Conclusion—HDAd is superior to FGAd for expression of transgenes in atherosclerosis-prone arteries.

Methods and Results—Vascular gene transfer is a powerful tool for investigating and treating vascular diseases; however, its utility is limited by brevity of transgene expression and vector-associated inflammation. Helper-dependent adenovirus (HDAd), an advanced-generation adenovirus that lacks all viral genes, is superior to first-generation adenovirus (FGAd) in normal rabbit arteries. We compared HDAd to FGAd in arteries of cholesterol-fed rabbits, a model of early atherogenesis in which transgene expression might be decreased, and inflammation increased.

HDAd expressed a transgene at least as well in arteries of cholesterol-fed rabbits as in arteries of chow-fed rabbits and expressed more durably than FGAd. In arteries of cholesterol-fed rabbits, HDAd stimulated less intimal growth, lipid deposition, and inflammation than FGAd. Neither vector affected phenylephrine-induced contraction or nitroprusside-mediated relaxation; however, both vectors decreased maximal acetylcholine-stimulated vasorelaxation. The relative absence of intimal growth in HDAd arteries could interfere with the utility of this model for testing atheroprotective genes; however, both coinfection of FGAd and extension of cholesterol feeding yielded larger intimal lesions, on which atheroprotective genes could be tested.

Conclusion—HDAd is superior to FGAd for expression of transgenes in atherosclerosis-prone arteries. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis ■ carotid arteries ■ gene therapy ■ vasodilation

In vivo vascular gene transfer is a powerful tool for investigating the roles of genes and their protein products in vascular homeostasis and disease.1-3 Vascular gene transfer has several advantages compared with the alternative approach of germ-line transgenesis with vascular targeting using cell-type-specific promoters. First, because vascular gene transfer is site-specific, unmanipulated vascular segments in the same animal can serve as experimental controls. Second, vascular gene transfer confines transgene expression to a specific site. In contrast, germ line transgenesis yields expression throughout the vasculature, making it difficult to determine the precise location(s) at which the transgene acts. Third, because vascular gene transfer cannot cause embryonic lethality, it can be used reliably to express transgenes in blood vessels of adult animals. Fourth, it is far easier to adjust gene expression levels using vascular gene transfer than using germ-line transgenesis. Finally, vascular gene transfer can be applied as a human therapy, whereas germ-line transgenesis cannot.

Despite its promise, the application of vascular gene transfer for both investigational and therapeutic purposes has been limited by brevity of transgene expression and vector-associated inflammation.4-7 These are significant limitations because many vascular diseases and biologically important phenotypes (e.g., atherosclerosis and aneurysm formation) develop over prolonged periods and are affected by inflammation. Accordingly, a vector used to investigate or treat these diseases must achieve prolonged transgene expression and should not cause significant vascular inflammation. Until recently, no such vector was available.8-9

Helper-dependent adenoviral (HDAd) vectors, advanced-generation adenovirus (Ad) vectors that lack all viral genes, are promising agents for vascular gene transfer. Recombinant HDAd are relatively easily prepared10,11 and transfer genes efficiently to many tissues.12 HDAd can express therapeutic genes for 2 to 3 years in rodents (essentially a rodent lifetime) and for more than 2 years in livers of nonhuman primates.12-14 To begin to test the suitability of HDAd for vascular gene transfer, several years ago we tested the performance of HDAd in normal rabbit carotid arteries. HDAd expressed a transgene far longer than first-generation Ad (FGAd) (>8 weeks versus 1 to 2 weeks for FGAd)5,6 and caused only

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minimal vascular inflammation. These data were promising and suggested that HDAd will be broadly useful for both investigational and therapeutic purposes. However, atherosclerosis and other vascular diseases develop and progress on a background of hyperlipidemia and associated arterial pathology. Before using HDAd to investigate or treat these diseases, the performance of HDAd in arteries of hyperlipidemic animals must be tested. Ideally, HDAd would express a transgene persistently and at high levels in arteries of hyperlipidemic animals, with little or no associated inflammation and no detrimental effects on arterial physiology. However, studies with FGAAd suggest that in arteries with early atherosclerosis, vector-mediated transgene expression could be significantly lower and vector-related inflammation and lesion formation significantly higher than in normal arteries. Here, we report experiments that test the performance of HDAd in a rabbit model of early atherosclerosis.

