Efficient transduction of vascular smooth muscle cells with a translational AAV2.5 vector: a new perspective for in-stent restenosis gene therapy

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Coronary artery disease represents the leading cause of mortality in the developed world. Percutaneous coronary intervention involving stent placement remains disadvantaged by restenosis or thrombosis. Vascular gene therapy-based methods may be approached, but lack a vascular gene delivery vector. We report a safe and efficient long-term transduction of rat carotid vessels after balloon injury intervention with a translational optimized AAV2.5 vector. Compared with other known adenovirus-associated virus (AAV) serotypes, AAV2.5 demonstrated the highest transduction efficiency of human coronary artery vascular smooth muscle cells (VSMCs) in vitro. Local delivery of AAV2.5-driven transgenes in injured carotid arteries resulted in transduction as soon as day 2 after surgery and persisted for at least 30 days. In contrast to adeno-virus 5 vector, inflammation was not detected in AAV2.5-transduced vessels. The functional effects of AAV2.5-mediated gene transfer on neointimal thickening were assessed using the sarco/endoplasmic reticulum Ca2+ ATPase isofrom 2a (SERCA2a) human gene, known to inhibit VSMC proliferation. At 30 days, human SERCA2a messenger RNA was detected in transduced arteries. Morphometric analysis revealed a significant decrease in neointimal hyperplasia in AAV2.5–SERCA2a-transduced arteries: 28.36 ± 11.30 (n=8) vs 77.96 ± 24.60 (n=10) μm², in AAV2.5–green fluorescent protein-infected, P<0.05. In conclusion, AAV2.5 vector can be considered as a promising safe and effective vector for vascular gene therapy.

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INTRODUCTION

Coronary heart disease represents the leading cause of mortality and morbidity in the developed world, accounting for approximately one of every six deaths in the United States in 2006. It is estimated that every 25 s someone in the United States will have a coronary event, with one in four of these events being fatal.1 Atherosclerosis leads to the development of flow-limiting lesions that result in clinical symptoms such as angina pectoris or intermittent claudication. Moreover, unstable lesions undergoing plaque rupture and thrombosis result in myocardial infarction. Percutaneous coronary intervention involving coronary stenting corresponds to the major medical intervention in the developed world for both acute coronary syndromes and symptomatic chronic coronary artery disease and remains refractory to pharmacological therapy.2,3 Bare-metal stents efficacy was severely hampered by proliferating vascular smooth muscle cells (VSMCs), and the resultant neointimal hyperplasia, which is the only mechanism responsible for in-stent restenosis (ISR) after metal stent placement.4 The advent of drug-eluting stents (stent eluting drugs targeting VSMC proliferation) significantly reduced neointimal proliferation. However, anti-proliferative properties of drug-eluting stent impair and/or delay re-endothelialization, hence leading to late stent thrombosis. Consequently, there is a medical need for improvements in percutaneous coronary intervention outcomes and gene therapy is considered as a promising approach for the prevention of ISR and late-state thrombosis.

Numerous genes were identified using in vitro proof of concept experiments as a potential target for gene therapy of ISR.2,3 Inhibiting cell proliferation and migration by arresting VSMCs in G0/G1 phase of the cell cycle has been a common approach using cell cycle regulatory proteins, or manipulation of mitogens, transcription factors, cytokines, and growth factors, promoters of apoptosis or antioxidants. Preclinical gene therapy studies targeting intimal/neointimal hyperplasia in multiple diverse animal models have demonstrated the feasibility and potential of a gene therapy approach.2,5 Among potential gene therapy targets, the sarco/endoplasmic reticulum calcium ATPase isofrom 2a (SERCA2a) (SERCA2a gene was approved for gene therapy use in human; two clinical trials have been initiated aimed using SERCA2a as a target to treat heart failure6,7) was reported to prevent neointimal thickening in a rat carotid injury model and in a human ex vivo ISR model performed in an organ culture of the internal mammary artery.8,9 The molecular mechanism responsible for this effect involves the normalization of calcium cycling in VSMCs and shutting of the calcium-dependent transcription factor nuclear factor of activated T lymphocyte (NFAT), resulting in cell cycle arrest in G0/G1.8,10 SERCA2a expression is specific for contractile VSMCs. Forced expression of
SERCA2a in contractile VSMCs prevents injury-induced de-differentiation toward a synthetic/proliferating/inflammatory/migratory phenotype, whereas forced expression of SERCA2a in synthetic VSMCs has no effect on phenotype but prevents the proliferative/migratory response to extracellular stimuli.\textsuperscript{3,9,11}

The limiting factor in the development of local gene therapies for ISR is the lack of a safe and efficient vector system to transduce vascular cells within the arterial wall.\textsuperscript{2} The optimal delivery vector for vascular tissue should be efficient in transducing target vascular cells with minimal transduction of non-target cells, have low toxicity and immunogenicity and allow sufficient longevity of transgene expression so that an adequate and sustained clinical response can be obtained.\textsuperscript{2} Successful gene transfer in vascular cells \textit{in vivo} and \textit{in vitro} has been reported using adenovirus vector serotype 5 (Ad5), causing it to be the principal vector of choice in many preclinical studies.\textsuperscript{7,12}

