Efficient Gene Transfer and Durable Transgene Expression in Grafted Rabbit Veins

Liang Du,^{1,*} Jingwan Zhang,^{1,*} Alexander W. Clowes,² and David A. Dichek¹

Abstract

Venous bypass grafts are useful treatments for obstructive coronary artery disease. However, their usefulness is limited by accelerated atherosclerosis. Genetic engineering of venous bypass grafts that prevented atherosclerosis could improve long-term graft patency and clinical outcomes. We used a rabbit model of jugular vein-to-carotid interposition grafting to develop gene therapy for vein-graft atherosclerosis. Rabbit veins were easily transduced *in situ* with a first-generation adenoviral vector; however, most transgene expression (~80%) was lost within 3 days after arterial grafting. This rapid loss of transgene expression was not prevented by transducing veins after grafting or by prolonged *ex vivo* transduction. However, delaying vein-graft transduction for 28 days (after the vein had adapted to the arterial circulation) prevented this early loss of transgene expression. We used the delayed transduction approach to test the durability of expression of a therapeutic transgene (apolipoprotein A-I) expressed from a helper-dependent adenoviral (HDAd) vector. HDAd DNA and apolipoprotein A-I mRNA were easily detectable in transduced vein grafts. Vector DNA and mRNA declined by 4 weeks, and then persisted stably for at least 6 months. Delaying transduction for 28 days after grafting permitted initiation of vein-graft neointimal growth and medial thickening before gene transfer. However, vein-graft lumen diameter was not compromised, because of gradual outward remodeling of grafted veins. Our data highlight the promise of HDAd-mediated gene therapy, delivered to arterialized vein grafts, for preventing vein-graft atherosclerosis.

Introduction

DESPITE ADVANCES in the medical treatment of atherosclerosis and the development of percutaneous coronary interventions, coronary artery bypass grafting (CABG) remains a mainstay in the treatment of coronary artery disease. More than 200,000 patients underwent CABG in the United States in 2010,¹ and studies that compare CABG with percutaneous coronary intervention continue to show advantages for CABG in important patient groups, especially those with complex multivessel disease.^{2–4} Nevertheless, the clinical benefits of CABG are limited by bypass graft occlusion leading to recurrent ischemia, infarction, and death.⁵

Arterial-derived coronary artery bypass grafts, usually constructed with the left internal mammary artery, have excellent long-term patency rates (>95% after 10 years).⁶ Other arterial-derived conduits are less well proven. For this reason—and because CABG is now used largely to treat multivessel coronary disease⁴—virtually all modern CABG operations include placement of at least one aortocoronary saphenous vein graft (SVG).⁷ SVG patency is inferior to that

of arterial conduits, and is estimated at 80–90% at 1 year and only 50% at 10 years.^{5,8} Despite evidence that both aspirin and lipid-lowering therapy improve SVG patency^{9,10}—as well as widespread implementation of these therapies⁷—there is no evidence that SVG patency rates have increased since 1968.¹¹ One study suggested that modern SVG patency rates may be decreasing (~55% by 18 months) and confirmed that loss of SVG patency is associated with poor clinical outcomes.¹² Moreover, SVG stenosis and occlusion are difficult to treat: percutaneous intervention is often unsuccessful,¹³ and the morbidity of both percutaneous intervention and redo CABG is high.¹⁴ For these reasons, interventions that improve SVG patency are needed.

Gene therapy is an attractive approach for improving SVG patency because it offers the possibility to treat a vein segment (potentially *ex vivo*) before it is grafted into the arterial circulation.^{11,15} Delivery of a durable biological therapy at the time of vein grafting might prevent the processes that cause SVG occlusion (thrombosis, intimal hyperplasia, and accelerated atherosclerosis).^{5,16,17} However, because SVG disease (especially atherosclerosis) develops

¹Department of Medicine/Cardiology and ²Department of Surgery, University of Washington, Seattle, WA 98195.

*These authors contributed equally.

over many years, optimal SVG gene therapy will likely require a vector that can express a therapeutic transgene in blood vessels for years. Until recently, no such vector had been described. We have shown that helper-dependent adenovirus (HDAd) can express a therapeutic transgene in rabbit arteries for at least 48 weeks, with stable expression from 4 to 48 weeks.¹⁸ HDAd is therefore a promising vector for use in developing gene therapy for SVG disease. Here we report experiments that test whether HDAd can achieve long-term, stable transgene expression in rabbit vein grafts.

Materials and Methods

Adenoviral vectors

FGAdCMVnLacZ is a first-generation E1/E3-deleted vector that expresses a β -galactosidase transgene.¹⁹ HDAd-gApoAI and HDAdNull are third-generation or "helper-dependent" adenoviral vectors that lack all viral genes.^{18,20,21} Vectors were amplified in 293 cells (FGAdCMVnLacZ) or (for HDAd) 293Cre4 cells²² as described²³ and purified by CsCl ultracentrifugation. We used a single preparation of FGAdCMVnLacZ (1.2×10^{13} viral particles [VP]/ml; measured by spectrophotometry).²⁴ The HDAd preparations ranged from 5×10^{11} to 2×10^{12} VP/ml. E1A-containing genomes and helper-virus contamination (for HDAd) were measured by quantitative PCR (qPCR), with reference to standard curves constructed with plasmid or viral DNA containing the target sequences. Primers and probes were as reported.²⁵ Helper virus contamination was $\leq 1\%$ in the HDAd preparations, and E1A copies were <1 in 10^6 vector genomes for all preparations. All vectors were diluted to 2×10^{11} VP/ml with Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) for both intravascular infusion and *ex vivo* transduction.