**Materials and Methods**

**Adenoviral Vectors**

We used 6 vectors: FGAduPA, FGAdNull, FGAdCMVlacZ, HDAduPA, HDAdNull, and HDAdGFP. FGAduPA, FGAdNull, and FGAdCMVlacZ are first-generation E1/E3-deleted vectors that contain a rabbit urokinase plasminogen activator (uPA) cDNA, an identical expression cassette without the uPA transgene, and a nucleus-localized β-galactosidase gene, respectively. HDAduPA, HDAdNull, and HDAdGFP are helper-dependent vectors lacking all viral open reading frames. HDAduPA and HDAdNull contain the same expression cassettes as FGAduPA and FGAdNull, respectively. HDAdGFP expresses green fluorescent protein (GFP). All 6 vectors include the cytomegalovirus (CMV) promoter and SV40 polyadenylation signal. Viral stocks were propagated in either human embryonic kidney (HEK) 293 cells (for FGAd) or 293 Cre cells (for HDAd). Viral concentrations (viral particles [vp]/mL) were measured with a spectrophotometer. E1A-containing genomes and helper virus contamination (for HDAd) were measured by real-time polymerase chain reaction (PCR). Viral preparations were used only if E1A-containing genomes were below 1 in 105 vector genomes. Helper virus concentrations were below 1% in all HDAd preparations.

**Rabbit Common Carotid Artery Gene Transfer**

Male New Zealand White rabbits (2.5 to 3.5 kg, Western Oregon Rabbit Co.) were fed either normal chow or a high-fat diet containing 0.25% cholesterol and 3% soybean oil (Dyets, Inc.). Plasma cholesterol levels were measured with a colorimetric assay (Abbott Laboratories). In rabbits fed the high-fat diet, cholesterol levels were measured after 4 weeks on the diet, and rabbits were assigned to the experimental groups in a manner such that the mean cholesterol levels of rabbits in each group did not differ significantly. Gene transfer surgery was performed 4 weeks after starting the high-fat diet. Endothelium-specific gene transfer to common carotid arteries of hyperlipidemic rabbits (atherosclerosis-prone arteries) or Chow-fed rabbits (normal arteries) was performed as described. For infusion in vivo, viral stocks were diluted to 2 to 7.5×1011 vp/mL with phenol red-free Dulbecco’s modified Eagle’s medium (DMEM) containing 1 mg/mL rabbit serum albumin (Sigma-Aldrich). Control arteries were infused with this diluent alone. Arteries were harvested at 3, 14, 28, or 56 days after infusion and for most experiments were either perfusion-fixed in situ with 10% neutral-buffered formalin and embedded in paraffin, placed in O.C.T. medium for frozen sectioning, snap-frozen for RNA extraction, or placed in explant culture for measurement of uPA activity. Arteries infused with HDAdGFP were fixed ex vivo with 4% paraformaldehyde for 1 hour, rinsed with PBS, placed in 30% sucrose at 4°C overnight, and then embedded in O.C.T. medium. Arteries infused with FGAdCMVlacZ were fixed for 2 hours in 0.2% glutaraldehyde in 0.1 mol/L PBS with 5 mmol/L EGTA, stained with X-gal for 4 hours at 37°C, and then embedded in paraffin. For vascular reactivity measurement, arteries were removed, rinsed, and placed immediately in ice-cold physiological saline solution (see below).

**Histochemistry, Immunohistochemical Staining, and Fluorescence Microscopy**

Sections of paraffin-embedded arteries were stained with Verhoeff–van Gieson stain for planimetry of intimal and medial areas. Serial frozen sections were stained with antibodies to macrophages (RAM-11, 1:50 dilution, Dako), vascular cell adhesion molecule-1 (VCAM-1), or intercellular adhesion molecule-1 (ICAM-1) (Rab1/9 and Rab2/3, respectively, at 1:50 dilutions; from Dr Myron Cybulsky, University of Toronto). Bound antibodies were detected with the Vectastain ABC kit (PK-4002, Vector Laboratories) and aminothiol carbazol substrate (Invitrogen). The specificity of primary antibodies was confirmed by substituting an isotype-matched primary antibody (eBiosciences). Lipid was visualized by Oil Red O staining of frozen sections. GFP expression was detected by fluorescence microscopy of frozen sections. β-Galactosidase expression was detected by light microscopic examination of nuclear fast counterstained sections.

**Histological and Morphometric Analyses**

Planimetry of intimal and medial areas was performed with ImagePro Plus 5.0 (Bethesda, MD). At least 4 evenly spaced sections from each artery were measured, and mean areas were computed. 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. Percentages of intimal RAM-11 and Oil Red O staining were calculated by dividing the stained areas by the total intimal area. The intensity of antibody staining for VCAM-1 and ICAM-1 was graded semiquantitatively using the following scale: 0, no staining; 1, rare positive cells or staining barely visible at low power (×100); 2, focal staining or faint diffuse staining clearly visible at low power; 3, multifocal staining or moderate diffuse staining; 4, intense diffuse staining. In most experiments, grading was done by 2 independent observers, whose scores were highly correlated (r2=0.82 for VCAM-1 and r2=0.84 for ICAM-1). The remainder of the scoring was done by a single observer, whose results were highly reproducible (r2>0.8 for 2 independent sets of scores). All observers were blinded to the identity of the specimens.