However, Ad vectors induced immunological responses to transduced cells along with an extensive inflammation in the vessel wall.\textsuperscript{13,14} Transduced cells are rapidly eliminated through cytotoxic T-cell-mediated clearance resulting in a transient \textit{in vivo} gene expression, which peaks at 7–14 days and is lost by 28 days.\textsuperscript{15–17} Moreover, clinical efficacy of Ad5 vectors is hampered by pre-existing immunity (neutralizing antibodies) and high interactions with cellular and non-cellular blood factors. Indeed, intravascular administration of Ad5 vectors has been found to induce high levels of cytokines, tumor necrosis factor \textit{z}, interleukin-6 (IL-6), IL-12, interferon \textit{y}, IL-1 and the monocytes chemoattractant protein-1.\textsuperscript{18–20}

Recombinant viral vectors based on the nonpathogenic parvovirus, adeno-associated virus (AAV), which has gained popularity as a vector for gene therapy applications, have a number of attractive features for vascular gene therapy: (1) AAV can infect a wide range of host cells, including both dividing and non-dividing cells; (2) AAV has not been associated with any human or animal diseases and are relatively non-immunogenic; (3) AAV evokes sustained gene expression \textit{in vivo}.\textsuperscript{3,11} AAV-based vectors are approved to use in humans and several clinical trials have been undertaken using AAV-directed gene transfer.\textsuperscript{6,7,22}

A total of 12 different AAV human-tissue-derived serotypes are known (AAV1 through AAV12); each AAV serotype, determined by its capsid proteins, interacts with specific receptors and may account for the differential tissue tropism.\textsuperscript{21} For example, tropism of AAV2 is defined by binding to the heparan sulphate proteoglycan receptor.\textsuperscript{22} rAAV2 has been shown to transduce VSMCs and endothelial cells (ECs) \textit{in vitro} but its use in the vasculature \textit{in vivo} has produced conflicting results, including relatively insufficient transduction.\textsuperscript{24–27} rAAV1 and rAAV5, which bind to sialic acid residues from the cell surface, have demonstrated to be more effective than rAAV2 to transduce primary ECs and VSMCs \textit{in vitro}.\textsuperscript{28–30} Genetic engineering of the AAV capsid allows modifications of its immunogenicity, tissue-retargeting and efficiency, allowing the possibility for the development of novel vectors optimized for therapeutic administration.

Recently, a chimeric AAV capsid variant (designated AAV2.5) was developed by a rational design strategy\textsuperscript{22,31} intended to improve the muscle transduction capacity of AAV1 with reduced antigenic cross-reactivity against both parental serotypes, while maintaining AAV2 receptor binding. The initial development of AAV2.5 capitalized on the fact that AAV2 was the only serotype approved for clinical use, and AAV1 was the only other AAV serotype under serious consideration for clinical studies. AAV2.5 was generated from the AAV2 capsid with five mutations from AAV1, namely, four substitutions of AAV1 amino acids in the AAV2 VP1 background along with one insertion (Q263A, N705A, V708A, T716N, T265, AAV2 numbering).\textsuperscript{31} Recently, the AAV2.5-mediated minidystrophin delivery to skeletal muscle provided a preliminary insight to the clinical tolerability of this approach, with no vector related adverse events observed.\textsuperscript{22} Although the AAV2.5 vector now appears to have significant potential for direct skeletal muscle gene transfer, it is still unclear whether this vector will be able to provide sufficient transduction efficiency in vascular muscle cells, which would make it the vector of choice for clinical trials in ISR.

In this study, we report that AAV2.5 demonstrated low latency and the highest efficiency in terms of \textit{in vitro} transduction of human coronary artery VSMCs. In the rat carotid artery model, AAV2.5 mediated a safe, effective and long-term transduction of medial VSMCs. Furthermore, we report that AAV2.5-driven gene transfer of SERCA2a following injury of the rat carotid artery prevented neointimal hyperplasia in the injured segments.

RESULTS

Vascular healing in the rat carotid artery model of restenosis

The rat carotid injury model is a widely used animal model that reproduces the vessel wall changes seen in ISR. Balloon injury destroys the EC layer and places medial VSMCs in contact with blood serum and growth factors, which induces VSMCs dedifferentiation, migration and proliferation, resulting in neointimal thickening (The intima is the tunic of internal arterial vessel. In normal arteries the intima consists of an epithelium squamous single layer (endothelium) positioned on the basal internal elastic lamina (IEL). The neointima typically refers to scar tissue that forms within blood vessels post injury as a result of VSMC migration through IEL followed by proliferation in the luminal space.) Vascular healing consists of re-endothelialization of the injured segments and re-differentiation of VSMCs to contractile/quiescent phenotype. In order to identify the critical period for successful gene therapy intervention, we elucidate step-by-step processes of post-injury healing in a rat carotid model (Figure 1). We monitored the processes of re-endothelialization by immunolabelling with CD31 (platelet/EC adhesion molecule 1, a marker of ECs) and trans-differentiation of VSMCs by immunolabelling with smooth muscle myosin heavy chains 1 and 2 (a-SMMs) from day 2 to day 30 post injury. As expected, in control carotids the luminal part of the vessel is covered by an impenetrable EC layer (red) positioned directly on the IEL (Figure 1, left panel). Medial VSMCs, located between IEL and external elastic laminas, exhibited a migratory contractile phenotype, as indicated by immunolabelling with a-SMMs (red; Figure 1, right panel). As expected, balloon injury destroyed the EC layer and damaged the IEL: indeed, 2 days after injury CD31 was undetectable in injured segments and several gaps were observed on the IEL, giving the possibility for blood growth factors to infiltrate the media. (In rat model of carotid dilatation by a PCTA balloon catheter, the first step in allowing VSMC proliferation and migration to the intima is the occurrence of IEL rupture.)\textsuperscript{32}