Animal surgeries

Fifty-nine male New Zealand White rabbits (3–3.5 kg; Western Oregon Rabbit Company, Philomath, OR) were assigned to six experimental groups and fed normal chow. All animal studies were approved by the University of Washington (Seattle, WA) Office of Animal Welfare. Gene transfer and vein-grafting procedures were carried out under general anesthesia (induction with ketamine [30 mg/kg] and xylazine [3 mg/kg] and maintenance with inhaled isoflurane [1–3%]). In general, approximately 3 cm of both external jugular veins and both common carotid arteries were exposed through a vertical midline incision and mobilized by gentle dissection and ligation of branches. After administration of intravenous heparin (500 units), group-specific surgical procedures were carried out. In all cases, vector was incubated in the vein lumen for 20 min, and then removed by aspiration.

Rabbits in the "Cuffed Anastomosis" group ($n=6$) received bilateral external jugular vein infusions of FGAdCMVnLacZ. Briefly, a segment of the left external jugular vein (approximately 3 cm long) was isolated between vascular clamps. A venotomy was made with a bent 19-gauge needle and blood was flushed from the vein lumen by injection of DMEM through a syringe and plastic catheter followed by gentle milking of the vein contents toward the venotomy. The lumen was then filled with FGAdCMVnLacZ and distended to a normal physiological caliber. After 20 min, the vector so-

lution was aspirated, the venotomy was repaired with a 7-0 polypropylene suture, blood flow was restored, and the vein was left *in situ*. This gene transfer procedure was also performed for the right external jugular vein. After transduction, the right jugular vein segment was excised and placed as an end-to-side interposition graft in the ipsilateral common carotid artery. The vein-to-artery anastomoses were constructed with two polymer cuffs (1–1.5 mm long) that were crafted from a 4-Fr introducer sheath (Terumo Medical, Elkton, MD). Each end of the transduced vein segment was pulled through a cuff, everted, and fixed with 8-0 silk suture. A segment of right carotid artery was isolated from the circulation, using vascular clamps, and a longitudinal arteriotomy (2-cm length) was made with scissors. The cuffed vein was anastomosed in reverse orientation with an 8-0 silk suture, essentially as described.²⁶

Rabbits in the "Sewn Anastomosis" group ($n=6$) underwent the same procedures described for the Cuffed Anastomosis group except that the transduced right jugular vein segment was sutured directly into the right carotid artery as a reversed end-to-side graft, using interrupted 7-0 polypropylene sutures, essentially as described.²⁷ Transduced but ungrafted veins in these initial Sewn Anastomosis and Cuffed Anastomosis groups are referred to as group I "Ungrafted" veins and the grafted veins in these two groups are referred to as group II "Transduced then Grafted" veins.

Rabbits in group III (the "Grafted then Transduced" group; $n=7$) underwent carotid interposition grafting of an untransduced right external jugular vein segment, using the same surgical procedure described for the Sewn Anastomosis veins. Immediately after completion of the anastomoses, the grafted vein segment was transduced with FGAdCMVnLacZ by infusing the vector solution via an arteriotomy in the caudal right carotid. The first rabbit in this group had a clotted graft at harvest. The remaining six grafts were patent.

Rabbits in group IV (the "Overnight Transduction" group; $n=12$) underwent two survival surgeries: one for vein harvest and a second for vein grafting. One rabbit died during the second surgery and was therefore excluded. At the first surgery, segments of either the right ($n=7$ rabbits) or both external jugular veins ($n=4$ rabbits) were excised, the incision closed, and the rabbit allowed to recover and then returned to its cage. Excised vein segments ($n=15$) were incubated overnight with FGAdCMVnLacZ (2×10^{11} VP/ml in serum-free DMEM) in a tissue culture incubator (37°C, 5% CO₂). The next day the rabbit was reanesthetized, the neck incision opened, and the transduced vein segment(s) placed as end-to-side interposition grafts either to the right carotid artery only ($n=7$ rabbits) or to both common carotid arteries ($n=4$ rabbits), using sewn anastomoses. All 15 grafts were patent at harvest.

Rabbits in group V (the "Delayed Transduction" group; $n=7$) also underwent two survival surgeries. During the first surgery, a segment of right external jugular vein was excised and placed as an end-to-side interposition graft to the right carotid artery, using the sewn anastomosis technique. Four weeks later, each rabbit was anesthetized, the neck was opened, and the grafted vein segment was transduced by infusion of FGAdCMVnLacZ via a caudal arteriotomy. During this surgery, a segment of left external jugular vein was also transduced with FGAdCMVnLacZ, using

procedures described previously, and left *in situ*. All rabbits survived with both of their transduced veins patent.

Twenty-one rabbits were enrolled in a time course study and infused with one of the HDAd vectors or DMEM. Two rabbits died during the grafting surgery. Details for the remaining 19 rabbits are given below.