**RNA Extraction and Quantitative Real-Time PCR**

Total RNA was extracted with the RNeasy Fibrous Tissue Mini Kit (Qiagen). Specific RNA were measured using quantitative reverse transcriptase–mediated (qRT) PCR with an Applied Biosystems 7500 Real-Time PCR System, the Verso 1-step QRT-PCR low ROX mix kit (Thermo Scientific), and 100 ng of RNA template in a 25-μL reaction. A no-template control was included in all experiments, and a no-reverse-transcriptase control was included in many experiments. Baseline levels of cytokine expression were determined by qRT-PCR performed on RNA extracted from unmanipulated arteries of Chow-fed rabbits. RNA levels were calculated using the ΔΔCT method and were normalized to GAPDH mRNA. Primer and probe sequences are in Supplemental Table I, available online at http://atvb.ahajournals.org.

**Plasminogen Activator Activity Assay**

To measure plasminogen activator activity in medium conditioned by explanted arteries, aliquots of conditioned medium were incubated with human Gla-plasminogen (0.87 μmol/L, American Diagnostica) and the plasmin substrate S-2251 (0.8 mmol/L, diaPharma) at 37°C. The change in absorbance at 405 nm was measured, and uPA concentration was calculated by reference to a human scuPA standard (American Diagnostica), with normalization to wet artery weight.
Measurement of Vascular Reactivity

Two weeks after infusion, carotid arteries were excised and connective tissue removed with the aid of a dissecting microscope. Arteries were cut into 3-mm rings and transferred to an organ bath containing physiological saline solution, equilibrated with 95% O2 and 5% CO2. Physiological saline solution contains the following: NaCl, 119 mmol/L; KCl, 4.7 mmol/L; MgSO4, 2.4 mmol/L; KH2PO4, 1.2 mmol/L; CaCl2, 3.3 mmol/L; NaHCO3, 25 mmol/L; EDTA, 0.03 mmol/L; dextrose, 6 mmol/L. Buffer was maintained at 37°C, pH 7.4. Arterial rings were attached to 2 probes in a Multi Wire Myograph System, 610 mol/L (DMT), and the transducer was interfaced to a Powerlab 4/26 recorder for measurement of isometric force. Rings were placed under an initial tension of 20 mN and equilibrated for 1 hour. Ring contraction was measured using phenylephrine hydrochloride (Sigma-Aldrich), and endothelium-dependent and -independent relaxations were measured using acetylcholine and sodium nitroprusside, respectively. Vessel relaxation was expressed as the percentage of contraction induced by phenylephrine.26

Statistical Analysis

Results are reported as mean±SEM. For comparison of 2 groups, we used an unpaired t test for normally distributed data with equal group variances and a Mann-Whitney rank-sum test for other data. Comparison of 3 or more groups (for plasma cholesterol values) was by 1-way ANOVA, with Dunn’s correction for multiple comparisons. Vascular reactivity was analyzed by repeated-measurement 2-way ANOVA. Concentration-response curves were fitted with a nonlinear regression program (GraphPad Prism) to obtain values of maximal effect, which were compared by 1-way ANOVA.

Results

Transgene Expression by HDAd and FGAd in Arteries of Chow-Fed and Cholesterol-Fed Rabbits

Three days after infusion of FGAduPA or HDAduPA in arteries of chow-fed rabbits, uPA mRNA levels in FGAduPA arteries were 10-fold higher than in HDAduPA arteries (Figure 1A; P<0.001). In cholesterol-fed rabbits, the increase in uPA mRNA in FGAduPA versus HDAduPA arteries was far more modest (2-fold; P=0.003). Measurements of plasminogen activator activity in medium conditioned by explanted arteries yielded similar results: plasminogen activator activity was 16-fold higher in medium from FGAduPA versus HDAduPA arteries of chow-fed rabbits but only 3-fold higher in medium from arteries of cholesterol-fed rabbits (Figure 1B; P≤0.006 for both). The smaller difference in transgene expression from FGAduPA versus HDAduPA in arteries of cholesterol-fed versus chow-fed rabbits appeared to result both from reduced expression by FGAduPA (60% to 80% less) and increased expression by HDAduPA (50% to 70% more) in arteries of cholesterol-fed rabbits. By 28 days after gene transfer, uPA expression was significantly higher in HDAduPA than FGAduPA arteries (Figure 1C and 1D), similar to results obtained in chow-fed rabbits.15 Repeat qRT-PCR (in a single batch) of the 3-day and 4-week cholesterol-fed rabbit arteries confirmed relatively persistent transgene expression from HDAduPA (Supplemental Figure I). Differences in transgene expression level and persistence between FGAd and HDAd were not due to transduction of different cell types because FGAd and HDAd both primarily transduce luminal endothelial cells (Supplemental Figure II).

