields large lipid- and macrophage-rich carotid lesions within 6 months. These lesions are a useful substrate for testing whether HDAd vectors—delivered to existing lesions—can promote lesion regression. Ligation of carotid side branches (routinely performed during the initial surgery) facilitates intraluminal vector delivery at this later time point.

Discussion

Our goal was to develop improved rabbit models for testing vascular-wall-targeted gene therapy that prevents or reverses atherosclerosis. Our major findings were as follows: (1) 7 months of diet-induced hyperlipidemia is inadequate to generate carotid atherosclerotic lesions in New Zealand White rabbits; (2) large lipid- and macrophage-rich carotid intimal lesions are generated within 6 months by a combination of diet-induced hyperlipidemia and infusion of medium (with or without HDAd) into the surgically isolated carotid lumen; (3) large lipid- and smooth muscle cell-rich lesions can be generated within 1 month by a combination of diet-induced hyperlipidemia, infusion of HDAd into the surgically isolated carotid lumen, and periarterial collar placement; (4) HDAd persists in the artery wall at substantial levels during lesion development in both of these models, allowing their use for testing gene therapy interventions that prevent atherosclerosis; and (5) formation of large intimal lesions within 6 months in carotids infused with buffer alone yields a model for testing gene therapy that promotes lesion regression.

Human atherosclerosis develops over decades. Animal models for testing atheroprotective therapies must yield reasonably representative lesions in far less time. The lesions in our models develop over 1–6 months, a practical period for preclinical studies. Moreover, these lesions develop in the common carotid artery, which is easily accessible for local delivery of HDAd as well as any other genetic, cellular, or molecular therapy that can be delivered directly to the blood vessel wall. If experimental therapies

continue to be effective when delivered with the surgical approach used in these models, delivery of these therapies via more clinically relevant percutaneous catheter-based technologies would be a logical next step in preclinical development. A recent study suggests that local delivery of a therapeutic device (i.e., a stent) to diseased coronary artery segments is more effective than systemically delivered “optimal medical therapy” in preventing future myocardial infarctions (Wald *et al.*, 2013). We speculate that a combination of device deployment and locally delivered gene therapy might prevent infarctions even more effectively than device therapy alone. The animal models described here will facilitate identification of atheroprotective gene therapy.

We were able to accelerate atherosclerotic lesion growth without resorting to interventions (such as balloon injury, cryoinjury, and electrical injury) that cause either complete loss of the endothelium, severe damage and disintegration of the media, or both (Uint *et al.*, 1989; Richardson *et al.*, 1990; Aliev *et al.*, 1998; Chiesa *et al.*, 2001). This is important because human atherosclerosis develops in normal arteries, not in severely injured arteries. Accordingly, we anticipate that relatively normal arteries will be more useful experimental settings than severely injured arteries for development of atheroprotective gene therapy. The approaches of luminal infusion and collar placement that are described here preserve both the endothelium and the underlying media (Kockx *et al.*, 1993; Schneider *et al.*, 2000). Vessels subjected to luminal infusion and/or collar placement have a relatively normal histologic appearance with intact vascular media (Supplementary Figs. S1A and S1B and S2D and S2E) and likely have more normal physiology than do severely injured blood vessel segments that have lost an entire subpopulation of cells (endothelium), large numbers of smooth muscle cells, or both cell types. We cannot exclude a low level of endothelial turnover in these models; however, light microscopy revealed intact endothelium in all groups, and large-scale loss of endothelial cells in group I and II arteries is virtually excluded by the presence of large numbers of adenoviral genomes in harvested arteries. Because gene transfer after luminal incubation of Ad occurs almost exclusively to luminal endothelial cells (Schulick *et al.*, 1995; Jiang *et al.*, 2011), the presence of ~ 0.5 vector genomes per vascular wall cell in both group I and II arteries translates into several vector copies per luminal endothelial cell. This suggests a high initial level of endothelial gene transfer and persistence of the transduced cells.

Although model development was our primary goal, our data provide additional insights. First, although we showed previously that HDAd-mediated gene expression persists for at least 4 weeks after coinfection with FGAd (Jiang *et al.*, 2011), we now find that both HDAd and FGAd are eliminated from the artery wall by 8 weeks. This finding confirms that HDAd—not FGAd—is suitable for long-term transduction of endothelial cells. Second, detection of large amounts of HDAd genomes in group I arteries and the equivalence of vector genomes in group I (6-month) and group II (1-month) arteries (Fig. 4) confirms our previous results showing persistence of HDAd genomes at a stable and high level after the first month postinfusion (Flynn *et al.*, 2011). Third, equivalence of intimal area as well as macrophage and lipid accumulation in group I arteries (infused with HDAd) and group V arteries (infused with

DMEM) supports and extends (from 4 weeks to 6 months) our previous finding that arterial infusion of HDAd at 2×10^{11} vp/ml does not accelerate atherosclerosis (Jiang *et al.*, 2011).

The animal models described here were developed primarily for testing HDAd-mediated gene therapy; however, we speculate that they will be useful both for testing other vectors and for testing other types of molecular therapies that are delivered directly to the vessel wall. The models used for groups I and II could be adapted for testing other vectors/therapies simply by substituting an appropriate control infusion for HDAdNull. Lesions would be expected in these control groups based on the group V results showing that lesion formation does not depend on HDAdNull infusion and data showing lesion formation in collared rabbit arteries in the absence of HDAdNull infusion (Booth *et al.*, 1989; Kockx *et al.*, 1992).

In summary, we developed new rabbit models of accelerated atherosclerosis that are well suited for preclinical testing of gene therapy vectors. Our data also continue to support the use of HDAd for gene therapy strategies that require long-term transgene expression in the blood vessel wall.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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