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Cornerstone for an efficient cardiac gene therapy is the need for a vector system, which enables selective and long-term expression of the gene of interest. In rodent animal models adeno-associated viral (AAV) vectors like AAV-6 have been shown to efficiently transduce cardiomyocytes. However, since significant species-dependent differences in transduction characteristics exist, large animal models are of imminent need for preclinical evaluations. We compared gene transfer efficiencies of AAV-6 and heparin binding site-deleted AAV-2 vectors in a porcine model. Application of the AAVs was performed by pressure-regulated retroinfusion of the anterior interventricular cardiac vein, which has been previously shown to efficiently deliver genes to the myocardium (3.5×10^{10} viral genomes per animal; $n = 5$ animals per group). All vectors harbored a luciferase reporter gene under control of a cytomegalovirus (CMV)-enhanced 1.5 kb rat myosin light chain promoter (CMV-MLC2v). Expression levels were evaluated 4 weeks after gene transfer by determining luciferase activities. To rule out a systemic spillover peripheral tissue was analyzed by PCR for the presence of vector genomes. Selective retroinfusion of AAV serotype 6 vectors into the anterior cardiac vein substantially increased reporter gene expression in the targeted distal left anterior descending (LAD) territory ($65\,943 \pm 31\,122$ vs control territory 294 ± 69, $P < 0.05$). Retroinfusion of AAV-2 vectors showed lower transgene expression, which could be increased with coadministration of recombinant human vascular endothelial growth factor (1365 ± 707 no vascular endothelial growth factor (VEGF) vs $38\,760 \pm 2448$ with VEGF, $P < 0.05$). Significant transgene expression was not detected in other organs than the heart, although vector genomes were detected also in the lung and liver. Thus, selective retroinfusion of AAV-6 into the coronary vein led to efficient long-term myocardial reporter gene expression in the targeted LAD area of the porcine heart. Coapplication of VEGF significantly increased transduction efficiency of AAV-2.
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Introduction

Efficient long-term gene expression may be of crucial importance for successful gene therapy of cardiac diseases. A prerequisite for a clinically applicable myocardial gene transfer is cardio-selective expression of the therapeutic gene, minimizing systemic side effects. Selective myocardial gene expression might be addressed at different levels, that is, by a specific application system, the use of a vector efficiently transducing myocardium and transcriptional targeting using a cardio-selective promoter. An application system is required which allows a selective and homogenous gene delivery to the target territory. It has been previously demonstrated that transvascular (intracoronary or retrograde coronary venous) application of vectors for gene therapy is feasible.[1–14] We have chosen pressure-regulated retroinfusion of the coronary veins, as a catheter-based delivery system, which has been previously proven to be safe for human application.[15,16] Recently, it has been shown to provide efficient gene transfer to ischemic and nonischemic myocardium. This was demonstrated in case of cDNA transfer of vascular endothelial growth factor (VEGF) and heat shock protein 90 in a chronic porcine ischemia/reperfusion model.[17,18] With regard to application of an angiogenic protein (fibroblast growth factor-2)[19] and adenoviral gene transfer,[13] pressure-regulated retroinfusion was superior to intracoronary antegrade delivery. Furthermore, a 'head to head' comparison with percutaneous and surgical intramyocardial injection highlighted the more efficient and much more homogenous gene transfer by pressure-regulated retroinfusion.[14] With an effective regional application method a long-expressing vector is necessary

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to achieve a sufficient long-term myocardial gene transfer. Viral vectors based on the adeno-associated virus (AAV) offer the unique possibility of obtaining high level and long-term gene expression in the heart of small animal models without a significant immune or inflammatory reaction.[20,21] Nine AAV serotypes with heterogeneous organ tropism have been compared regarding their myocardial transduction efficiency so far exclusively in rodents.[22–29] However significant species-dependent differences in transduction characteristics exist and large animal models are of imminent need for preclinical evaluation of adeno-associated viral vectors.