Intimal Growth and Macrophage and Lipid Accumulation Are Increased by FGAd but Not HDAd

Two weeks after infusion of FGAdNull in arteries of cholesterol-fed rabbits, intimal lesions were 3- to 4-fold larger than in control DMEM-infused arteries (Figure 2A; 0.011±0.003 versus 0.003±0.001 mm2; P=0.01). In contrast, HDAdNull infusion did not significantly increase intimal area.
expression of VCAM-1, ICAM-1, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6). Arteries infused with FGAAdNull had significantly higher expression of VCAM-1 than control DMEM-infused arteries (Figure 3A and Supplemental Figure III; \( P<0.01 \)). ICAM-1 expression was also modestly increased by FGAAdNull; however, this increase was not statistically significant (Figure 3B and Supplemental Figure III; \( P<0.1 \)).

HDAdNull-infused arteries of cholesterol-fed rabbits tended to have higher expression of VCAM-1 and ICAM-1 than control DMEM-infused arteries, but these increases were also not statistically significant (Figure 3A and 3B; \( P<0.3 \) and 0.1, respectively).

Compared with unoperated arteries from chow-fed rabbits, expression of the atherogenic cytokines MCP-1, TNF-α, and IL-6 was substantially elevated in arteries harvested from cholesterol-fed, operated rabbits, regardless of whether arteries were infused with DMEM, FGAAdNull, or HDAdNull (6- to 60-fold mean increases; Figure 3C to 3E; \( P<0.05 \) for all). Compared with DMEM-infused arteries, FGAAdNull-infused arteries had modest, statistically insignificant increases in MCP-1 and TNF-α mRNA (1.5- to 2-fold; \( P<0.2 \) and 0.1, respectively). Infusion of HDAdNull significantly increased expression of MCP-1 (2-fold; \( P<0.03 \)) but not TNF-α. IL-6 expression was similar among all 3 groups of infused arteries.

To determine whether HDAd-induced intimal growth and inflammation becomes more evident at a later time point, we infused arteries of cholesterol-fed rabbits with HDAdNull (7.5 \( \times 10^{11} \) vp/mL) or DMEM alone and harvested the arteries 4 weeks later. Compared with DMEM arteries, HDAdNull arteries had larger intimas, a higher percentage of lesion occupied by macrophages and lipid, increased expression of intimal VCAM-1 and ICAM-1, and higher expression of TNF-α mRNA (Figure 4; \( P<0.04 \) for all). These results were striking because we found no appreciable intimal growth in a separate study in which HDAdNull was infused a lower dose (2 \( \times 10^{11} \) vp/mL).\(^{27} \) Therefore, to test the hypothesis that HDAdNull-induced intimal growth at 4 weeks is dose dependent, we cut new sections of arteries both from that study\(^{27} \) and from the present experiment and stained them all in a single batch. Analysis of these sections confirmed that intimal area, macrophage and lipid content, and ICAM-1 and VCAM-1 expression were all increased by the higher dose of HDAd (\( P<0.06 \) to 0.008) but were not significantly increased by the lower dose (\( P<0.3 \) to 0.6; Supplemental Figure IV).

**Similar Vascular Reactivity in Arteries Infused With FGAAd and HDAd**

Vascular reactivity was measured in rings cut from the same arteries in which we measured inflammation, lipid accumulation, and intimal growth. Arteries infused with DMEM, FGAAdNull, or HDAdNull (both at 5 \( \times 10^{11} \) vp/mL) contracted similarly to phenylephrine and relaxed similarly to sodium nitroprusside (Figure 5A and 5B). Arteries of all 3 groups also relaxed significantly in response to acetylcholine (\( P<0.001 \) for acetylcholine dose-responsive relaxation for all 3 groups), confirming the presence of functional endothelium in arteries from all groups (Figure 5C). Across the full range of acetylcholine doses, the responsiveness of the 3 groups of arteries to acetylcholine did not differ significantly (\( P>0.05 \).
by 2-way repeated measures ANOVA). Nevertheless, at higher acetylcholine doses, arteries infused with FGAdNull or HDAdNull relaxed less completely than did DMEM-infused arteries. Comparison of values of maximal effect among the 3 groups revealed less relaxation for both FGAd-Null and HDAdNull-infused arteries compared with DMEM-infused arteries ($P < 0.05$ for both).