Harvest and processing of vein segments transduced with FGAdCMVnLacZ

All FGAdCMVnLacZ-transduced veins were harvested 3 days after transduction. The third day after transduction was also 3 days after grafting in groups I–IV, but was 4.5 weeks after grafting in group V. Excised veins were rinsed with DMEM and cut transversely into three segments of equal length. The middle segment was snap-frozen in liquid nitrogen. The cranial and caudal segments were fixed with 10% formalin for 30 min and stained with 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-Gal) for 30 min to detect β -galactosidase expression (from FGAdCMVnLacZ). The cranial segments were opened longitudinally, pinned to wax boards (lumen up), and imaged next to a ruler. The caudal segments were stored in 70% ethanol, processed into paraffin, and sectioned. For each paraffin-embedded vein segment, three or four sets of 6- μ m-thick serial sections were collected along the vein at steps of at least 100 μ m, covering 200–300 μ m along each vein segment.

Planimetry and histology of vein segments transduced with FGAdCMVnLacZ

Pinned vein segments were photographed with a Leica DFC295 image system (Leica Microsystems, Buffalo Grove, IL). Blue (X-Gal-stained) and total surface areas were measured by computer-assisted color thresholding (Image-Pro 5.0; Media Cybernetics, Rockville, MD). The percentage blue surface area was calculated by dividing the blue area by total area. Serial sections taken at ≥ 100 - μ m steps were stained with hematoxylin and eosin (H&E) and nuclear fast red (Vector Laboratories, Burlingame, CA). Step sections from groups III and V were also stained with Verhoeff–Van Gieson (VVG) stain. Stained sections were examined by an observer blinded to group identity. The location (luminal, medial, or adventitial) and quantity of X-Gal-stained blue cells were assessed semiquantitatively, using nuclear fast red-stained sections. Intimal, medial, and luminal areas were measured/calculated using H&E or VVG-stained sections. The intimal area of group V veins was measured directly on three or four step sections per vein, using computer-assisted planimetry. The medial area of group I–V veins was calculated by measuring circumferences at the intimal–medial and medial–adventitial borders, calculating the area within both circumferences (assuming circular geometry *in vivo* and using the formula $\text{Area} = C^2/4\pi$) and subtracting the area within the intimal–medial circumference from area within the medial–adventitial circumference. Luminal area was calculated, using the same sections, by measuring the luminal circumference and using the formula $\text{Area} = C^2/4\pi$. Medial area measurements were easily made on the majority of group I (ungrafted) and group V (delayed transduction) veins. Sections of most of the group II, III, and IV veins (all grafted only 3 days before harvest) could not be used for measurement of medial area either because the sections

were fragmented or because the medial–adventitial border could not be identified with confidence. Sections of several group I and group II veins were stained with an antibody to CD31 (Dako, Carpinteria, CA),²⁸ to identify endothelial cells.

Quantification of LacZ mRNA

Frozen vein segments were pulverized in liquid nitrogen and total RNA was extracted (RNeasy; Qiagen Sciences, Germantown, MD) and quantified by spectrophotometry (NanoDrop; Thermo Scientific, Wilmington, DE). After treatment with DNase I (Thermo Scientific), LacZ mRNA was measured by quantitative reverse transcriptase-mediated PCR amplification, using 50 ng of RNA as a template. Primers were ATCAGGATATGTGGCGGATG (forward) and TGCATAAACCGACTACACAAATCA (reverse), and the probe sequence was CGGCATTTTCCGTGACGTCTCGTT. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA measured in the same extracts.¹⁸ At the time of harvest, the walls of the group V (Delayed Transduction) veins were noticeably thicker than walls of veins in the other groups. Increased wall thickness (and cellularity) is expected in veins exposed to the arterial circulation for 1 month versus 3 days,^{26,29} and was confirmed by histology (see below). Consistent with this increased cellularity, RNA yields from group V grafted veins were higher than RNA yields from external jugular veins that were transduced (but not grafted) and harvested 3 days later (4.6-fold higher; similar to the 5-fold increase in DNA reported by others in 1-month vein grafts).²⁹ This hyperplasia—and accompanying cellular RNA—reduces the proportion of LacZ mRNA in 50 ng of total vein mRNA and if not accounted for would lead to a relative underestimate of LacZ mRNA in group V veins. To correct for this, we multiplied the LacZ mRNA measurements in group V veins by 4.6.

Time dependence of ex vivo transduction

Segments of untransduced internal jugular vein were removed from three rabbits in group IV at the end of their terminal surgeries. The segments were each further cut into 12 or 13 pieces 2–3 mm in length. The pieces were equilibrated in DMEM with 10% autologous rabbit serum in an incubator at 37°C and 5% CO₂ for 1 hr, and then incubated with FGAdCMVnLacZ vector (2×10^{11} VP/ml) for intervals of 20 min, 60 min, 120 min, or 24 hr. At each time point, three or four pieces (at least one piece from each rabbit) were removed from the vector solution and placed in DMEM with 10% fetal bovine serum (FBS) for 24 hr. The pieces were then fixed, stained with X-Gal, and pinned on a white wax board with the luminal surface up. The percentage blue surface area was calculated by color thresholding and planimetry, as described previously.