In this study, we combined for the first time regional retroinfusion-mediated myocardial gene transfer in a large animal model (pig) with long-expressing adeno-associated viral vectors. We compared heparin binding-deficient AAV-2 vectors with AAV-6 vectors which both previously demonstrated to efficiently transduce murine myocardium after systemic application.[22,23] Additionally coapplication of vascular permeability factor (rhVEGF) for enhancement of myocardial transduction efficiency[23,30] was tested. For transcriptional targeting we have used the rat myosin light chain (MLC) 2v promoter fused with the cytomegalovirus immediate-early (CMV) enhancer which previously enabled a predominant expression in murine myocardium upon systemic vector administration.[22,31]

Results

Transduction efficiencies of heparin binding-deficient AAV-2 vectors and AAV-6 vectors

In the present study we analyzed the suitability of an AAV-mediated gene transfer via retroinfusion. We compared transduction efficiencies of AAV-2 vectors devoid of binding to its primary receptor heparan-sulfate proteoglycan with AAV-6-pseudotyped vectors. In all groups, the adeno-associated viral vectors (3.5×10^{10} viral genomes(vg) per animal) were applied to the left anterior descending (LAD)-dependent myocardium by selective pressure-regulated retroinfusion of the anterior interventricular vein. The groups were comparable in regard to body weight and hemodynamics at baseline and day 28. Coronary angiograms performed at day 0 and day 28 showed no evidence for compromised coronary flow. After 28 days retroinfusion-facilitated application of the vectors luciferase reporter gene expression in the LAD target territory was measured with a luciferase assay. Modified AAV serotype 2 showed moderate mean transgene expression in the target area (mean luciferase activity target area: 1365 ± 707 relative light units (RLU) per mg protein). AAV serotype 6 vectors enabled significant higher reporter activities in the target territory ($65\,943 \pm 31\,122$ RLU per mg protein) compared to AAV 2 ($P = 0.05$) (Figure 1). We further tested whether coapplication of recombinant human vascular endothelial growth factor (rhVEGF) facilitates gene transfer with the modified AAV serotype 2. Coapplication of VEGF significantly increased mean transgene expression in the target LAD territory (1365 ± 707 RLU per mg protein without VEGF vs $38\,760 \pm 2448$ RLU per mg protein with VEGF, $P = 0.0079$) (see Figure 1). Modified AAV serotype 2 with coapplication of VEGF almost achieved mean target area luciferase expression levels obtained after treatment with AAV serotype 6 ($38\,760 \pm 2448$ RLU per mg protein) (see Figure 1). There were no signs of systemic hypotension during and after application of rhVEGF (data not shown). Although not systematically tested, preliminary data indicate that VEGF also increases transduction with AAV-6 vectors as previously shown for mice.[23]

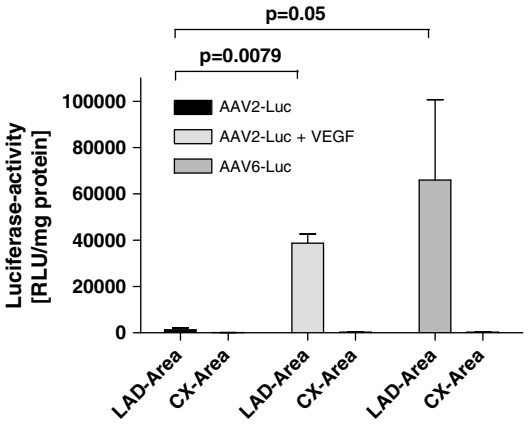

Figure 1 Luciferase activity in the left anterior descending (LAD) target area and the CX control territory 28 days after retroinfusion of viral vectors (3.5×10^{10} vg per animal). Adeno-associated viral (AAV)-2-Luc = AAV-2/CMV_{enh}-MLC1.5-Luc, AAV-2-Luc+ vascular endothelial growth factor (VEGF) = AAV-2/CMV_{enh}-MLC1.5-Luc+100 μg rhVEGF, AAV-6-Luc = AAV-6/CMV_{enh}-MLC1.5-Luc. Values are presented as means ± s.e.m.; $n = 5$ per group.

Selective gene transfer to the target LAD territory after retrograde delivery of modified AAV serotype 2 and AAV serotype 6 was confirmed by very low amounts of luciferase expression in all myocardial homogenates of the control region (CX territory) (see Figure 1).

In pigs treated with modified AAV serotype 2 with or without coapplication of VEGF and pigs treated with AAV serotype 6, a gradient from epimyocardial probes to endomyocardial probes and from proximal to distal LAD regions was observed (see Figure 2).

Hematoxylin and eosin (HE) staining of myocardial territory with high luciferase expression revealed intact myocardium without signs of leukocyte infiltration (Figure 3).

