Stent-based delivery of adeno-associated viral vectors with sustained vascular transduction and iNOS-mediated inhibition of in-stent restenosis

I Fishbein¹,², DT Guerrero¹, IS Alferiev¹,², JB Foster¹, NG Minutolo¹,², M Chorny¹,², AM Monteys¹, KH Driesbaugh², C Nagaswami² and RJ Levy¹,²

INTRODUCTION
Stent angioplasty, that is, percutaneous placement of balloon-expandable or self-expandable metallic or polymeric scaffolds in the vascular conduit narrowed by atherosclerotic plaque is a treatment of choice in symptomatic patients with coronary and peripheral atherosclerosis. Approximately 1 million patients in the USA alone undergo this treatment each year.¹ Sophistication of peripheral atherosclerosis. Approximately 1 million patients in the treatment of choice in symptomatic patients with coronary and the vascular conduit narrowed by atherosclerotic plaque is a expandable or self-expandable metallic or polymeric scaffolds in Stent angioplasty, that is, percutaneous placement of balloon-

In-stent restenosis remains an important clinical problem in the era of drug eluting stents. Development of clinical gene therapy protocols for the prevention and treatment of in-stent restenosis is hampered by the lack of adequate local delivery systems. Herein we describe a novel stent-based gene delivery platform capable of providing local arterial gene transfer with adeno-associated viral (AAV) vectors. This system exploits the natural affinity of protein G (PrG) to bind to the Fc region of mammalian IgG, making PrG a universal adaptor for surface immobilization of vector-capturing antibodies (Ab). Our results: 1) demonstrate the feasibility of reversible immobilization of AAV2 vectors using vector tethering by AAV2-specific Ab appended to the stent surface through covalently attached PrG, 2) show sustained release kinetics of PrG/Ab-immobilized AAV2 vector particles into simulated physiological medium in vitro and site-specific transduction of cultured cells, 3) provide evidence of long-term (12 weeks) arterial expression of luciferase with PrG/Ab-tethered AAV2Luc, and 4) show anti-proliferative activity and anti-restenotic efficacy of stent-immobilized AAV2iNOS in the rat carotid artery model of stent angioplasty.

INTRODUCTION
Stent angioplasty, that is, percutaneous placement of balloon-expandable or self-expandable metallic or polymeric scaffolds in the vascular conduit narrowed by atherosclerotic plaque is a treatment of choice in symptomatic patients with coronary and peripheral atherosclerosis. Approximately 1 million patients in the USA alone undergo this treatment each year.¹ Sophistication of vascular stent designs, especially those which combine mechanical support with local drug delivery, has expanded clinical indications for stent angioplasty resulting in a decrease of bypass surgery and target lesion revascularization procedures. Nonetheless, even with 3rd generation drug eluting stents (DES), stent failure caused by in-stent restenosis (ISR) remains a significant problem, especially in certain patient populations, such as diabetics.²

Gene therapy delivered from stent struts directly to the acutely remodeled vessel wall presents an advance compared to DES. The benefits of localized gene therapy implemented in the form of gene delivery stents (GDS) over DES are prolonged modification of the vascular substrate, the selectivity of exerted effects towards smooth muscle cells (SMC) versus endothelium, and a potential combination of the anti-ISR therapy with the interventions preventing progression of atherosclerotic process in the stented artery. Driven by this rationale, attempts have been made to overexpress reporter and therapeutic genes in the stented vasculature of experimental animals using both non-viral³–⁵ and viral⁶–¹² vectors immobilized on stent struts. While generally successful in demonstrating the initial presence of the encoded gene product in the arterial tissue, these studies have typically failed to achieve sustained expression of therapeutic transgenes. Early discontinuation of anti-restenotic treatment was linked to the loss of therapeutic gain at the later time points after the intervention.¹³ The studies in the field have identified vector-triggered inflammatory and immune responses resulting in promoter shut-down and vector destruction as the principal causes for the abrogated transgene expression in vivo.¹⁴,¹⁵ Additionally, inadequacy of the stent-based delivery system, allowing almost immediate release of the stent-immobilized vector depot, presents a major deficiency in GDS design.

The nature of therapeutic genes capable of inhibiting deleterious remodeling of the stented arteries has been investigated in many studies.¹⁶,¹⁷ We¹⁸ and others⁵,¹¹ have previously demonstrated that augmented nitric oxide production with overexpression of endothelial and inducible isoforms of nitric oxide synthase encoded by gene vectors delivered from the stents inhibits restenosis though multiple mechanisms related to reduced SMC proliferation¹⁶ and migration,¹⁹ enhanced endothelial re-growth,²⁰ and decreased ingress of inflammatory cell types into the vascular wall.²¹ Our prior work on stent-mediated delivery of inducible nitric oxide synthase (iNOS) for augmenting nitric oxide availability in the stented vasculature employed adeno viral (Ad) vectors.⁷–⁹ AdiNOS demonstrated anti-restenosis efficacy in the rat carotid model that was optimally enhanced with dietary supplementation of the iNOS substrate, arginine, and its prosthetic co-factor, tetrahydrobiopterin (BH₄).²² Nevertheless, Ad vectors have multiple shortcomings when developed as human therapeutics,³⁰ and thus are inapplicable for GDS clinical translation beyond the proof of concept phase.