Duration of transgene expression from veins transduced with HDAd

We used the delayed transduction method for a time course study that investigated the persistence of HDAd vector DNA and HDAd-mediated transgene expression in grafted veins. Grafted veins were infused with HDAdgApoAI (11 rabbits), HDAdNull (6 rabbits), or DMEM (2 rabbits) and harvested 3 days, 4 weeks, 12 weeks, or 24 weeks later. Infusions were

done via an arteriotomy in the adjacent carotid wall. Other aspects of the gene transfer and surgery protocols were as described for group V. At harvest, vascular clamps were placed on the carotid adjacent to the vein graft and arteriotomies were made between the clamps and the anastomoses. DMEM (3 ml) was used to rinse the lumen and to remove residual blood. The vein was then excised and divided into five pieces of equal length. Segments 2 and 4 were fixed with 4% paraformaldehyde for 24 hr, placed in 70% ethanol, and then processed into paraffin. Segments 1, 3, and 5 were divided longitudinally into two equal pieces. One of the two pieces was placed in RNAlater (Life Technologies) for 24 hr, and then trimmed to remove the adventitia. Trimmed pieces were stored at -80°C , and then pulverized in liquid nitrogen to extract RNA. RNA was extracted with the RNeasy kit (Qiagen) and quantified with the NanoDrop spectrophotometer. apoA-I mRNA was measured by quantitative reverse transcriptase-mediated PCR, as described.¹⁸ The other halves of pieces 1, 3, and 5 were snap-frozen and stored at -80°C . They were later thawed, the adventitia was trimmed, and DNA was extracted with the DNeasy kit (Qiagen). Vector copy number was measured by qPCR amplification of noncoding HDAd “stuffer” DNA, as described.¹⁸ A “background” qPCR signal (above the signal in the no-template or no-reverse transcriptase controls) was detected in DNA from veins infused with DMEM. The highest qPCR signal obtained from DNA extracted from a DMEM-infused vein was used to define the “no-vector” background in this assay and DNA from an HDAd-infused vein segment was considered “positive” for vector DNA only if a signal above this background level was detected.

Planimetry and histology of vein segments transduced with HDAd

As described previously, two segments from each of the veins transduced with HDAdgApoAI or HDAdNull were processed into paraffin blocks. Four 6- μm -thick sections of each segment (at approximately 170- μm steps) were stained with H&E and used for planimetry. One segment in each of 2 veins (of 33 total veins) contained valves; these were not used for planimetry. If the medial–adventitial border was indistinct on the H&E-stained slides, another set of sections (four per vein segment; separated by the same step distance as the H&E-stained slides) was stained with VVG and used for planimetry. Digital images and computer-assisted planimetry were used to measure circumferences of the lumen, the intimal–medial border, and the medial–adventitial border. Assuming circular geometry *in vivo*, these circumferences were used (as described previously) to calculate lumen area, intimal area, and medial area. Planimetry was done on three to eight sections per vein, with seven or eight sections for most veins.

Statistics

Unless otherwise indicated, data are presented as means $\pm$ SD. Two groups were compared by unpaired *t* test when data were normally distributed and group variances were equal. The Mann–Whitney rank-sum test was used when these conditions were not met. More than two groups were compared by one-way analysis of variance (ANOVA) when data were normally distributed with equal group variances and by Kruskal–Wallis

ANOVA when these conditions were not met. Statistical analyses were performed with the SigmaStat program (Systat, San Jose, CA). $p < 0.05$ was considered significant.

Results

Transgene expression in veins is rapidly lost after grafting

We first tested whether rabbit jugular veins could be efficiently transduced *in situ* and whether grafting into the arterial circulation affected transgene expression. We transduced the left and right external jugular veins of 12 rabbits with a first-generation adenoviral vector, FGAdCMVn-LacZ.¹⁹ After transduction, the left vein was left in place (group I: Ungrafted) and the right vein was placed as an end-to-side carotid interposition graft (group II: Transduced then Grafted). The vein graft anastomoses were constructed either with a synthetic cuff (Cuffed Anastomosis; $n=6$) or by direct sewing (Sewn Anastomosis; $n=6$). Three days after transduction, all transduced veins (both grafted and ungrafted) were patent. All veins in group I (Ungrafted) had easily measurable LacZ transgene expression (Fig. 1A and B). Blue surface area (indicating nuclei of transduced cells) averaged 20–30% (Fig. 1C and D), consistent with efficient *in vivo* gene transfer. LacZ gene expression was significantly lower in grafted versus ungrafted veins (84 and 81% decreases vs. ungrafted veins for cuffed and sewn anastomosis, respectively; $p=0.004$ for both; Fig. 1A and B). Measurement of percentage blue area on the vein-graft surfaces yielded similar results (73 and 75% decreases in blue surface area compared with ungrafted veins, respectively; $p=0.2$ and 0.02 ; Fig. 1C and D, and Fig. 2A and B). Sections of ungrafted veins showed transduced cells predominantly along the lumen and in the adventitia (likely from leakage during vector infusion), with fewer in the media (Fig. 2C). Compared with ungrafted veins, the grafted veins had fewer transduced cells in all three layers, with loss of transduced cells most pronounced along the luminal surface (Fig. 2D). Cells along the luminal surface of both ungrafted and grafted veins were identified as endothelial cells by CD31 staining (Supplementary Fig. S1; supplementary data are available online at www.liebertpub.com/hum).

Because of the similarity of results with both the cuffed and sewn anastomoses, our observation that a longer segment of vein could be obtained from grafts that were sewn (providing more tissue for analysis), and the higher clinical relevance of sewn anastomoses, we used sewn anastomoses for the remainder of the studies.