Transduction of extracardiac organs

Transduction of noncardiac tissue was measured by PCR analysis for vector genomes by PCR amplification of a distinct region of the luciferase reporter gene 28 days after gene transfer. In animals treated with AAV serotype 6, significant amounts of vector genomes were detected in liver and lung, but not aorta and skeletal muscle (Figure 4). Further PCR analyses of brain and gonads could not detect vector genomes. Although vector genomes were detectable in liver and lung, reporter gene expression after delivery of AAV-2 vectors without and with coapplication of VEGF as well as AAV-6 vectors was close to background in any extracardiac tissue compared to saline-treated animals. The cardiac expression pattern is most likely explained by the cardiac-specific activity of the CMV-MLC2 promoter (see Figure 5).

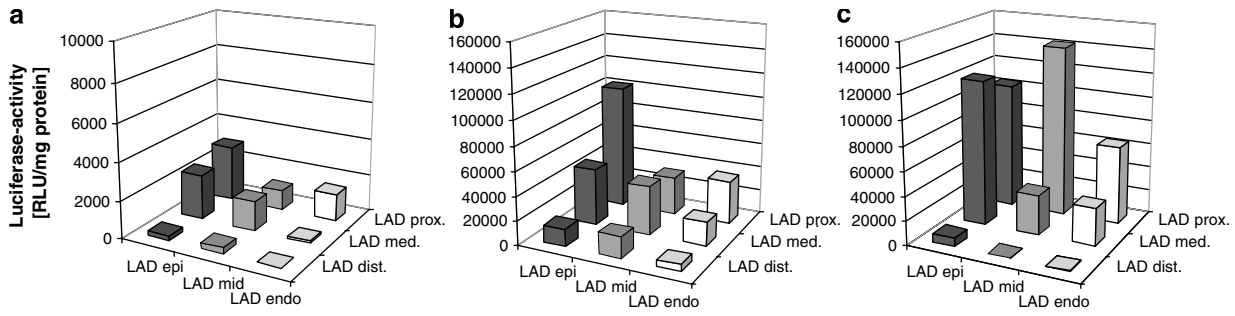

Figure 2 Luciferase activity in the left anterior descending (LAD) target area 28 days after retroinfusion of AAV-2/CMV_{enh}-MLC1.5-Luc (**a**), AAV-2/CMV_{enh}-MLC1.5-Luc+100 μg rhVEGF (**b**) or AAV-6/CMV_{enh}-MLC1.5-Luc (**c**). Note the different scale between pigs treated with AAV-2-Luc and pigs treated with AAV-2-Luc in combination with VEGF. Values are presented as means.

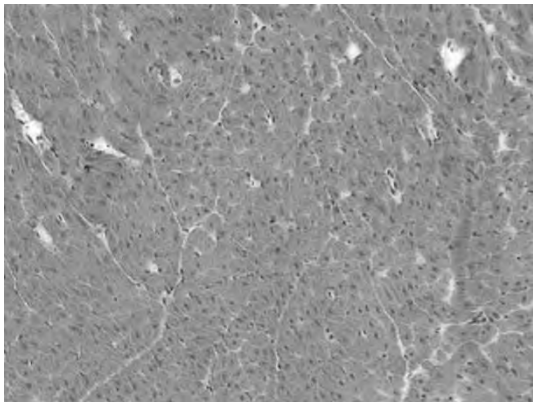

Figure 3 HE staining of a representative LAD area with high luciferase activity after AAV-6-Luc delivery (AAV-6/CMV_{enh}-MLC1.5-Luc, 3.5×10^{10} vg per animal).

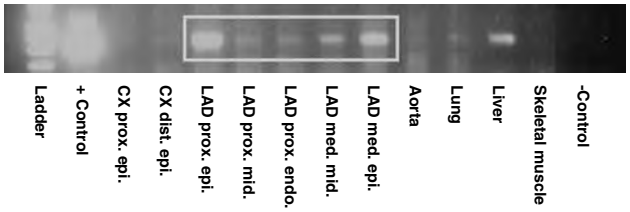

Figure 4 Detection of vector genomes by PCR in the target area (light-blue box) and nontarget area as well as extracardiac samples (aorta, lung, liver, skeletal muscle) using luciferase primers after retroinfusion of 3.5×10^{10} genomic particles of AAV-6/CMV_{enh}-MLC1.5-Luc. The 677 bp luciferase band is faint in cardiac nontarget tissue, but clearly visible in samples from the target area and liver.