Herein we report on the formulation, characterization and in vitro and in vivo investigations of a novel stent-based platform
for iNOS-encoding adenoviral associated viral vector (AAV2iNOS) delivery to the stented vasculature. Recently AAV has emerged as the preferred vector in human gene therapy trials due to their low immunogenicity and minimal activation of inflammatory signaling cascades. To immobilize transduction-competent AAV particles on the stent struts we re-formulated our previously described chemical methodology for the bare metal surface functionalization with thiol-reactive, pyridylthio groups, thus allowing for covalent immobilization of thiolated protein G, a universal affinity binding adaptor for Fc fragment of mammalian IgG. The presence of antibody-binding moiety on the stent surface enables further binding specificity of vector-capturing antibodies and subsequent vector tethering. In addition, we identified an optimized monoclonal mouse antibody to intact AAV serotype 2 (AAV2) particles (clone A20) with sufficient affinity to support vector tethering. We then used this antibody to reversibly attach AAV2 vector particles on the PrG-functionalyzed model surfaces and stainless steel stents to characterize the system in terms of maximal vector capacity, vector release rate, transduction activity in vitro and in vivo, and therapeutic effects of stent immobilized AAV2iNOS in ISR prevention.

RESULTS
Reversible immobilization of AAV vectors on stainless steel surfaces
A novel vector immobilization strategy illustrated in Figure 1 was used to reversibly append AAV2 to the surface of stainless steel foil, mesh disk and stent samples. Vector tethering optimization experiments confirmed that IgG attachment to the metal surface is dependent upon the presence of surface immobilized PrG, as evidenced by preferential binding of fluorescently labeled antibody to the PrG-primed surface (Supplementary Figure 1A) and by depletion of fluorescently labeled IgG from the solution incubated with PrG-treated foil samples, but not with the samples prepared with the omission of PrG (Supplementary Figure 1B). Additionally, we investigated the effectiveness of vector immobilization as a function of antibody concentration and established the optimal input amounts of the capturing antibody per surface area (Supplementary Figure 1C).

Formation of an AAV vector monolayer on the surface of stainless steel mesh was confirmed with SEM that showed complete surface coverage with spherical objects of 30–40 nm diameter (Figure 2b), supposedly representing AAV capsids coated with a 12-nm layer of gold-palladium. Some of these entities appeared to be clustered into groups of 3-5 objects. No such artifacts were observed on the surface of unmodified mesh disks (Figure 2a).

Release kinetic of PrG–Ab–tethered AAV2 vector in vitro
To determine whether antibody tethering of AAV2 particles to the metal surface provides adequate affinity forces to prevent immediate release of immobilized vector, the amount of AAV2iNOS genomes associated with stainless steel foils formulated using the PrG-A20 vector immobilization strategy was determined immediately upon the samples preparation (unreleased samples) and at 7 time points during a 1-month release experiment. Release of vector was carried out at 37 °C in simulated medium comprised of 5% human serum albumin/DMEM. Shaking (70 RPM) of the samples was used to simulate the shearing effect of blood flow. Results of the release experiment imply that there are two linear phases of fast (day 0–day 2) and slow (day 2–day 31) release of AAV2 particles from the surface of model foils (Supplementary Figure 2). Approximately 24% of viral load is released during the first 2 days, followed by additional release of 43% of the initial virus load between days 2 and 31. Overall, two thirds of the viral cargo was dissociated from the metal surface during the first month of in vitro release (Supplementary Figure 2).

In vitro transduction with PrG-Ab–tethered AAV2 vector
To assure that antibody-mediated immobilization of AAV2 vectors does not compromise their transduction capacity, stainless steel
mesh disks were formulated with $5 \times 10^8$ vector genomes (VG) of AAV2-eGFP or $3.5 \times 10^8$ VG of AAV2-iNOS. The samples formulated with omitting the A20 antibody incubation step or both PrG and A20 exposures served as controls in the eGFP expression experiments. The metal disks properly formulated with AAV2-capturing antibody, but lacking exposure with AAV2-iNOS were employed as controls in INOS over-expression studies. Robust eGFP expression was demonstrated 3 days after the placement of PrG/A20-AAV2-eGFP meshes in subconfluent HEK cultures, while only minimal transduction was observed in the cell cultures treated with control meshes (Figures 3a and b). Intensity of eGFP expression in cells transduced with properly immobilized AAV2 vector was further increased by day 12, while no substantial increase of eGFP expression was detected in wells treated with control meshes (Figures 3a and b). Notably, the cells transduced with AAV2-eGFP eluting meshes were located almost exclusively under the mesh and within 100 μm of mesh disk circumference (Figure 3a), thus demonstrating site-specificity of gene transfer by Ab-tethered vector.

Inducible nitric oxide synthase activity in HEK293 cells, similarly transduced with mesh-immobilized AAV2-iNOS was detected with Griess assay that demonstrated high levels of a stable NO end-product, nitrite, in the media conditioned with mesh-immobilized AAV2-iNOS, but not in the media from untreated cells or cells treated with PrG/A20-modified mesh samples that were not exposed to AAV2-iNOS (Figure 3c).