**Increased Intimal Growth in HDAd-Infused Arteries 8 Weeks After Infusion or 4 Weeks After Coinfusion of FGAd**

Analysis of arteries harvested 4 weeks after vector infusion (Figure 4 and Supplemental Figure IV) suggested that infusion of HDAd in arteries of cholesterol-fed rabbits causes dose-dependent intimal growth, macrophage and lipid accumulation, and adhesion molecule expression. However, even at the higher dose, the effect of HDAd on intimal growth and inflammation is small compared with effects we found previously in 4-week FGAd arteries.17,18 Relatively limited inflammation and intimal growth in HDAd arteries (especially at $2 \times 10^{11}$ vp/mL) bodes well for HDAd as a clinical and experimental tool. However, there is a problematic aspect to these results. The utility of this cholesterol-fed rabbit carotid artery model for rapidly testing atheroprotective gene therapy depends on its ability to generate a lesion soon after vector infusion. This lesion, which forms in response to the combination of hyperlipidemia and inflammation associated with Ad vector infusion,17 is the target on which atheroprotective gene(s) expressed from the infused vector are tested. If—unlike FGAd-infused arteries—HDAd-infused arteries of cholesterol-fed rabbits form only small intimal lesions, it will be difficult to use this model to test the efficacy of HDAd-expressed atheroprotective transgenes.

We therefore formally compared FGAd and HDAd (both at $7.5 \times 10^{11}$ vp/mL) for their ability to induce lesions at 4 weeks, and we tested whether HDAd-induced lesions would become larger by 8 weeks. Mean cholesterol levels in the 4 weeks groups did not differ significantly either at the time of DMEM/Ad infusion or at the time of harvest (Supplemental Table II and data not shown). Intimal area was significantly increased by FGAdNull compared with both DMEM and HDAdNull (0.067 ± 0.015 versus 0.017 ± 0.004 mm² and 0.029 ± 0.008 mm² for DMEM and HDAdNull-infused arteries, respectively; $P < 0.001$ and 0.01; Figure 6). By 8 weeks after infusion, intimal areas of HDAdNull arteries had increased (0.077 ± 0.024 mm²; $P = 0.04$ versus 4-week HDAdNull) and were similar to intimas of 4-week FGAdNull arteries. Intimal growth in 8-week HDAdNull arteries was not due only to prolonged cholesterol feeding and the surgical procedure because DMEM-infused carotid arteries of hyperlipidemic rabbits have virtually no intimal growth 8 weeks after infusion (Figure 6).

To enable testing of HDAd-expressed atheroprotective genes within 4 weeks of vector infusion, we tested whether coinfusion of FGAdNull ($7.5 \times 10^{11}$ vp/mL) with an HDAd (HDAdNull, $2.0 \times 10^{11}$ vp/mL) would accelerate intimal growth at 4 weeks. This experiment is based on our previous work showing that human-like early atherosclerotic lesions form in arteries of hyperlipidemic rabbits infused with FGAdNull.17 Intimal areas of the coinfused arteries (Figure 6) were similar to intimas of 4-week FGAdNull arteries (3-fold; $P = 0.002$).

**Transgene Expression in Arteries Coinfused With FGAdNull**

We next asked whether coinfusion of an HDAd with FGAdNull would cause rapid loss of HDAd-mediated gene expres-
sion, as reported by others in a hepatic gene transfer model.13 Chow-fed rabbits were used for this experiment. As expected,15 when HDAd-mediated uPA expression was measured 3 days after infusion (HDAduPA was infused at $2.0 \times 10^{11}$ vp/mL), coinfusion of FGAdNull ($7.5 \times 10^{11}$ vp/mL) increased uPA expression 20- to 30-fold at both RNA and protein levels (Supplemental Figure V). After 4 weeks, uPA expression in HDAduPA arteries coinfused with FGAdNull decreased substantially but was still detectable above background ($P = 0.04$ for uPA mRNA and $P = 0.01$ for uPA activity versus control, HDAdNull-infused arteries). Moreover, at 4 weeks, both uPA mRNA and activity were higher in HDAduPA arteries coinfused with FGAdNull than in HDAduPA arteries coinfused with HDAdNull. Although these increases were not statistically significant ($P = 0.09$), the higher levels of uPA mRNA and activity in FGAdNull-infused arteries do not support the hypothesis that coinfusion of FGAdNull will cause rapid loss of HDAd-mediated transgene expression.