De-differentiation of VSMCs from a contractile to a synthetic phenotype was already initiated by arterial injury with a resultant loss of SMMs (Figure 1, right panel). Four days after injury, most of the medial VSMCs acquired a synthetic/proliferating/inflammatory phenotype. At day 4, CD31-positive cells appeared to be sitting or hanging on the IEL, suggesting proliferation of remaining ECs or homing circulating EC precursors in the site of the injury. Seven days after injury, the luminal part of the injured vessel was entirely recovered by CD31-positive cells; importantly, the medial cells from the luminal part of the vessel were also identified as CD31-positive, suggesting trans-differentiation of a part of medial VSMCs into CD31-positive endothelial-like cells (Figure 1). However, the majority of medial VSMCs still exhibited a synthetic phenotype (SMMs-negative). At day 14, the luminal part of the vessel was recovered by a thin CD31-positive cell layer situated on the neointima; the majority of medial and neointimal VSMCs were SMMs-positive, suggesting growth arrest.
and re-acquisition of contractile phenotype. No difference was detected between 14 and 30 days post injury for these analyzed parameters (Figure 1).

This dynamic of vascular healing was confirmed by real-time quantitative reverse transcription-PCR analysis for α-smooth muscle actin, another marker of contractile VSMCs (Figure 2a). Relative expression of α-smooth muscle actin in injured segments dramatically decreased from 2 to 4 days after injury and slowly recovered at day 7 reaching the normal level at day 14. As expected, histological examination revealed a significant increase in intimal thickness associated with a slight increase in medial thickness at day 30 post injury, suggesting the proliferation of VSMCs in both the media and neointima.

Altogether these data demonstrated that in the rat carotid model the formation of neointimal lesions and vascular healing were completed at day 14. The decisive period for neointimal proliferation occurs within 2–14 days after injury, when the EC layer is destroyed and medial VSMCs undergo dedifferentiation. Therefore, this period can be considered suitable for gene therapy intervention.

Identification of the virus vector appropriate for gene therapy of restenosis
To identify the AAV vector appropriate for vascular gene therapy in humans, we compared the efficiency of different natural AAV vectors.
Transduction of the rat carotid artery with local delivery of AAV2.5 and Ad5 virus vectors

To compare the particularities of AAV2.5–GFP and Ad5–GFP in vessel transduction in vivo, we performed balloon injury of the rat left carotid artery followed by transduction with equivalent genome particles of both vectors (10^10 particles per rat). The animals were killed 2, 4, 7 and 30 days after surgery. The right carotid arteries were used as controls of non-injured, non-infected arteries. As elastin and collagen, both abundantly present in the vessel wall, exhibit green auto-fluorescence when excited at 488 nm, we analyzed the efficacy of GFP transduction by immunolabelling with an anti-GFP antibody (red; Figure 4, Supplementary Figures 1S and 2S). GFP expression was never observed in the non-injured, non-infected arteries. In injured and AAV2.5-transduced arteries, GFP expression was observed only in the extreme luminal part of the vessel 2 days following injury (Figure 4, left panel). In contrast, when the arteries were transduced with Ad5, all the medial cells were GFP-positive (Figure 4, right panel). Four days after surgery, GFP was expressed in the entire medial layer in the arteries transduced with both vectors (Figure 4, Supplementary Figures 1S and 2S). In AAV2.5-transduced arteries, a high level of GFP expression was observed throughout the media in all the experimental animals, but only a few positive cells were present in the adventitia and in the neointimal layer (Figure 4, left panel), confirming high affinity of AAV2.5 for muscular cells. In contrast, transduction of the vessels with Ad5 was not specific to VSMCs, as GFP-positive cells were also present in the adventitia (Figure 4, right panel).

Transduction of the medial cells with AAV2.5 remained stable up to 30 days after injury (the last time point examined), as attested by GFP expression detected only in the medial layer (Figure 4, left panel). In contrast to the AAV2.5 vector, vessels transduced with Ad5–GFP exhibited a transient expression pattern: 7 days after injury, only ~50% of medial cells were GFP-positive and at day 14 only few GFP-positive cells were detected in injured vessels (Figure 4, right panel). These differences in vessel transduction for both vectors were also observed by quantitative real-time reverse transcription-PCR analysis of GFP expression in injured vessels (Figure 5a). The relative expression of GFP messenger RNA (mRNA) levels was increased at day 2 and day 4 post injury for both vectors. At day 7 post infection, Ad5-mediated GFP expression was low and was undetectable at day 14, whereas AAV2.5-mediated GFP expression was still detectable 30 days after vector delivery (Figure 5a).