Persistence of AAV2 stent load after arterial catheterization and in vivo release

To determine physical resilience of the submicronial PABT/PrG/Ab-AAV layer to withstand mechanical impact inherent to vascular catheterization, we quantified with RT-PCR residual AAV2 load in three groups of similarly formulated stents that were surface-modified with AAV2-eGFP: 1) unmanipulated stents; 2) stents inserted via the external carotid arteriotomy site into the mid-segment of the common carotid artery in rat model and immediately retrieved without arterial deployment, and 3) stents deployed in the mid-segment of the common carotid artery and harvested 2 days after implantation. The results of this experiment demonstrate that despite inevitable brushing related to introduction of gene-eluting stents through the unyielding plastic introducer and narrow vascular conduit, approximately 70% of the initial AAV2 load remains associated with the stent (Figure 4). Moreover, abundant surface AAV2 can still be quantitated after arterial deployment and 2-day release in vivo (Figure 4).

Time course of site-specific arterial expression after implantation of AAV2-Luc-tethered stents in rats

Prior gene delivery stent studies typically reported limited duration of transgene expression in the stented vasculature.3,4 To assess kinetics of vascular transduction with our new stent-based AAV2 delivery system, stents formulated with $7.5 \times 10^8$ VG of AAV2-Luc appended to the bare stainless steel surface via PABT/PrG/A20 linking system were deployed in the common carotid location in a rat model. The animals then underwent serial bioluminescence imaging at 1, 2, 4 and 12 weeks after stent implantation (Figure 5a). Localized expression of luciferase spatially corresponding to the location of deployed stent was detected in all animals at all time points (Figure 5a). Importantly, the signal intensity increased over time reaching a maximum of $4.35 \times 10^6$ photons/min/cm²/steradian at 12 weeks post-stenting (Figures 5a and b).

AAV2-iNOS stents attain site-specific transgene expression in vivo and enhanced nitric oxide production ex vivo

To demonstrate functional competence of locally delivered AAV2 encoding our therapeutic transgene of interest, INOS, bare metal stents and stents formulated with $4.5 \times 10^8$ VG of AAV2-iNOS immobilized via PrG/A20 tethering were deployed in the common carotid arteries in a rat model. Ongoing expression of human INOS in the stented arterial segments explanted 8 days after surgery was determined by RT-PCR and analyzed by the ∆ΔCt method, demonstrating baseline expression levels in the arteries treated with BMS. In comparison, a 23-fold higher occurrence of human INOS transcript was determined in vascular tissue of animals treated with AAV2-iNOS eluting stents (Figure 6a).

In a separate experiment, PrG/A20-modified stents formulated with or without $8 \times 10^8$ VG of AAV2-iNOS were deployed in the common carotid arteries of rats supplemented with arginine and BH4 via drinking water as previously published,22 to prevent uncoupling of iNOS with the undesirable formation of superoxide and peroxynitrite. Nitric oxide production was determined with Griess assay of the media conditioned by the stented carotid segments harvested at euthanasia (day 8) and cultured ex vivo for 72 h. Compared with arterial tissue from animals treated with PrG/A20-only stents, a 16-fold higher NO production was found in the medium conditioned with AAV2-iNOS stent-treated arteries (Figure 6b).

AAV2-iNOS stents inhibit ISR in the rat model of stent angioplasty through mitigation of cell proliferation in stented arteries

To investigate the mechanistic aspects of stent-based delivery of AAV2-iNOS to stent-injured arteries and to validate the therapeutic potential of this intervention, bare metal stents and gene eluting stents ($5 \times 10^8$ genomic particles of AAV2-iNOS immobilized via the PrG/A20 tether) were deployed in two groups of rats supplemented with Arg and BH4. Morphometric analysis of the explanted arteries treated with the stents capable of local arterial delivery of AAV2-iNOS gene vector revealed a 31% reduction of the neointimal area (Figures 7a and b), without significant changes in the area of the media (Figures 7a and c) thus resulting in a 34% decrease of

Figure 2. Scanning electron microscopy. Representative electron microscopy images of (a) bare metal stainless steel mesh disks and (b) mesh samples formulated according to the PrG/A20 tethering protocol and incubated with AAV2 vector demonstrating surface coverage with viral capsids. Original magnification is 80 000x.
the neointima to media ratio (Figures 7a and d). The antirestenotic effect of AAV2iNOS-eluting stents was accompanied by a 47, 57 and 23% decreases of Ki-67 labeling in the neointima, media and adventitia, respectively, of the GDS-treated compared with BMS-treated arteries (Figures 8a and b).

To confirm effective iNOS transduction of AAV2iNOS stent-treated rat arteries, we assessed expression of the DYKDDDDK epitope (FLAG-tag) cloned into human iNOS construct expressed by the AAV vector using immunohistochemistry. This approach allowed us to avoid ambiguity related to cross-reactivity between endogenous rat iNOS upregulated by vascular intervention27 and the antibody generated to human iNOS. Massive staining with a cellular pattern was observed in rats treated with AAV-iNOS-eluting stents, while no staining was detected in BMS-treated animals (Supplementary Figure 3). To elucidate what
cell type is predominantly targeted by stent-eluted AAV vectors we used dual immunofluorescence (for FLAG and cell type-specific markers). We found that the vast majority of FLAG-expressing cells are neointimal SMC, although adventitial macrophages are also notably transduced (Supplementary Figure 4).

Stent-based delivery of AAV2iNOS did not increase stent-triggered inflammation, as inferred from equal prevalence of macrophages in the lesions induced by gene-eluting and control stents (434 ± 81/mm² and 422 ± 87/mm², respectively; Supplementary Figure 5).