Transducing veins after grafting does not increase transgene expression

We hypothesized that transgene expression was lost in grafted veins because of vein manipulation during the grafting procedure that damaged or removed transduced cells. We tested this hypothesis in a new group of rabbits (group III: Grafted then Transduced; $n=7$) by first grafting the veins and then immediately transducing the grafted vein segments (Supplementary Fig. S2). At harvest 3 days later, one graft was thrombosed. This graft was used to measure LacZ mRNA but was neither pinned for planimetry nor sectioned. Measurement of both LacZ mRNA and blue

FIG. 1. Transgene expression in ungrafted and grafted veins. Rabbit external jugular veins were transduced *in situ* with FGAdCMVnLacZ and either left in place (Ungrafted) or placed as carotid interposition grafts (Transduced then Grafted). Grafts were anastomosed to the arterial circulation either by a cuff technique (left) or a sewing technique (right). Three days later the veins were removed and segments were processed for measurement of either (A and B) LacZ mRNA or (C and D) percentage blue luminal surface area coverage, measured after X-Gal staining. Data points represent individual veins; bars represent group means.

surface area revealed uniformly low levels of transgene expression in all group III vein grafts, similar to the low levels in the group II (Transduced then Grafted) grafts ($p \geq 0.4$ for both; Figs. 3 and 4).

Prolonged ex vivo transduction does not increase transgene expression after grafting

We hypothesized that transgene expression in grafted veins would be higher if we increased the efficiency of the gene transfer procedure by transducing the vein segments

ex vivo. We reasoned that if loss of transgene expression in grafted veins is inevitable, then residual vein transgene expression might be increased if expression was lost from a higher initial level. To attempt to increase transgene expression we incubated vein segments *ex vivo* with FGAdCMVnLacZ for up to 24 hr, and then fixed and stained them with X-Gal. Percent blue surface area (median [interquartile range]) was 0.51% (0–2.8%) at 20 min, 0.79% (0.6–5.2%) at 1 hr, 1.3% (0.61–5.3%) at 2 hr, and 17% (2.4–43%) at 24 hr ($p = 0.003$ by ANOVA; Supplementary Fig. S3A). Therefore, prolonged *ex vivo* vein transduction

FIG. 2. Loss of transgene expression after grafting. (A and B) *En face* images and (C and D) cross-sectional views of vein segments that were either (A and C) transduced *in situ* and not grafted or (B and D) transduced *in situ* and grafted immediately into the arterial circulation. Veins were harvested 3 days later and stained with X-Gal. (A and B) One segment of each vein was pinned with the luminal side up; (C and D) another segment was embedded in paraffin, sectioned, and stained with H&E. Rulers (A and B) indicate millimeters; scale bars (C and D) represent 250 μm .

FIG. 3. Expression of LacZ transgene in ungrafted and grafted veins, detected by RNA analyses. LacZ mRNA was measured in extracts of vein segments that were transduced with FGAdCMVnLacZ. Individual veins were either transduced *in situ* and not grafted (Ungrafted), transduced *in situ* and grafted immediately (Transduced then Grafted), grafted and then transduced *in situ* immediately (Grafted then Transduced), incubated overnight *ex vivo* with vector and then grafted (Overnight Transduction), or grafted and then transduced *in situ* 28 days later (Delayed Transduction). In all cases veins were removed and RNA was extracted 3 days after transduction. Data points represent individual veins; bars represent group means. Twelve of the 19 Ungrafted data points (many are hidden) and all 12 of the Transduced then Grafted data points are reproduced from Fig. 1 and are shown here to facilitate comparison with other groups.

yielded variable transgene expression as judged by X-Gal staining; however, some of the 24-hr transduced veins had high levels of transduction (Supplementary Fig. S3A and B).

We therefore transduced additional vein segments by incubation *ex vivo* for 24 hr (group IV: Overnight Transduction; $n=15$ veins), grafted them, and compared transgene expression 3 days after grafting with transgene expression in veins that were transduced *in situ*, and then grafted (group II). Neither blue surface area nor LacZ mRNA differed significantly between groups II and IV (Figs. 3 and 4; $p \geq 0.2$ for both).

Delaying transduction until 4 weeks after grafting increases transgene expression

We next hypothesized that early (3 day) loss of transgene expression in grafted veins was a consequence of cell turnover that occurs during adaptation of venous tissue to arterial pressure and flow, and that this loss of transgene expression could be avoided by transducing vein grafts after adaptation to the arterial circulation.²⁹ To test this hypothesis, we placed unilateral vein grafts at an initial surgery and then reoperated 4 weeks later. During this second surgery, we infused FGAdCMVnLacZ both in the grafted vein and in

FIG. 4. Expression of LacZ transgene in ungrafted and grafted veins, detected by X-Gal staining and *en face* planimetry. Planimetry was used to measure the percentage of luminal surface area staining blue after X-Gal staining. Individual veins were either transduced *in situ* and not grafted (Ungrafted), transduced *in situ* and grafted immediately (Transduced then Grafted), grafted and then transduced *in situ* immediately (Grafted then Transduced), incubated overnight *ex vivo* with vector and then grafted (Overnight Transduction), or grafted and then transduced *in situ* 28 days later (Delayed Transduction). In all cases veins were removed, fixed, and stained 3 days after transduction. Data points represent individual veins; bars represent group means. Twelve of the 19 Ungrafted data points and all 12 of the Transduced then Grafted data points are reproduced from Fig. 1 and are shown here to facilitate comparison with other groups.