Next, we assessed the inflammatory response associated with the carotid injury and virus transduction in both experimental protocols by analyzing the relative expression of IL-1β (Figure 5b). As expected, vascular injury induced a local inflammatory response, as attested by increased IL-1β expression in injured segments compared with control segments. In accordance with

Transduction (Figure 3a). Flow cytfluorimetric analysis demonstrated that at this time-point AAV2.5-induced transduction of hCASMCs 10 times higher compared with the most vascular-tropic AAVs, which include AAV1, AAV5 and AAV2. Next, we compared the latency of AAV1, AAV2.5 and Ad5 vectors in hCASMCs. The cells were transduced with 10^5 MOI of the indicated AAV vector or with 100 MOI of Ad5 vector. The percentage of GFP-positive cells was determined 48 h after transduction (Figure 3b). As expected, a robust efficiency in the transduction of hCASMCs was observed with the Ad5 vector (~80% of cells). The AAV2.5 vector demonstrated in vitro a low latency, because it was detected at high levels (50% of GFP-positive cells) as soon as 48 h after infection compared with only ~10% with AAV1 hCASMCs-transduced cells (Figures 3b and c). Next, we compared the efficacy of AAV2.5 and Ad5 vectors in in vivo vessel transduction in the rat carotid injury model.

serotypes (AAV1, AAV2, AAV5, AAV8 and AAV9) with the synthetic AAV2.5 serotype and Ad5 to transduce human coronary artery smooth muscle cells (hCASMCs). Cultured hCASMCs were transduced with 10^5 multiplicity of infection (MOI) of different rAAV viruses, all carrying green fluorescent protein (GFP). The transduction efficiency of hCASMCs with different rAAV serotypes was estimated as a percentage of transduced cells on day 7 after gene therapy of restenosis.

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Figure 2. Transient trans-differentiation and neointima formation in rat carotid model of restenosis. (a) Relative expression of α-SMA (α-smooth muscle actin, a marker of contractile VSMC) in injured carotid segments at different time point after injury. Real-time quantitative PCR analysis. At least three different rat carotid samples were pooled for each time-point. (b) Representative hematoxylin/eosin staining of carotid artery cross-section. Objective ×10 (upper panel), ×60 (lower panel). (c) Morphometric analysis of carotid artery cross-sections. Bars represent the mean ± s.e.m. of mean values obtained for each animal. At least five individual measures were performed for each animal on different carotid cross-sections. a, adventitia; m, media; ni, neointima; lm, lumen.
Ad5 transduction is responsible for an extensive inflammation observed within 2–7 days in injured and infected vessels. Importantly, AAV2.5 did not cause an additional inflammatory response (Figure 5b). Thus, these results showed a high in vivo VSMC transduction efficacy of AAV2.5 with two considerable advantages compared with the Ad5 vector: a long-term expression and an absence of inflammatory response.

Effect of AAV2.5–SERCA2a gene transfer on cultured rat VSMCs

We have previously demonstrated that Ad5-driven SERCA2a gene transfer to synthetic cultured VSMCs restored SERCA2a expression to the levels observed in contractile VSMCs and prevented their proliferation and migration through the inhibition of the transcription factor NFAT.8,11 Here we tested whether a similar physiological effect could be obtained by using AAV2.5-driven SERCA2a gene transfer. Cultured rat aortic VSMCs were transduced with $10^5$ MOI of either AAV2.5–SERCA2a or Ad5–beta-galactosidase (βGal; control virus, Supplementary Figure 3S). In accordance with previous observations,8,13 un-infected (data not shown) and AAV1–βGal-transduced synthetic VSMCs exhibited low to undetectable SERCA2a expression (Figure 6a). Following AAV2.5–SERCA2a gene transfer, SERCA2a protein expression was markedly increased at day 4 (Figure 6a). Two weeks after the beginning of the experiments, SERCA2a expression in AAV2.5–SERCA2a-transduced cultures was almost as high as in contractile VSMCs of the rat aorta (Figure 6a). Notably, immunofluorescence analysis demonstrated that SERCA2a-positive cells poorly express NFAT downstream signaling protein cyclin D1 (Cyclin D1 mediates SMC proliferation and migration in NFAT-dependent manner.34) (Figure 6b), confirming the previous observations obtained with Ad5–SERCA2a-transduced VSMCs.8 As expected, AAV2.5-mediated SERCA2a gene transfer inhibited rat aortic VSMC proliferation and NFAT transcriptional activity (Figures 6c and d). Altogether these data demonstrated that in cultured rat aortic VSMCs, AAV2.5–SERCA2a gene transfer restored a stable long-term expression of functional SERCA2a protein to the levels observed in contractile VSMCs in vivo and prevented NFAT transcriptional activation and VSMC proliferation in the presence of serum.

Effect of AAV2.5-directed SERCA2a gene transfer on post-injury healing

We have previously reported that Ad5-mediated human SERCA2a gene transfer prevented neoimtimal proliferation in the rat carotid
injury model.8,10 Here we tested the functional effect of AAV2.5-mediated human SERCA2a gene transfer on neointimal proliferation in the same model. Balloon injured left carotid arteries were infected with AAV2.5–SERCA2a or AAV2.5–GFP (control virus) for 30 min directly after the injury and the animals were killed 30 days later. At the end point the endothelial regeneration of injured segments was complete in all groups of animals (Supplementary Figure 4S). No difference in endothelial regeneration was observed within saline-treated, AAV2.5–GFP or AAV2.5–SERCA2a-transduced vessels. Expression of human SERCA2a mRNA in injured and infected arteries was attested in all infected animals (n = 9) by real-time PCR performed with primers designed for the human SERCA2a isoform (Figure 7).