**DISCUSSION**

The main findings of our study are: 1) demonstration of the feasibility of labile immobilization of AAV vectors on bare metal surfaces through affinity interaction-mediated molecular bridges established between covalently immobilized PrG and the vector-binding antibody; 2) sustained release of AAV2 particles in simulated physiologic medium in vitro and localized transduction of cultured cells with AAV2 vector immobilized via PrG/anti-AAV2 antibody tethering; 3) confirmation of reasonable resistance of the AAV2 monolayer established on the surface of a stent to the mechanical abrasion inherent to angioplasty procedure; 4) transduction of the arterial wall with a sustained site-specific expression of encoded transgene by the AAV2 vector eluted from the stent and 5) ISR inhibition in rats treated with AAV2iNOS eluting stents that was at least partially related to the suppression of cell proliferation in the injured vessel wall.

The implementation of human gene therapy protocols for a range of vascular disease, such as atherosclerosis, pulmonary hypertension and restenosis, requires elaboration of clinically acceptable methods for local delivery of gene vectors to the arterial wall, which are yet to be developed. Substrate mediated gene delivery, a gene therapy technique based on improved biodistribution and enhanced effectiveness of gene vectors positioned on the interface between the carrying device and the tissue is well suited for cardiovascular applications involving stent implantation. Thick polymeric coatings initially used for bulk immobilization of the vector depot were shown to be highly pro-inflammatory and thus unsuitable in the context of therapeutic GDS. This problem motivated us and others to develop techniques for stent immobilization of gene vectors that rely on reversible tethering of vector particles directly to modified metal surfaces of stents thus avoiding the inflammatory reactions triggered by polymeric coatings. Unlike our prior work that used direct attachment of vector-binding moieties to polylallyamine bisphosphate-modified steel, our present studies employed a universal adaptor, PrG that exhibits a strong affinity to the Fc fragments of mammalian IgG and can thus be used as a molecular bridge between the stent surface and vector binding antibody. Engagement of the Fc domain of antibody into affinity pairing with PrG allows for the optimal presentation of antibody molecules with their antigen-binding F(ab) domains opposing the material surface. This arrangement makes the epitope binding sites maximally exposed for vector tethering. Furthermore, due to the system modularity, it can be readily modified to immobilize a variety of gene vector types, provided that the antibodies to vector capsid or envelopes are available. This versatility of PrG/Ab-based delivery system is exemplified by transduction of primary rat aortic SMC with adenoviral vector immobilized on model meshes with PrG/anti-Ad hexon antibody tether (Supplementary Figure 6). It is noteworthy that antibodies used for vector immobilization cannot act as neutralizing antibodies, since being themselves immobilized, they dissociate from the vector when the vector is released from the stent (Figure 1, Step 8).

The number of viral genomes detected in association with the metal samples by two independent assays (PCR and Pico Green assay) is comparable and 10–20 times lower than calculated based on a maximal geometrical capacity of 20–25 nm spherical particles attached to the surface in a monolayer arrangement. This discrepancy is consistent with data reporting that up to 98% of AAV2 preps consist of empty particles devoid of genome. Since antigenically indistinguishable from the DNA-containing counterparts, the empty capsids occupy space on the surface proportional to their prevalence in the prep, but are not registered by viral DNA-based enumeration methods.

Two phase zero-order release kinetics profile of the vector into simulated physiologic medium was demonstrated in our in vitro experiments (Supplementary Figure 2). It is feasible that a fraction of non-specifically bound AAV2 vector held on the surface through the weak hydrophobic forces is responsible for the rapid (burst) release phase (day 0–day 2). When this rapidly mobilizable fraction is exhausted, the further release (day 2–day 31) is mediated solely by the dissociation of the AAV2 from the PrG/A20 tether; a process that is characterized by a slower rate than the initial burst release of the non-specifically bound fraction. Overall, approximately 67% of surface-associated vector genomes are released during the first month, which is comparable to the release of Ad vector appended to a bare metal surface through hydrolysable cross-linkers. While not specifically addressed in the present study, it is feasible to assume that release kinetics can be modulated by the usage of anti-AAV2 antibodies with different affinity (Kd). To that end, dissimilar transduction time course was
demonstrated in our prior studies with Ad vectors immobilized through the adapters with different Kd. Moreover, concurrent tethering of several antibody linkers with different Kd on the same stent provides a potential strategy for vector release optimization. Additionally, this modular system is consistent with the simultaneous immobilization of different classes of viral vectors, such as Ad and AAV, or viral and non-viral vectors on the same stent. These intriguing possibilities will be investigated in our future studies.

To study the general aspects of transduction with the metal substrate-immobilized AAV2 vector we used a non-vascular cell type (HEK293) due to its susceptibility to AAV2 transduction (Figure 3). Remarkably, presence of AAV2-capturing antibody on the surface of mesh disks exposed to AAV2-eGFP increased transduction of cells in culture (Figures 3a and b), substantiating the proposed immobilization and release paradigm (Figure 1). The majority of transduced cells were located within 100 microns (3-5 cell diameters) from the mesh borders, consistent with the intended local delivery application of stent-immobilized AAV2 vector. The fluorescence signal emitted by cells transduced with PrG/A20-tethered AAV2-eGFP increased 5.5-fold between days 3 and 12. This expression pattern may reflect continuous transduction events mediated by infection-competent AAV2 particles released from the meshes after the day 3 time point. Similar prolongation of transduction competence upon exposure to the cell culture media at 37°C was demonstrated earlier for Ad vector immobilized on the metal substrate.