the ungrafted contralateral external jugular vein. Both veins were harvested 3 days later. The grafted veins were assigned to a new group (group V: Delayed Transduction; $n=7$) and the ungrafted veins were added to the other group I (Ungrafted) veins from the first experiments (Fig. 1). Measurement of LacZ mRNA and *en face* planimetry both revealed that delaying transduction for 4 weeks yielded increased transgene expression compared with veins that were transduced immediately after grafting (i.e., vs. group III; Figs. 3 and 4; $p \leq 0.01$ for both measures of transgene expression). When compared with group I (ungrafted veins; $n=19$, including 12 veins from the first experiment [Fig. 1]) the delayed transduction veins had equivalent transgene expression measured both by LacZ expression and percentage blue surface area ($p \geq 0.3$ for both measures; Figs. 3 and 4, and Supplementary Fig. S4).

Consistent with our hypothesis that vein grafts adapt to the arterial circulation during the first 4 weeks after grafting, veins from group V (Delayed Transduction) had thicker walls (intima plus media; both grossly and microscopically) than veins in any of the other groups (Fig. 5A–E). Whereas veins harvested 3 days after grafting (groups II–IV; Fig. 5B–D) had virtually no measurable intimal area and thin medias,

FIG. 5. Intimal and medial growth without lumen loss in veins after 28 days in the arterial circulation. (A) Vein transduced *in situ* and not grafted; (B) vein transduced *in situ* and then grafted; (C) vein grafted and then immediately transduced; (D) vein transduced *ex vivo* and then grafted; (E) vein grafted and then transduced 28 days later. (F) Medial areas of veins that were transduced *in situ* and not grafted (Ungrafted), transduced *in situ* and grafted immediately (Transduced then Grafted), grafted and then transduced immediately (Grafted then Transduced), incubated overnight *ex vivo* with vector and then grafted (Overnight Transduction), or grafted and then transduced 28 days later (Delayed Transduction). (G) Luminal cross-sectional area of veins from three of the groups in (F). All veins were removed, fixed, and stained with X-Gal 3 days after transduction. (A–E) H&E stain; scale bar, 50 μm (same scale for all panels). Arrows, medial–adventitial border; arrowheads, luminal surface. Dashed line in (E) indicates the intimal–medial border. Data points in (F) and (G) represent individual veins; bars represent group means.

group V veins had an easily measurable neointima ($41 \pm 15 \times 10^4 \text{ mm}^2$) as well as considerable medial growth (medial area = $16 \pm 2.4 \times 10^5 \text{ mm}^2$; 10- to 30-fold greater than groups II–IV; Fig. 5F). However, this neointimal and medial growth did not compromise lumen area in the group V veins, which was significantly larger than the lumen area of both the group I ungrafted veins (Fig. 5G; $p < 0.001$) and the group III grafted veins (grafted, immediately transduced, and then harvested 3 days later) (Fig. 5G; $p < 0.05$).

Delayed transduction achieves persistent expression of a therapeutic transgene without compromising lumen diameter

We next tested whether vector DNA and transgene expression persisted in grafted veins after delayed transduction. Veins ($n = 38$) were infused 28 days after grafting with either a helper-dependent adenoviral vector or with DMEM, and then harvested 3 days, 4 weeks, 12 weeks, or 24 weeks later. Infusates were as follows: HDAdgApoAI (four to eight veins per time point), HDAdNull (two to four veins per time point), or DMEM (four veins; all harvested at the 4-week time point). All grafts were patent at harvest except one 4-week HDAdgApoAI graft, which was excluded from analysis. Vector DNA was detectable in 31 of 33 HDAd-infused grafts (Fig. 6A). apoA-I mRNA was present in all HDAdgApoAI-transduced grafts ($n = 21$) and was undetectable in all HDAdNull and DMEM controls ($n = 16$; Fig. 6B). Vector DNA and apoA-I expression declined from 3 days to 4 weeks, and then stabilized (no significant decline in vector DNA or apoA-I expression between 4 and 24 weeks by ANOVA).

Delaying transduction with FGAdCMVnLacZ for 4 weeks after vein grafting allowed initiation of intimal and medial growth (Fig. 5E and F). Although this intimal and medial growth did not cause lumen loss 4 weeks after grafting (Fig. 5G), we were concerned that it might progress and cause lumen loss at later time points. We therefore measured intimal, medial, and luminal areas in HDAd-transduced grafts harvested 3 days to 24 weeks after transduction (4–28 weeks after grafting; Fig. 7A–C). Mean intimal and medial areas of HDAd-transduced grafts did not change significantly from 3 days to 4 weeks after transduction ($p \geq 0.08$), but both were increased at 24 weeks ($p \leq 0.01$). Despite this, mean luminal area increased from 3 days to 24 weeks, due to gradual outward remodeling of the vein grafts ($p = 0.001$; Fig. 7C and D).