Discussion

We tested the performance of HDAd in a rabbit model of early atherosclerosis. Our major findings were that (1) HDAd expresses a transgene at least as well in atherosclerosis-prone arteries as it does in normal arteries; (2) transgene expression from HDAd is more durable than that from FGAd; (3) HDAd stimulates less intimal growth, lipid and macrophage accumulation, and VCAM-1 expression than FGAd; (4) HDAd induces only minimal lesion formation and inflammation, especially when it is infused at a submaximal dose; (5) both FGAd and HDAd have similar, relatively minor effects on vascular reactivity; and (6) limited intimal growth in HDAd-infused arteries may decrease the utility of this animal model for testing atheroprotective genes expressed by HDAd; how-

Figure 4. High-concentration HDAd increases intimal growth, lipid accumulation, and artery wall inflammation 4 weeks after infusion. Rabbits were fed a cholesterol-enriched diet for 1 month, and their carotid arteries infused with either DMEM or HDAdNull ($7.5 \times 10^{11}$ vp/mL) and harvested 4 weeks later. A, Intimal area. B, Percentage of Oil Red O (ORO)-positive area in the intima. C, Percentage of RAM-11-positive area in the intima. D and E, VCAM-1 and ICAM-1 expression using semiquantitative immunostaining. F to H, Expression of MCP-1, TNF-α, and IL-6, respectively, using qRT-PCR. Data points are from individual arteries; bars indicate group means.
ever, this limitation may be circumvented by examining later time points or by “spiking” HDAd with FGAd. Our results support the utility of HDAd as a tool for investigation and treatment of atherosclerosis and other vascular diseases.

To be useful for investigating, preventing, and treating atherosclerosis, a vector must be able to express transgenes at high levels in arteries that are at risk for developing atherosclerosis. An earlier in vivo study reported lower transgene expression from FGAd in atherosclerotic versus nonatherosclerotic balloon-injured rabbit arteries; however, a subsequent ex vivo study reported increased expression from FGAd in atherosclerotic versus normal rabbit arteries. In the present study, HDAd expressed at least as much transgene mRNA and protein in atherosclerosis-prone as in normal arteries (Figure 1). Our results are therefore similar to the ex vivo results cited above, potentially because in both this earlier study and the present study the transduced cells were endothelial cells and the vectors contained the CMV promoter. In contrast, in the in vivo study the transduced cells were likely smooth muscle cells and the vector contained a different promoter.

The relatively robust expression of HDAd in atherosclerosis-prone versus normal arteries (Figure 1) is encouraging; however, as we found earlier, HDAd-mediated transgene expression was initially far less than that obtained with the same cassette in FGAd (Figure 1). Increased expression from FGAd is likely due to upregulation of the CMV promoter by FGAd-stimulated, host-derived inflammatory factors because HDAd expression is increased in vivo (but not in vitro) by codelivery of FGAd (Supplemental Figure V). It was possible that vascular inflammation in cholesterol-fed rabbits would upregulate HDAd expression as effectively as FGAd; however, it did not (Figure 1). Achievement of higher expression from HDAd in this model will likely require new expression cassettes that function independently of local inflammatory stimuli.

The most important and encouraging findings of this study are that in arteries of hyperlipidemic rabbits (a setting that accentuates pathogenic effects of FGAd), HDAd causes less intimal growth, lipid and macrophage accumulation, and VCAM-1 expression than FGAd (Figures 2 and 3 and Supplemental Figure III). Moreover, when infused at a submaximal dose (2 × 10^11 vp/mL, a dose that yields near-maximal transgene expression) HDAd causes little if any significant arterial pathology (Supplemental Figure IV). Therefore, HDAd is both a “cleaner” experimental tool than FGAd and a safer vector for gene therapy. Improved performance of HDAd in this model is likely due to deletion of all viral genes in HDAd, which conceals transduced cells from the adaptive immune system and avoids cellular toxicities of viral gene products.