Mechanical forces inherent to the stent introduction into the vasculature through percutaneous or arteriotomy access, and stent advancement via tortuous, narrowed and often calcified vascular conduit to the deployment site present a challenge for the integrity of the vector depot of GDS. Physical dislodgement of the vector en route to the target site decreases the applied vector dose and promotes off-target tissue inoculation. To model vector shearing involved in stent positioning at the target site, groups of similarly formulated AAV2-eGFP stents were compared with or without insertion via the introducer tubing and a passage though the common carotid artery, demonstrating that approximately 70% of initial vector dose is still associated with the undeployed stents after their retrieval from the vasculature (Figure 4). This is an expected result since the vector depot is not coated with any protective polymer layer to avoid additional inflammatory stimuli. We did not investigate the fate of the dislodged AAV2-eGFP in these experiments. Based on published reports, most of the systemically administered vector ends up in liver. The dose of the vector released into circulation (ca 2.2x108 VG) is at least 1000-fold lower than a tolerated dose in published systemic AAV administration studies and is not expected to cause toxicity and systemic inflammation. Nonetheless, our future studies will explore a possibility of AAV2 depot stabilization using chemical cross-linking of surface-bound AAV2.

Approximately 75% of the dose that was associated with the stents at the time of deployment was found to be released during the first 48 h. Faster vector release in vivo (Figure 4) compared to the cell-free in vitro system (Supplementary Figure 2) might be attributed to more aggressive release medium, decreased pH of tissue fluid due to acute inflammation at the site of stent deployment, the shearing effect of blood flow, and vector interaction with vascular and blood-borne cells. Overall, in situ release of the vector is more sustained than reported for non-viral and Ad vectors. Since restenotic neointima is not present at the time of stent implantation, the capability of GDS to sustain release of the transduction-competent vector is crucial if neointimal SMC are considered as the target cell type for GDS intervention.

![Figure 7. ISR inhibition by AAV2iNOS - eluting stents in the rat carotid model. Representative images of Verhoeff-van Gieson stained arterial sections (a) obtained from the BMS-treated (n = 10) and AAV2iNOS-treated (n = 10) rat carotid arteries. Neointimal area (b), medial area (c) and neointima/media area ratio (d) as determined by morphometry of the BMS-treated and AAV2iNOS eluting stent-treated rat carotid arteries. L, N, M, A and S denote lumen, neointima, media, adventitia and spaces left by dissolved stent struts, respectively.](image-url)
To validate both transduction of arterial tissue with AAV2iNOS vector and the anticipated physiological effects of iNOS overproduction, we constructed AAV2 vectors with human iNOS sequence to be able to distinguish a vector-overexpressed transgene from the endogenous rat iNOS, which was shown to be actuated by vascular injury.27 Super-physiological levels of iNOS production in the vascular tissue can result in relative deficiency of Arg and BH4 with ensuing iNOS decoupling which leads to production of damaging reactive oxygen species.42 To prevent this, animals in our AAV2iNOS stent studies were supplemented with Arg and BH4 through the drinking water. Human iNOS-specific primers were able to support amplification of DNA sequence that was reverse-transcribed using RNA extracted from the carotid arteries treated by AAV2iNOS GDS, but not by BMS (Figure 6a). Moreover, the over-expressed human iNOS was translated in a protein that maintained functionality in rat vasculature, as evidenced by highly elevated NO production in the media conditioned with AAV2iNOS GDS-transduced arteries compared to the media conditioned with stents that were similarly formulated aside from the vector acquisition (Figure 6b). The optimal levels of NO production for the prevention of restenosis in balloon injured or stented arteries remains largely unknown. In a similar ex vivo assay nitrite accumulation in the media conditioned by the rat carotid arteries transduced with free AdiNOS (2x10^6 pfu) was an order of magnitude lower than in our experiments, which is reasonable, considering much lower vector titer and a limited contact time of the vector with the vessel wall. A 34% inhibition of ISR was observed in our therapeutic study in Arg/BH4-supplemented animals treated with the AAV2iNOS GDS compared to BMS-treated counterparts (Figure 7). Our previously published experiments with AdiNOS-eluting stents formulated with Coxackie-Adenovirus receptor D1 domain-mediated affinity binding of the vector8 and with covalent vector attachment via hydrolysable cross-linkers2 demonstrated a ~50% reduction of ISR. Moreover, in a model of indwelling delivery of free AdiNOS suspension to Fogarty balloon injured rat arteries a subtotal 95% inhibition of restenosis was observed,44 supporting the role of nitric oxide as a principal anti-restenotic mediator.