Discussion

We tested whether adenovirus-mediated transgene expression in veins persisted after grafting and whether HDAd could achieve long-term stable transgene expression in vein grafts. Our major findings are as follows: (1) rabbit external jugular veins are efficiently transduced by adenoviral vectors; (2) transgene expression is rapidly lost when these veins are grafted into the arterial circulation; (3) transgene expression in vein grafts is not increased either by grafting before *in vivo* transduction or by prolonged transduction *ex vivo* before grafting; (4) transgene expression in vein grafts is increased significantly by delaying transduction for 28 days; during this time vein grafts undergo neointimal growth and medial thickening without lumen loss; (5) infusion of HDAd 28 days after vein grafting yields transgene

FIG. 6. Persistence of HDAd vector DNA and transgene expression in grafted veins. Twenty-eight days after grafting, veins were infused with HDAdgApoAI, HDAdNull, or DMEM and then harvested 0.5, 4, 12, or 24 weeks later. (A) Vector DNA; dotted line indicates highest PCR signal in a DMEM-infused vein; (B) apoA-I mRNA was measured in vein graft extracts. Data points represent group means; variances indicate the SEM; *n*, number of veins.

expression that persists for at least 24 weeks, with stability from 4 to 24 weeks; and (6) delaying gene transfer for 28 days after grafting does not result in lumen loss at later time points.

There are many reports of successful gene transfer to vein grafts.^{11,30} However, we could find only one study that compared transgene expression in ungrafted and grafted veins.³¹ This study reported results similar to ours: transgene expression at 3–7 days was 50–85% lower in grafted versus ungrafted veins. Despite these results, included in one of the earliest vein-graft gene transfer papers, the problem of rapid loss of transgene expression in grafted veins does not seem to have been addressed further. Possible explanations for lack of attention to this problem are that most studies were aimed at generating short-term efficacy data and most used first-

generation adenoviral vectors, which lose expression rapidly, independently of grafting.^{19,32–34}

Short-term efficacy studies in animals have been valuable in proving the concept that vein-graft gene therapy can produce sufficient recombinant protein to alter vein-graft biology in potentially therapeutic ways (e.g., by decreasing neointimal growth, inflammation, or oxidative stress).^{35–37} However, human vein-graft disease, especially aortocoronary vein-graft disease, develops far more slowly than does neointimal growth and inflammation in animal vein-graft models.⁵ Most aortocoronary vein grafts fail because of occlusive atherosclerosis, which develops over many years.^{38,39} Accordingly, gene therapy aimed at preventing human vein-graft atherosclerosis will likely require years of transgene expression. It is difficult to imagine that an

FIG. 7. Intimal and medial growth, lumen enlargement, and outward remodeling in grafted veins. Twenty-eight days after grafting, veins were infused with either HDAdgApoAI (open circles) or HDAdNull (solid circles) and harvested at the indicated time points. Vein segments were fixed, embedded in paraffin, sectioned, stained, and analyzed by computer-assisted planimetry to determine (A) intimal area, (B) medial area, and (C) luminal cross-sectional area. Data points represent individual veins; bars represent group means. (D) Representative sections of veins harvested at the indicated time points after transduction. VVG stain (0.5, 4, and 12 weeks); H&E stain (24 weeks); scale bars, 500 μm . Color images available online at www.liebertpub.com/hum

approach in which ~80% of transgene expression is rapidly lost, simply due to grafting, would be successful either in achieving durable transgene expression or in preventing vein-graft atherosclerosis. Although it would be fortuitous if a few days of gene therapy had durable (i.e., years-long) therapeutic effects, the failure of the PREVENT III and IV trials (in which short-term exposure to decoy oligonucleotides did not prevent late vein-graft stenosis)^{40,41} suggests that more durable molecular interventions are needed to reduce vein-graft disease.

By transducing vein grafts 28 days after placement in the arterial circulation and using HDAd, we achieved durable transgene expression in grafted veins. The 24 weeks of vein-graft transgene expression reported here is several months longer than we could find in any previous study, including those using adeno-associated virus (AAV) and lentivirus.^{42,43} Both AAV and lentivirus are potentially useful vectors for preventing vein-graft disease. AAV has the advantage of low immunogenicity and an excellent safety record in humans; lentivirus could possibly provide longer term expression due to its ability to integrate in the genome. Nevertheless, both vectors are relatively unproven for vascular applications and have uncertain tropism for vascular cells. AAV has a limited cloning capacity and lentivirus—as with all integrating vectors—carries a risk of insertional mutagenesis. The optimal gene therapy vector for vein-graft disease would transduce vascular cells at high efficiency, not be targeted by a robust preexisting human immune response, cause minimal if any vascular inflammation, and persist indefinitely. HDAd fits almost all of these requirements^{44,45} with the exception that immunity to the Ad5 capsid is highly prevalent.⁴⁶ Whether AAV or lentivirus could satisfy all of these requirements remains to be tested.

Delayed transduction with HDAd appears to be a promising platform for developing durable gene therapy that could prevent late vein-graft failure. Despite its promise, however, delayed gene transfer has both technical and biological shortcomings. The most obvious shortcomings of delayed gene transfer—compared with the usual protocol of gene transfer *ex vivo* at the time of graft placement—are that the vein must be reaccessed *in vivo* at a later time point and the vector must be delivered *in vivo* rather than *ex vivo*. Reoperation (as done in this study) is unattractive clinically; however, grafted veins could be accessed for gene delivery via percutaneous catheter-based intervention.⁴⁷ The complete absence of side branches in vein grafts would facilitate incubation of vector in the vein lumen and minimize systemic vector escape. Relevant to this, in a surgical focal arterial gene transfer model we found no vector DNA outside the transduced artery, despite sampling many organs and tissues with a highly sensitive PCR assay.¹⁸ Therefore, catheter-based vector infusion into an isolated vein-graft lumen followed by aspiration and rinsing of the lumen would likely avoid significant systemic exposure and could prevent serious systemic immune responses.⁴⁸