For HDAd to serve as an optimal investigational and therapeutic tool, it should have minimal effects on vasomotor...
function. In chow-fed rabbits, several groups reported that FGAd impairs endothelial-dependent relaxation.\textsuperscript{6,31,32} In cholesterol-fed rabbits, a single study showed baseline, diet-related impairment of endothelial-dependent relaxation that was not worsened by FGAd infusion.\textsuperscript{33} This impairment was, however, reversed by infusion of an FGAd expressing endothelial nitric oxide synthase. In the present study, also performed in cholesterol-fed rabbits, both FGAd and HDAd reduced maximal endothelial-mediated dilation (Figure 5). This result suggests that the Ad capsid (common to FGAd and HDAd) impairs endothelial-mediated vasodilation, consistent with a report of impaired endothelial-mediated vasodilation only 6 hours after FGAd infusion.\textsuperscript{9} We considered why in our study, in contrast to the study in cholesterol-fed rabbits cited above,\textsuperscript{33} both FGAd and HDAd decreased maximal endothelial-dependent relaxation. This inconsistency might be because in the present study, a shorter duration of cholesterol feeding (6 versus 11 to 12 weeks) and a lower percentage of dietary cholesterol (0.25% versus 0.5 to 1.0%) caused less impairment of endothelial function. With less diet-related impairment of endothelial function, a detrimental effect of Ad on endothelial function may be unmasked. The apparent inhibitory effect of HDAd on maximal endothelium-mediated vasodilation (Figure 5) seems to be a limitation shared by both HDAd and FGAd and could likely be avoided either by lowering the vector dose or by including an endothelial nitric oxide synthase expression cassette in the vectors.\textsuperscript{6,33}

The lack of significant intimal lesion growth after HDAd infusion at a submaximal dose ($2 \times 10^{11}$ vp/mL; Supplemental Figure IV) and the relatively small lesions in 2 to 4-week arteries infused at a higher dose ($7.5 \times 10^{11}$ vp/mL; Figures 2, 4, and 6) bode well for HDAd as an investigational and therapeutic tool. However, this result has a problematic aspect because the intimal lesions that form in this model after Ad infusion are the substrate on which Ad-encoded transgenes are tested for atherogenic or atheroprotective activities.\textsuperscript{17,18} Atherogenic activities of transgenes expressed from HDAd will likely still be revealed because they would increase lesion growth above a low background; however, atheroprotective activities of transgenes might not be detected. Fortunately, we found that both continuing the experiment for an additional 4 weeks and spiking HDAd with FGAdNull increased intimal lesion size (Figure 6). Both approaches improve the suitability of this model for detecting atheroprotective transgene effects.

In conclusion, in atherosclerosis-prone arteries, HDAd is superior to FGAd for both experimental and therapeutic purposes because it expresses transgenes more durably than FGAd (Figure 1 and Supplemental Figure I) and causes less lesion growth, lipid and macrophage accumulation, and vascular inflammation (Figures 2, 3, and 6). Future work will be directed at addressing the remaining limitations of HDAd for vascular gene transfer, including an initially low level of transgene expression, mild impairment of maximal endothelium-mediated vasodilation, and possible atherogenic effects at later time points.

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Disclosures
None.

References


Helper-Dependent Adenovirus Is Superior to First-Generation Adenovirus for Expressing Transgenes in Atherosclerosis-Prone Arteries
Bo Jiang, Kun Qian, Liang Du, Ian Luttrell, Kanchan Chitaley and David A. Dichek
Supplemental Figure Legends

**Figure I.** Relatively persistent transgene expression from HDAd. Arteries of cholesterol-fed rabbits were infused with either FGAduPA or HDAduPA (both at $7.5 \times 10^{11}$ vp/mL) and harvested 3 d or 4 wk later. uPA mRNA was measured in arterial extracts by quantitative RT-PCR. Results are expressed as arbitrary units (AU), with the mean of the group with the lowest expression (FGAduPA at 4 wk) assigned a value of 1. Data points are from individual arteries; bars are group means.

**Figure II.** Transgene expression in arteries infused with FGAd or HDAd. FGAdCMVnLacZ (A and B) or HDAdGFP (C and D) were infused in arteries of either chow-fed (A, C) or cholesterol-fed (B, D) rabbits and harvested 3 d later. Sections of HDAdGFP arteries were viewed with fluorescence microscopy. FGAdCMVnLacZ arteries were stained with X-gal, embedded, sectioned, and counterstained with nuclear fast red. Transgene expression is primarily in luminal endothelium. Limited transgene expression in the adventitia (arrows) is likely due to leakage of vector during the surgical procedure.

**Figure III.** Histochemical and immunohistochemical detection of lipid, macrophages, and adhesion molecule expression in arteries of hyperlipidemic rabbits. Arteries were infused with DMEM, FGAdNull, or HDAdNull (both at $7.5 \times 10^{11}$ vp/mL) and harvested 14 d later. Lipid content was detected with oil red O staining. Sections were stained for expression of RAM-11 (to identify macrophages), ICAM-1, or VCAM-1.