Neointimal formation triggered by stent implantation in healthy rat arteries is limited and typically results in a 20–25% luminal narrowing with a 30–80 μm layer of neointimal tissue over the struts. While stent-based delivery of AdiNOS achieved a stronger neointimal inhibition than was observed in our present study with AAV2iNOS-eluting stents, it was associated with a high percentage of uncovered struts, which in humans is a proven risk factor of stent thrombosis.45 The extent of ISR is much greater in human atherosclerotic arteries, where subtotal (>90%) occlusions of the stented segment with neointimal tissue is a common finding.46 Since nitric oxide is deficient in atherosclerotic arteries, it is feasible that in the atherosclerotic milieu, stents designed to deliver gene therapy for nitric oxide augmentation will show a larger therapeutic gain than in healthy rats. To this end, the ultimate clinical translatablebility of AAV2iNOS GDS should be investigated in a diseased animal model, such as the atherosclerotic rabbit iliac artery48 or the coronary artery of diabetic hypercholesterolemic swine.49

Some limitations of our studies should be mentioned. The antirestenotic effects of a single fixed dose of AAV-iNOS were investigated in vivo. It is possible that the therapeutic effects can be enhanced with the adjustment of the vector dose. The impact of stent-based delivery of AAV2iNOS on recovery of denuded endothelium in the stented artery was not determined, and this should be the subject of advanced investigations. Off-target transduction of perivascular tissue and distant organs was also not studied. The rat carotid model used in these experiments, involving healthy animals, is known to be more permissive in terms of therapeutic effectiveness than more stringent human-like porcine models involving hypercholesterolemia and diabetes.

In conclusion, the present studies demonstrate immobilization of viable AAV2 vectors on bare metal surfaces through the affinity bridges consisting of PrG and an AAV2-specific antibody. The amount of a surface-immobilized vector is consistent with a monolayer arrangement of the vector particles on the surface.

**Figure 8.** AAV2iNOS - eluting stents decrease proliferative activity in stented vasculature. (a) Representative immunohistochemical Ki-67 staining of BMS-treated (n = 4; left) and AAV2iNOS GDS-treated (n = 4; right) arteries. (b) Ki-67 labeling density (number of positive cells/mm²) in adventitia, media and neointima of stented arteries. L, N, M, A and S denote lumen, neointima, media, adventitia and spaces left by dissolved stent struts, respectively.
Antibody-mediated binding bestows sustained release properties to immobilized vector and provides site-specific transduction in vitro and in vivo. In the rat carotid model, stents formulated with surface-bound AAV2 demonstrate escalating expression of encoded transgene (luciferase) for 12 weeks and ISR mitigation with overexpression of inducible nitric oxide synthase.

**MATERIALS AND METHODS**

**Materials**

Stainless steel (grade 316) foil sheets were purchased from Goodfellow (Corapolis, PA, USA). Stainless steel (grade 316) mesh disks (3 mm diameter) were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Multilink stainless steel (grade 304) stents were custom made by Laserage (Waukegan, IL). The angioplasty catheters were donated by NuMED (Hopkinton, NY). Polyallylamine bisphosphonate with latent thiol groups (PABT) and pyridyldithio-engrafted polyethyleneimine (PEI-PDT) were synthesized in-house according to previously published methods. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP.HCl) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). The thiolated form of protein G (PrG-SH) was purchased from Protein Mods (Madison, WI, USA). Anti-AAV2 (clone A20), anti-Ad hexon and anti-Ki67 antibodies were obtained from GeneTex (Irvine, CA, USA), Biodesign International (Saco, ME, USA) and Novus Biologicals (Littleton, CO), respectively. All other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and VWR International (Radnor, PA, USA).

All reporter gene vectors used in the present studies (AAV2eGFP, AAV2Luc, and AdeGFP) were constructed and produced in the Vector Core facility of the University of Pennsylvania. AAV2Luc, was produced by the Research Vector Core at the Center for Cellular and Molecular Therapeutics of the Children’s Hospital of Philadelphia (CHOP).

**Vector immobilization protocol (Figure 1)**

Stainless steel stents, mesh disks and foils were cleaned with isopropanol and incubated in a 0.15% aqueous solution of polyallylamine bisphosphonate with latent thiol groups (PABT) at 60 °C with shaking for 1 h (Figure 1, Step 1). Acidity of the solution was adjusted with potassium bicarbonate to pH of 4.5. After washing the samples in double distilled water (DDW), thiol groups in the side chains of PABT were deprotected with tris (2-carboxyethyl) phosphine hydrochloride (TCEP.HCl; 10 mg/ml in 0.1M acetate buffer, pH 4.5; at room temperature (RT) with shaking for 15 min; Figure 1, Step 2). The samples were then thoroughly washed with degassed DDW and reacted with an 0.5% aqueous solution of pyridyldithio-engrafted polyethyleneimine (PEI-PDT) at 28 °C with shaking for 1 h (Figure 1, Step 3) followed by the incubation with thiolated protein G (PrG-SH; 125 μg/ml in degassed PBS; at 28 °C with shaking for 1 h; Figure 1, Step 4). After extensive washing of the samples, an antibody of recombinant specificity, e.g., anti-AAV2 (clone A20), diluted in PBS to the volume sufficient to completely cover the samples and providing 2–5 μg of antibody/cm² of metal surface was added to the samples at 28 °C with mild shaking for 45 min (Figure 1, Step 5). The samples were washed in PBS and incubated with AAV2 suspension (10¹⁰ VG/ml PBS) at 28 °C with mild shaking for 45 min (Figure 1, Step 6), resulting in capturing of AAV2 particles on the surface of metal samples (Figure 1, Step 7) thus enabling subsequent release of the vector in vitro and in vivo (Figure 1, Step 8).