Morphometric analysis was performed on hematoxylin–eosin-stained carotid artery cross-sections (Figure 8). Adventitial thickness was increased in all injured arteries as a consequence of injury. One month after injury, abundant neointimal proliferation and medial thickening were observed in all injured saline-injected (n = 3) and AAV2.5–GFP-transduced vessels (n = 10). No differences were observed for these parameters between saline and GFP groups (neointima: 72.56 ± 19.26 vs 77.97 ± 24.59 μm², P = NS; media: 127.15 ± 20.99 vs 148.98 ± 16.05 μm², P = NS). The overall neointima area in injured and AAV2.5–SERCA2a-infected arteries was significantly less than in GFP-transduced arteries (28.36 ± 11.30 vs 77.96 ± 24.60 μm², P < 0.05, Mann–Whitney test; Figure 8b). The media area and total vessel wall area (media + neointima area) were also significantly decreased in SERCA2a-infected arteries compared with GFP-infected arteries. Thus, AAV2.5–SERCA2a expression significantly reduced post-injury proliferation and neointimal formation in the rat carotid injury model.

Figure 4. Transduction of vessels with AAV2.5–GFP (left panel) and Ad5–GFP (right panel) in rat carotid injury model. Confocal immunofluorescence showing GFP expression (red) on cross-sections of a rat carotid artery at different time-points after injury and transduction. Green, elastin autofluorescence. Typical pattern of spots observed in carotid artery cross-sections of all infected animals (at least three rats were used per time point). Bar = 20 μm. Total vessel wall transduction. a, adventitia; m, media; ni, neointima.
studies showing a stable (at least 1 month) rAAV transduction of
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36–38 Developed by rational design, the AAV2.5 vector
isolated with the use of phage display into the rAAV2 capsid have
the incorporation of VSMC-specific and EC-specific peptide ligands
ECs by genetic modification of the capsid proteins, which included
earlier studies undertaken to increase AAV tropism to VSMCs and
exposed cells were transduced with the above viruses. Numerous
mediated efficient gene transfer to VSMCs.

serotypes.
whereas maintaining AAV2 receptor binding,22,39 making it the
combines the improved muscle transduction capacity of AAV1,
whereas rAVV1 and rAAV5, which bind to sialic acid residues from
VSMCs of the vessel wall and to prevent exposure to blood flow, excessive
neointimal formation makes the neointima thick and rigid and prevents
injury, because the ECs and IEL might prevent AAV penetration into the
medial layer of the vessel.26,40 Furthermore, in agreement with previous observations made for rAAV2-mediated reporter gene expression in injured vessels, no expression of AAV2.5-mediated transgene was detected in the neointima of injured vessels.30,35 Restriction of AAV2.5-infected cells to the media layer of injured vessels suggests that there is no propagation of genetically modified cells.

In this study, efficient transduction of VSMCs from injured
segments was obtained by local administration of AAV2.5 vector
for only 30 min. We cannot exclude that direct intracoronary
injection of a virus vector during percutaneous coronary
intervention could incite systemic spread that can increase immune responses against the vector or its gene product, as well as increase the risk for side effects arising from gene expression in off-target regions. Local delivery of virus vectors from gene-
eluting stent surfaces could greatly increase AAV2.5 selectivity and
reduce possible off target gene transfer. Viral vectors can be
combined with biomaterials in coated stents either through
capsulation within the material or immobilization onto a
material surface. Subsequent biomaterial-based delivery can
increase the vector’s residence time within the target site, thereby
potentially providing localized delivery and enhancing selectivity of transduction.72 Bare-metal endovascular stents coated with a
synthetic complex for reversible binding of the virus vector were
already successfully tested on animal models of restenosis.42,43

Proliferation of VSMCs and resultant neointimal hyperplasia is
the only mechanism responsible for restenosis after stent
placement.2 Whereas some neointima formation is necessary for
vessel healing after stenting in order to imbed the stent within the
vessel wall and to prevent exposure to blood flow, excessive
neointima formation narrows the lumen.44 In both rats (this paper)
and humans,35 de-differentiation of medial VSMCs occurs within
2–4 days after surgery. In this paper, we demonstrated that
AAV2.5-driven proteins were already expressed in transduced
carotid vessels within the critical period for VSMC proliferation
induction: the first 2–4 days after surgery.