**Figure IV.** Infusion of high dose ($7.5 \times 10^{11}$ vp/mL), but not low dose ($2 \times 10^{11}$ vp/mL) HDAdNull causes intimal growth, accumulation of lipid and macrophages, and expression of adhesion molecules in carotid arteries of cholesterol-fed rabbits. Arteries were harvested 4 wk after infusion of DMEM or HDAdNull. A, Intimal area; B, Percentage of oil red O-stained area.
in the intima; C, Percentage of RAM-11-stained area in the intima; D and E, semiquantitative
immunohistochemistry for intimal expression of VCAM-1 and ICAM-1.

**Figure V.** Co-infusion of FGAd increases HDAd-mediated transgene expression at 3 d and does
not expedite loss of HDAd expression. Arteries of chow-fed rabbits were infused with the
indicated vectors alone or in combination and were harvested either 3d or 4 wk later. A, uPA
mRNA (expressed by HDAduPA) was measured by quantitative RT-PCR of artery RNA and
expressed as arbitrary units (AU). B, Plasminogen activator (PA) activity was measured in
medium conditioned by arteries explanted 3 d or 4 wk after vector infusion. PA activity (IU)
was normalized to wet artery weight.
Figure I

The figure shows a scatter plot comparing the levels of uPA/GAPDH (AU) in FGuPA and HDuPA at 3d and 4wk.

- At 3d, the comparison between FGuPA and HDuPA yields a P-value of 0.08.
- At 4wk, the comparison between FGuPA and HDuPA yields a P-value of 0.002.
Figure II

A

B

C

D
Figure III

<table>
<thead>
<tr>
<th></th>
<th>DMEM</th>
<th>FGAdNull</th>
<th>HDAdNull</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid (oil red O)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Macrophage (RAM-11)</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>VCAM-1</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>ICAM-1</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>Gene</td>
<td>Primers</td>
<td>Probe</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>GTGAAGAGGCTAATGAGCTATAGAAGAA</td>
<td>CAACACGACCAAGTGTCAC</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGCTGCACTTCAGGGTGATC</td>
<td>CCCTCAGGAGGAAGAGTC</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>GTCCTTGCTTGCGGAATTTC</td>
<td>TGGGCTCTGCTGCCAGGTC</td>
<td></td>
</tr>
<tr>
<td>uPA</td>
<td>TACGAAAACATACCATGCCCCA</td>
<td>CACAATTACTGCAGGAACCCAGGACCA</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>TCATTGACCTCCACTACATGGTCTA</td>
<td>TCCAGTATGATTCCCACCGGCA</td>
<td></td>
</tr>
</tbody>
</table>

**Table I. Primers and probes for qRT-PCR**
**Table II. Plasma cholesterol levels at time of gene-transfer surgery**

Experiments examining intimal growth, vascular inflammation, and vessel reactivity 2 wk after gene-transfer surgery (Figures 2, 3 and 5; n = 6 per group)

<table>
<thead>
<tr>
<th>Infusate</th>
<th>DMEM</th>
<th>FGAdNull</th>
<th>HDAdNull</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>298 ± 79</td>
<td>403 ± 160</td>
<td>351 ± 68</td>
</tr>
</tbody>
</table>

Experiments examining intimal growth at 4 or 8 wk after gene-transfer surgery (Figure 6; n = 5 – 8 per group)*

<table>
<thead>
<tr>
<th>Infusate</th>
<th>DMEM</th>
<th>FGAdNull</th>
<th>HDAdNull</th>
<th>HDAdNull</th>
<th>FGAd+HDAd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of harvest</td>
<td>4 wk</td>
<td>4 wk</td>
<td>4 wk</td>
<td>8 wk</td>
<td>4 wk</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>266 ± 34</td>
<td>284 ± 36</td>
<td>285 ± 35</td>
<td>333 ± 52</td>
<td>442 ± 104</td>
</tr>
</tbody>
</table>

Experiments examining effects of low dose and high dose of HDAd at 4 wk after gene-transfer surgery (Figure 4; n= 4 – 6 per group)

<table>
<thead>
<tr>
<th>Infusate</th>
<th>DMEM</th>
<th>HDAdNull</th>
<th>HDAdNull</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(2 × 10^{11} vp/mL)</td>
<td>(7.5 × 10^{11} vp/mL)</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>469 ± 170</td>
<td>500 ± 129</td>
<td>505 ± 126</td>
</tr>
</tbody>
</table>

*Primary data for the DMEM 8 wk group are not available; however, at the time these rabbits were operated, cholesterol levels of 400 – 700 mg/dl were required for entry to the study (see reference 17).