A more theoretical shortcoming of delayed gene transfer is that it misses a potential window of opportunity to prevent vein-graft disease before it begins.^{11,49} Although few investigators would expect occlusive vein-graft atherosclerosis to develop in the first 28 days after grafting, some hold the opinion that *any* vein-graft intimal growth is a morbid precursor either to occlusive intimal growth or to late vein-graft

atherosclerosis.^{50,51} According to this view, early intimal growth in vein grafts must be suppressed in order to ensure long-term patency. Indeed, most vein-graft gene therapy studies gauge their “success” by measuring the effect of gene therapy on early intimal growth.^{11,30,52} An opposing view holds that early intimal growth is part of “arterialization” (physiological adaptation of a thin-walled vein to the elevated flow, pressure, and shear of the arterial system) and is not per se a disease that needs treatment.^{11,49} This controversy is relevant to delayed gene transfer because the veins in our study developed a neointima between the time of grafting and the time of (delayed) transduction. Adherents of the former point of view would see this as a serious problem. However, adherents of the latter viewpoint would focus on our observation that this early intimal growth does not reduce lumen area; instead, lumen area increases during the first 28 days and continues to increase afterward. Our finding of large patent veins 6 months after gene transfer makes it difficult to categorize early (1 month) vein-graft neointimal growth as serious pathology.

We believe there is merit in both of the views described previously. The presence of an intima (that includes more than a single endothelial cell layer) is likely a prerequisite for atherosclerosis. However, the presence of an intima is not sufficient for atherosclerosis development. All human elastic and muscular arteries have intimas, but not all human arteries develop atherosclerosis, and many human intimas remain disease-free for nine or more decades. Moreover, in an atherogenic environment an intima (and atherosclerosis within it) could develop long after vein grafting, just as it does in normally “intima-free” mouse and rabbit arteries that are exposed to high plasma cholesterol. For these reasons, an approach that maintains intimal health and durably prevents intimal accumulation of lipid and inflammatory cells appears more robust than an approach that aims only at limiting early intimal growth. As our next step in moving this gene therapy toward the clinic, we propose using this vein-graft model in fat-fed rabbits to test whether expression of atheroprotective genes such as apolipoprotein A-I¹⁸ can prevent vein-graft atherosclerosis. Apolipoprotein A-I is a promising therapeutic gene because interventions that increase plasma apoA-I are uniformly atheroprotective in both animals and humans. Moreover, HDAdApoAI is atheroprotective in rabbit arteries,¹⁸ likely by increasing cholesterol transport out of the artery wall.⁵³ Other potentially therapeutic genes for vein-graft atherosclerosis include secreted decoys for vascular cell receptors that mediate inflammatory cell entry,⁵⁴ antiinflammatory proteins, and extracellular antioxidants.⁵⁵ All of these gene products could be delivered from transduced endothelium, which is the primary target of HDAd both in arteries⁴⁵ and in veins (this study). Prevention of atherosclerosis would be defined as a reduction in lipid and macrophage content of the vein-graft intima.²⁷ Because intimal lipid accumulation and inflammation along with subsequent plaque rupture and vessel occlusion is the pathology that underlies late vein-graft failure,³⁰ prevention of lipid and macrophage accumulation would likely prolong the patency of human aortocoronary SVG. Prevention of lipid and macrophage accumulation in vein grafts in fat-fed rabbits by gene therapy would provide a solid rationale for moving forward toward clinical trials of gene therapy for SVG disease.

An important issue that is not addressed in the present study is whether there is a significant inflammatory response to vector infusion in the vein-graft wall. This response, which would risk exacerbating vascular disease, has been a central issue in vascular gene therapy, particularly with adenovirus.^{33,56,57} The small number of “delayed transduction” vein grafts at any of the time points beyond 3 days precludes a confident assessment of HDAd-mediated vein-graft inflammation at this time. On the basis of our previous work, in which HDAd infusion into arteries of chow- or fat-fed rabbits caused only minimal inflammation,^{44,45} we anticipate that HDAd infusion into vein grafts will not cause a significant local inflammatory response. Nevertheless, we are currently addressing this question definitively with experiments that compare inflammation in a large number of grafted veins infused with DMEM, HDAdNull, or (as a positive control) a first-generation adenovirus.

In summary, we report significant progress in developing gene therapy for vein-graft disease. A translational pathway toward the clinic will include additional rabbit studies aimed at preventing the decline in gene expression between 3 days and 4 weeks (e.g., with expression cassette engineering or coexpression of immunomodulatory cytokines)^{23,25} and assessment of the efficacy of gene therapy in a vein-graft atherosclerosis model.²⁷ If successful, subsequent experiments would be performed in larger animals such as pigs, in which coronary bypass surgery is feasible,^{28,58} hyperlipidemia-induced atherosclerosis can be modeled,⁵⁹ limb veins more closely resemble human saphenous veins,^{60,61} and catheter-based delivery can be performed with clinical-grade materials.⁶²

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Author Disclosure Statement

The authors declare no competing financial interests.

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Address correspondence to:

Dr. David A. Dichek

Department of Medicine

University of Washington

1959 NE Pacific Street, Box 357710

Seattle, WA 98195-7710

E-mail: ddichek@uw.edu

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