The transition of VSMC phenotype from contractile to synthetic
is associated with downregulation of functional protein entities
associated with the contractile response; we refer to voltage
activated L-type calcium channels, SR calcium release channel Ryr
and fast’ isoform of SR calcium pump S100A9.45 On the other hand, the expression of the molecular entities modulating the plasma membrane store-operated channel functioning, such as
Orai1-3, STIM1 and to the IP3 receptor channel, is highly
expressed in VSMCs.46 We have previously reported that forced
SERCA2a expression prevents de-differentiation of contractile
VSMCs in injured vessels.46,47 It is worth mentioning that store-
operated channel influx following agonist stimulation is not
observed in contractile VSMCs, naturally expressing SERCA2a,46
once again highlighting the importance of the SERCA isoform(s)
expressed in VSMCs. Conversely, gene transfer of SERCA2a to synthetic cultured VSMCs had no effect on VSMC phenotype or on the expression of store-operated channel sub-units, but prevented functional association of STIM1 and ORAI1/2 thereby preventing store-operated channel functioning and NFAT activity.\textsuperscript{11} Transcriptional inhibition of NFAT is sufficient to prevent proliferation and migration of synthetic VSMCs.\textsuperscript{8,11} Efficient long-term transduction of rat VSMCs was observed with AAV2.5–SERCA2a, which was sufficient to translate the functional effects of SERCA2a, such as inhibition of NFAT transcriptional activity, reduction of VSMC proliferation and neointimal hyperplasia, reported previously with Ad5-driven SERCA2a transduction of VSMCs.\textsuperscript{8–11} It is worthy to mention that somatic overexpression of SERCA2a has no effect on the expression of endogenous SERCA2b isoform,\textsuperscript{8,11} supporting the hypothesis that distinct SERCA isoforms are implicated in different physiological functions.\textsuperscript{46} Considering that in this study the virus vector was administered locally and under pressure to denuded carotid vessels, we assume that in our model VSMCs were principally infected. On the other hand, we cannot exclude that ECs adjacent to the site of injury could also be transduced in this model. We have recently reported that AAV1-directed SERCA2a overexpression in ECs enhances endothelial nitric oxide synthase expression.
and activity. Exogenous nitric oxide was shown to inhibit VSMC proliferation by specifically changing the expression and activity of cell cycle regulatory proteins. Thus, the probable transduction of adjacent ECs in our model could also contribute to reducing neointimal thickening.

We also documented the formation of moderate neointima in AAV2.5–SERCA2a-transduced rat carotid arteries, suggesting that vascular healing and re-endothelialization could be possible when SERCA2a gene transfer is used. Indeed, in contrast to cytotoxic drugs delivered by drug-eluting stent, SERCA2a controls VSMC physiological functions, allowing the possibility of vascular healing. Significantly, rigorous assessment of the safety of the intracoronary injection of AAV1–SERCA2a vector was performed in numerous pre-clinical and clinical trials, demonstrating safety and suggested benefits on heart failure in the absence of any organ damage or inflammatory response.

Similarly, we considered the effect of balloon injury or balloon injury followed by transduction with Ad5 or AAV2.5 vectors on the pro-inflammatory changes in the vascular secretory phenotype, by using IL-1β expression in carotid arteries segments as an indicator. Indeed, increased production of pro-inflammatory cytokines,

![Figure 7](image_url)

**Figure 7.** Transduction of vessels with AAV2.5–SERCA in vivo: long-term RNA expression. Real-time reverse transcription (RT)-PCR analysis of SERCA2a mRNA expression in rat carotid arteries 1 month after injury and transduction. SERCA2a mRNA was normalized to β-actin mRNA and compared with mRNA in sham-operated carotid arteries.

![Figure 8](image_url)

**Figure 8.** Effect of AAV2.5-driven SERCA2a gene transfer on post-injury vascular healing. Two groups of animals were analyzed: sham-operated control, injured and AAV2.5–GFP (n = 10) or AAV2.5–SERCA2a (n = 8) infected carotid artery 1 month after surgery. (a) Representative hematoxylin–eosin staining of carotid artery cross-section. Objective × 10 (upper panel), × 60 (lower panel). (b) Morphometric analysis of carotid artery cross-sections. Bars represent the mean ± s.e.m. of mean values obtained for each animal. At least five individual measures were performed for each animal on different carotid cross-sections. a, adventitia; m, media; ni, neointima; lm, lumen.
including IL-1β, is a cell-autonomous mechanism that contributes to the pro-inflammatory changes in vascular wall, even in the absence of infiltrating immunocytes.51 We have demonstrated that in contrast to the Ad5 vector, AAV2.5 does not provoke any specific pro-inflammatory changes in the vessel wall. Our data are in agreement with a recent study demonstrating the absence of a cellular immune response to AAV2.5 capsid following vector injection into the bicep of patients.52,53 Thus, AAV2.5 can be considered as the vector of choice for clinical trials of ISR.

We recognize that the transition to the clinical trial will highlight the variations in responses to treatments because of differences between diseased human coronary arteries of patients and of the rat carotid artery. Specifically, human atherosclerotic plaques demonstrate certain advanced features, such as ruptures, erosions and hemorrhages that are not presently mimicked by an animal model. Furthermore, in advanced human atherosclerotic plaques, dense fibrous matrix may serve as a barrier for virus vector-delivery.54 Furthermore, in advanced human atherosclerotic plaques, the common carotid artery and hemorrhages that are not presently mimicked by an animal model. Therefore, it is important to consider the potential clinical outcome.

Several clinical trials have been undertaken to investigate the safety and feasibility of percutaneous intracoronary gene transfer in humans, using either vascular endothelial growth factor coding sequence or antisense-oligodeoxynucleotides to the cell-cycle inhibitor p27 to target proliferation.55-59 All these trials suggested safety and feasibility of ISRG gene therapy, however, were not efficient in preventing ISR. The main reason for the lack of efficacy likely includes poor transduction efficiency of VSMCs in human atherosclerotic lipid-rich lesions with liposomes or Ad5 resulting in a potentially harmful biodistribution of the therapeutic gene. In addition, the presence of neutralizing antibodies to the Ad vector and interaction with circulating blood components also accounted for the lack of efficacy in preventing ISR in above clinical trials.2

In conclusion, AAV2.5 demonstrated the highest transduction efficacy of VSMCs in both rats and humans, low latency and reduced immunogeneity after in vivo gene transfer in the rat ISR model. Although balloon injury in rat carotid arteries models only some aspects of human ISR, this study suggests that AAV2.5 has great potential to be a new vector for future clinical trials aiming gene transfer to the vasculature.