**Quantification of the vector load associated with metal surface**

To determine the number of AAV2 genomic particles on the surface of metal samples two methods were employed.

**qPCR of vector transgene sequence.** The vector genome (VG) number of AAV2eGFP attached to the metal surface was determined by qPCR as reported by us before. In brief, AAV2 was eluted from the surface and lysed using 2-h incubation at 56 °C in proteinase K diluted in ALT buffer. Viral DNA was extracted and purified using QiAmp DNA Mini kit per manufacturer instructions. Transgene sequence was amplified in a 5 μl reaction using eGFP-specific primers (forward: 5’ TCCTTGCCCTGCTCACC 3’; reverse: 5’ CAG TGG ATG TTG CTC TTA CTA G 3’). Serial dilutions of free AAV2eGFP of a known titer were similarly processed through the isolation and qPCR protocols to generate a calibration curve.

**Fluorimetric detection of AAV2 DNA with Pico Green assay.** Alternatively, AAV2 VG number was determined with Pico Green assay as recently reported with minor modifications. In brief, AAV2 particles immobilized on metal substrate were lysed in a buffer comprising 10 mM Tris.HCl, 100 mM NaCl and 0.1% SDS at 70 °C for 1 h, followed by a rapid cooling of lysate to 25 °C (5 min) to anneal single stranded DNA fragments. 20-μl aliquots of AAV2 lysates were added into individual wells of a 96-well fluoroimetry plate prefilled with 180 μl of Pico Green diluted 1:160 in Tris-EDTA buffer. Fluorescence was measured at 485/538 nm within 5 min of components mixing (Gemini EM, Molecular Devices, Sunnyvale, CA, USA). A calibration curve was constructed using the dilution series prepared from a free AAV2 of a known titer that were similarly processed.

**Scanning electron microscopy (SEM)**

AAV2eGFP was immobilized on the stainless steel mesh disks via the PrG/A20 tethering at the density of 5x10⁶ VG/mesh (per qPCR). Unmodified meshes were used as control. Immediately upon preparation the meshes were rinsed in cacodylate buffer, and fixed in 2% glutaraldehyde/cacodylate buffer followed by alcohol dehydration, HMDS drying and sputter-coating with gold palladium. The surface of meshes was then imaged at 80 000 magnification using a Quanta FEG 250 system (FEI, Hillsboro, OR) to visualize individual AAV2 particles on the metal surface.

**Cell transduction experiments**

Stainless steel mesh disks were modified with PABT, PEI-PDT, PrG and anti-AAV2 antibody as delineated in the Vector Immobilization Protocol section, prior to the exposure of the samples to 10¹⁰ VG of AAV2eGFP. The resulting mesh vector loading amounted to 5x10⁶ VG of AAV2eGFP as determined by qPCR. Control meshes were formulated omitting the PrG and/or capturing antibody attachment steps and were exposed to the same vector suspension as the properly formulated samples. Following PBS washing, the meshes were individually placed in the wells of 96-well plates with subconfluent cultures of HEK 293 cells. Transduction was assessed 3 and 12 days after mesh placement by fluorescence microscopy and fluorimetry.

**Engineering of a shuttle plasmid for production of AAV2-iNOS**

The human iNOS cDNA sequence was moved into an AAV shuttle vector using standard molecular biology techniques. Human iNOS cDNA sequence containing both a Myc and a DYKDDDDK tag (FLAG) epitope was expressed under the control of the CMV promoter sequence and cloned upstream of the BgH poly A sequence. The human iNOS expression cassette was flanked at each end by AAV serotype 2 145-bp inverted repeat sequences. AAV2 vectors were generated by triple transfection technique in the Research Vector Core at the Center for Cellular and Molecular Therapeutics (CHOP).

**Rat carotid model of stent angioplasty**

All animal procedures employed in the reported studies confer to federal regulations on laboratory consequently animal use and were preapproved by the Institutional Animal Care and Use Committee of CHOP. All rats were obtained from the same supplier and were used consecutively. No special randomization beyond successive use of animals was performed. The pre-established exclusion criteria in our animal studies were a significant deviation from the standard surgical protocol; morbidity beyond day 2 post-surgery, and thrombosis of the stented vessel. Male Sprague-Dawley rats (10–11 weeks of age; 375–425 gram) were pre-treated with aspirin (3 mg/kg via drinking water for 2 days prior the intervention) and were administered heparin 200 U/kg via the tail vein. Under ketamine (80 mg/kg, 3 mg/kg via drinking water for 2 days prior the intervention) and were preapproved for surgery. A 2 F Fogarty catheter was introduced into the common carotid artery through the left carotid artery and tunneled under the skin of the neck region. The endothelial layer of the common carotid artery was then abraded by three passages of the inflated balloon on the tip of the catheter. Teflon introducing tube was then placed in the external carotid artery over the Fogarty catheter and the Fogarty catheter was removed. A stent-loaded...
Angioplasty catheter (10 mm length, 1.5 mm nominal diameter; NuMED, Hopkinton, NY) was inserted through the introducer into carotid circulation, advanced to the mid-segment of the carotid arteries, and deployed at 14 atm. The operator was not blinded to the group allocation. After retrieving the angioplasty catheter, the external carotid artery was tied-off, the fascia and skin were repaired, and the animals were recovered.