MATERIALS AND METHODS

Virus vectors

Ad5 carrying GFP gene and β-gal gene under human cytomegalovirus early promoter was generated by RJ Hajjar.59 Adenovirus titer is expressed in MOI determined as the number of virus genome per cell. Following rAAV vectors were used: various AAV serotypes (rAAV1, rAAV2, rAAV2.5, rAAV6 and rAAV9, packaging genomes with AAV2 inverted terminal repeats) carrying GFP gene under cytomegalovirus promoter. Two AAV2.5 vectors (AAV2.5–GFP and AAV2.5–SERCA2a) carried GFP gene or human SERCA2a coding sequence, respectively under human cytomegalovirus promoter. AAV2.5 is a chimeric capsid described in detail previously.25 AAV2.5 is a chimeric AAV2 capsid variant in which four residues were substituted with AAV1 amino acid (Q263A, N705A, V706A, T716N, AAV2 numbering) and one AAV1 amino acid (T265, AAV1 numbering) was inserted.25,26 These mutations are all on the structurally variable regions of the virion surface (variable region I and variable region IX). AAV2.5 offers improved muscle transduction properties of AAV1 with minimal recognition by serum neutralizing antibodies.25 All these rAAV vectors were generated by Gene Therapy Center, University of North Carolina (Chapel Hill, NC, USA). AAV titer in units of vector genomes is expressed in MOI determined as the number of virus genome per cell.

Flow cyt fluorometric analysis

hCASMC (Lonza, Allendale, NJ, USA) were infected with 10⁵ MOI per cell with different rAAV–GFP serotypes or 100 MOI Ad5–GFP. Two or seven days after transduction cells were fixed with 1% paraformaldehyde-containing phosphate-buffered saline and subjected to flow cyt fluorometric analysis (Cell Quest Pro software, BD Biosciences, San Jose, CA, USA). GFP was excited by an argon laser and fluorescence was detected using a 530/30 nm band pass filter in the FL1 channel.

Rat carotid artery injury and gene delivery

The left external carotid artery from adult male Sprague–Dawley rats (Charles River, MA, USA) weighing 350 to 400 g was injured using a 2F Fogarty embolectomy catheter (Baxter Healthcare Corp., Maurepas, France) that was produced in-house. The common carotid artery through the external carotid and inflated to two atmospheres 3×20 s. After both the proximal common and the proximal internal carotid arteries were clamped, viral infusion mixtures containing ~10¹⁵ particles of virus vectors (Ad–GFP, AAV2.5–GFP or AAV2.5 SERCA2a), diluted to a total volume of 100 μl was instilled between the two clamps, and the external carotid artery was then ligated. The viruses were maintained in the artery for 30 min under pressure. Perfusion was restored through the internal and the common carotid artery after 30 min of instillation, and the neck incision was closed. At 2, 4, 7, 14 and 30 days after surgery the animals were killed. The left and right carotid arteries were dissected, flushed with saline, included in cymriatx and frozen at —80°C.

Real-time quantitative reverse transcription-PCR assays

Relative gene expression was determined using two-step quantitative real-time PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Cergy, France) following a easy step as described in the RNeasy Isolation Kit (Qiagen, Les Ulis, France) with on-column DNase I treatment to eliminate contaminating genomic DNA with RNase-free DNase Set (Qiagen). After annealing oligodT (1 μM) to template RNAs (0.5 μg) at 70°C for 5 min, primer extension was initiated by adding the Reverse transcriptase Moloney Murine Leukemia Virus enzyme plus 0.5 μM dNTP, 1 U RNasin and 10 mM dithiothreitol (DTT), and carried out for 45 min at 37°C. Quantitative PCR was performed using the LightCycler LC480 (Roche Diagnostics, Meylan, France). The PCR mix included 5 μl of each reverse transcriptase (diluted 1:25) and 300 μM of each primer in 1× LightCycler DNA SYBR Green 1 Master Mix. The forward and reverse primer sequences for complementary DNA were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA) according to European Molecular Biology Laboratory (Heidelberg, Germany) accession numbers: the human SERCA2a, 5′-CTGTCCATGTCACTCCACTCTC-3′ and 5′-AGGCGGTTACTCCAGTATTGCAG-3′; the rAAV–GFP: 5′-GGGAAATCGTGCGTGACATT-3′ and 5′-GGCCGAGTGCCATTCTCCT-3′; the rat hypoxanthine phosphoribosyltransferase, HPRT, gene: 5′-AGGACCTCTCGGAACTGTG-3′ and 5′-ATCCTCCAAGTGTCTTATATA-3′; the rat β-smooth muscle actin gene: 5′-ACCAGGATTTAGGACCC-3′ and 5′-CAGAGCCGACGAC-3′; the rat IL-1β gene: 5′-CAGTGTCAACGACACGACGAC-3′ and 5′-GGGTTGAGTGG-3′; the β-actin gene: 5′-CAGTGTCAACGACACGACGAC-3′ and 5′-GGGTTGAGTGG-3′. The PCRs were performed using the following thermal settings: denaturation and enzyme activation at 95°C for 5 min, followed by 40 cycles of 95°C (10 s), 60°C (15 s) and 72°C (15 s). Post-amplification dissociation curves were performed to verify the presence of a single amplification product and the absence of primer dimers. Controls and water blanks were included in each run; they were negative in all cases. Real-time quantitative PCR data represent the amount of each target mRNA relative to the amount of HPRT gene mRNA or β-actin, gene mRNA, estimated in the logarithmic phase of the PCR. Serial dilutions were used to determine the fit coefficients of the relative standard curve.

Morphometric analysis

Hematoxylin–eosin staining was performed on cross-sections. Specimens were measured by using Lucia G computer software (Laboratory imaging, Praha, Czech republic) and an optical microscope (Nanterre, France). We have measured the area of media and intima layers of vessels. The following groups were analyzed: (1) injured–non-infected (saline); (2) injured + AAV2.5 GFP; (3) injured + AAV2.5 SERCA2a; (4) the right carotid arteries were used as non-injured.

Confocal immunofluorescence

Immunostaining was performed using the following primary antibodies: a-GFP (Abcam, Paris, France), a-SERCA2a, a-cyclin D1 (556470, BD Biosciences); a-CD31 (Abcam); a-SMMS, smooth muscle myosin heavy chains 1 and 2 (Abcam); an-EOS, endothelial nitric oxide synthase (Abcam).
and secondary antibodies conjugated to Alexa-546 or Alexa-488. To avoid the auto-fluorescence of GFP, the arteries were fixed on acetone.9 Slides were examined with a Leica TCS4D confocal scanning laser microscope equipped with a 25 mW argon laser and a 1 mW helium-neon laser, using a Plan 10× objective or Plan Apochromat 63× objective (NA 1.40, oil immersion). Green fluorescence was observed with a 505–550 nm band-pass emission filter under 488 nm laser illumination. Red fluorescence was observed with a 560 nm long-pass emission filter under 543 nm laser illumination. Pinholes were set at 1.0 Airy units. Stacks of images were collected every 0.4 μm along the z axis. To allow comparison, all settings were defined on control arteries and were kept constant for all recording. For double immunofluorescence, dual excitation using the multitrack mode (images taken sequentially) was achieved using the argon and He/Ne lasers.

Cell culture, proliferation (BrdU) and NFAT-reporter assay
Rat aortic VSMC were isolated from the media of the thoracic aorta from male Wistar rats and cultured as described.8 To characterize rat aortic VSMCs, we used smooth muscle myosin heavy chain 1&2, smooth muscle alpha-actin, SM22, calponin and caldesmon, as previously described.8,61 Cells were infected with rAAV at 100 plaque-forming unit per cell. Proliferation was measured 4 days after transduction by BrdU incorporation during 48h using Cell Proliferation ELISA, BrdU (colori-meteric assay kit (Roche Diagnostics). For NFAT-reporter assay, cells were infected with AAV for 4 days, then transfected with NFAT promoter luciferase construct (NFAT-Luc, Stratagene, Amsterdam, Netherlands). The luciferase activity was measured by using commercial kit (Promega, Charbonnières, France) in relative luciferase units, normalized to the protein content and expressed as percentage of values in control wells. βGal activity was measured using commercial kit (Promega). All quantitative data presented as a means ± SEM of at least three independent experiments.

Protein preparation and immunoblotting analysis
Rat aortic VSMC were infected with 100 MOI per cell of AAV2.5–SERCA2a or AAV2.1–βGal and then cultured in Dulbecco’s modified Eagle’s medium supplemented with serum (10%). Cells were harvested 2, 4 and 14 days after transduction. Proteins were extracted by using Cell Extraction Kit (PromoKine, Promocell, Heidelberg, Germany). Protein concentration was determined using a Bradford assay. In all, 30 μg of proteins were then run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred on polyvinylidene difluoride membrane. Following primary antibodies were used for immunoblotting (α-GAPDH 1/2500, α-SERCA2a 1/2000). Proteins were visualized by using the Ettan DIGE Imager (GE Healthcare, Orsay, France).

Statistical analysis
All quantitative data are presented as mean of at least three independent experiments ± s.e.m. Data were analyzed by using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). A one-way analysis of variance followed by Tukey’s multiple comparisons test was performed for comparison of multiples values. Statistical comparison of two groups was done by a nonparametric Mann–Whitney test or an unpaired Student’s t-test. Differences were considered significant for values with P<0.05.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
A-ML, SWJMP, LL and RJH contributed to the conception, design, analysis and interpretation of all data; drafting the manuscript, revising it critically for important intellectual content and final approval of the manuscript. RJS and SWJMP designed and produced virus vectors. NM, A-ML, LL, EM, ZK and JC performed the in vivo gene transfer. A-ML, LL, EM and FA performed immunohistochemistry and morphometry experiments and the analysis of results. LL, EM and AM performed the in vitro data. LL, IY, RB and IL contributed to the quantitative reverse transcription-PCR analysis. LH led the fluorescence-activated cell sorting analysis.

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