Vector dislodgment en route to the deployment site and in vivo release

Twelve stents were formulated with PrG/A20 tethers and incubated with $10^{10}$ VG of AAV2$_{GFP}$ as specified in the Vector Immobilization Protocol section. One group of AAV2$_{GFP}$ loaded stents (n=4) was mock-deployed on the bench and the expanded stents were removed from the catheters and stored at -20°C pending viral DNA isolation and PCR. A second group of stents (n=4) was inserted via an introducer into the external carotid arteries of rats and advanced to the mid-segment of the common carotid arteries. The stents were then withdrawn from the carotid artery without deployment. Immediately after withdrawal, these stents were expanded, removed from the catheters and stored at -20°C. A third group of stents (n=4) was inserted through the introducer, advanced into the mid-segment of the common carotid artery and deployed in vivo. The stented arteries were harvested 2 days post-stenting and the stents were carefully separated from the arterial tissue using a dissecting microscope. Viral DNA was isolated from all stent samples with a QIAamp DNA mini-kit and amplified with RT-PCR using GFP-specific primers.

Bioluminescence imaging

Stents were formulated with $7.5 \times 10^6$ VG of AAV2$_{Luc}$ immobilized on the stent surface with PrG/A20 tethering. Stents were then deployed in the Fogarty balloon-injured common carotid arteries of the male Sprague-Dawley rats (n=4). One, two, four and twelve weeks after stent implantation, the animals were anesthetized with isoflurane and buprenorphine and their vessels were harvested and stored at -20°C for 2 days post-stenting. The stented carotid segments were photomicrographed at 40x. The extent of restenosis, expressed as the neointima to media areas ratio, was derived from the morphometric measurements performed using Image J software. The investigator performing morphometry was blinded to the group allocation of histological samples.

Ki-67 immunohistochemistry

A subset of slides from the animals treated with BMS (n=4) and AAV2$_{NOS}$ eluting stents (n=4) were immunostained with anti-Ki67 antibody (1:100) to determine the prevalence of proliferating cells in the neointima, media and adventitia of the stented arterial segments. A secondary horse anti-rabbit biotinylated antibody (Vector labs, BA-1100), Vectorstain ABC Elite peroxidase kit (Vector labs, PK-6100) and 3, 3’ diaminobenzidine chromogen (Vector labs, SK-4105) were used as recommended by the manufacturer for color development. The slides were counterstained with Gill-3 hematoxylin, mounted and photographed at 200x. The number of Ki-67-positive cells in individual arterial compartments was determined for each image (Image J) by counting cell nuclei after converting the image into a black and white 16-bit image and adjusting threshold to visually match the original image. The number of proliferating cells was then normalized by the area of the respective arterial compartment in the captured image, and averaged over 4 images obtained for each slide.

Arterial expression of transgene following AAV2$_{NOS}$ stent implantation

Arterial segments treated with BMS (n=4) and AAV2$_{NOS}$-eluting stents configured with $4.5 \times 10^6$ VG of AAV2$_{NOS}$ immobilized on the stent surface via PrG/A20 tethering (n=4) were harvested at the animals’ sacrifice (day 8 post-stenting) and homogenized under liquid nitrogen in an ALT lysis buffer. RNA was extracted from the lysed arterial tissue using a RNeasy fibrous tissue mini kit (Qiagen, Germantown, MD), reverse transcribed using a Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), and the cDNA was amplified using human NOS sequence-specific primers (forward: 5′ TTTCCTTACAGGGCCGAGAAG 3′, reverse: 5′ AT CATTGTGTGGCAGATCT 3′). To assure the specificity of the primer set to human versus endogenous rat iNOS sequence, the reverse primer was chosen to bind in the Myc-SDK tag portion of the transgene.

Quantification of nitric oxide production by the stented arteries ex vivo

Rats were maintained on medicated water providing supplementation of arginine (250 mg/kg/day) and BH4 (10 mg/kg/day) for 2 days prior to stent implantation and until sacrifice using a previously optimized protocol. The animals were euthanized 8 days post-surgery. The harvested stented arterial segments were intraluminally flushed with sterile saline and placed into individual wells of a 24-well plate prefilled with F12/DMEM medium supplemented with 10% FBS and antibiotics. Non-manipulated left and right carotid arteries from 2 donor rats harvested at the same time as the stented arteries were also cultured ex vivo as the additional controls. Griess assays were performed on the media conditioned with the individual arteries 3 days after establishing ex vivo cultures.

Morphometric studies

BMS (n=10) and gene eluting stents ($8 \times 10^8$ VG of AAV 2iNOS immobilized via the PrG/A20 tether; n=10) were deployed in the Fogarty balloon injured carotid arteries of male Sprague-Dawley rats treated with arginine (250 mg/kg/d) and BH4 (10 mg/kg/d) for the duration of the experiment, starting at day 2 before the intervention. The animals were sacrificed 2 weeks post-stenting. The stented carotid segments were harvested, fixed in 10% neutral formalin and acid-treated to dissolve stent struts prior to routine paraffin embedding and sectioning. Verhoeff-van Gieson stained sections were photomicrographed at 40x. The extent of restenosis, expressed as the neointima to media areas ratio, was derived from the morphometric measurements performed using Image J software. The investigator performing morphometry was blinded to the group allocation of histological samples.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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