Discussion

Aim of our study was to develop a delivery system for efficient long-term gene transfer into the myocardium in a large animal model. Combination of selective retroinfusion into the anterior interventricular vein with AAV vectors resulted in a significant luciferase reporter gene expression in the LAD target area, but not the LCX nontarget area after 4 weeks. We have chosen selective retroinfusion since this percutaneous technique is superior to intracoronary antegrade delivery of angiogenic proteins[19] and adenoviral vectors[13] and results in a more homogenous gene transfer than intramyocardial injections.[14] A major limitation of intracoronary infusion is the short adhesion time of the vector with the coronary endothelium. Thus, the intracoronary applied vector will be quickly distributed throughout the systemic circulation. Variables that can determine efficiency of viral gene delivery via the coronary circulation are coronary flow, vector concentration and endothelial permeability, as systematically analyzed in isolated hearts.[30,32,33] Interestingly, it has been previously demonstrated that an increased vector residence time and perfusion pressure in the coronary vessels enhance myocardial gene transfer.[30,34] Selective pressure-regulated retroinfusion of the coronary veins prolongs adhesion time of the vector with the cardiac endothelium and increases endothelial permeability.[14,19] The increased transduction efficiency in the VEGF approach might be explained by an additional increase in vascular permeability as observed previously in systemic transduction of mice with AAV vectors or intracoronary transfer in rabbits using adenoviral vectors.[23,30]

We have used AAV vectors since they enable a long-term gene expression in the heart in the absence of an immune or inflammatory reaction in small animal models.[8,20,21] Recent advances in AAV vector development resulted in vectors that can be applied efficiently via a transvascular route—at least in rodents.[2–5,8] These so called pseudotyped vectors take advantage of the ability of certain AAV serotypes to efficiently cross the blood vessel barrier. Systemic application of pseudotyped AAV-6, -8 or -9 vectors resulted in uniform and extensive transfer of a lacZ reporter gene in adult mice.[23–26] Intravenous injection of AAV-8 vectors enabled reconstitution of δ-sarkoglycan in δ-sarkoglycan-deficient TO-2 hamsters[27] and extended their lifespan by preventing heart failure in this animal model of dilated cardiomyopathy.

We used AAV-2 (R484E, R585E) and AAV-6 vectors since they lack heparin binding avoiding neutralization by heparin necessary for the retroinfusion protocol. We previously showed that inactivation of two heparin-binding motives at positions 484 and 585 resulted in detargeting from its primary receptor, heparan-sulfate proteoglycan.[35] Intravenous injections in mice enabled an increased transduction of the heart, while hepatic transduction was decreased. However, transduction efficiencies in the porcine model were close to background without additional administration of VEGF most

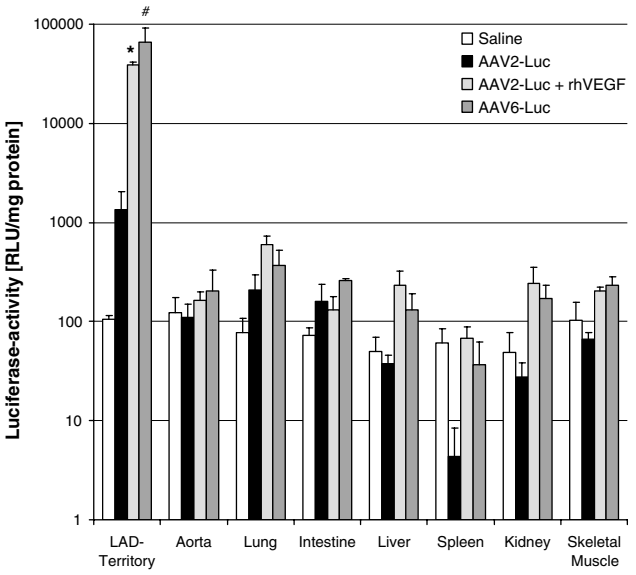

Figure 5 Luciferase activity in the left anterior descending (LAD) target area and selected systemic organs 28 days after retroinfusion of viral vectors (3.5×10^{10} vg per animal) or saline as a negative control. Adeno-associated viral (AAV)-2-Luc = AAV-2/CMV_{enh}-MLC1.5-Luc, AAV-2-Luc+ vascular endothelial growth factor (VEGF) = AAV-2/CMV_{enh}-MLC1.5-Luc+100 μg recombinant human vascular endothelial growth factor(rhVEGF), AAV-6-Luc = AAV-6/CMV_{enh}-MLC1.5-Luc (saline, saline-treated negative control group). Values are presented as mean ± s.e.m.; $n = 5$ per group for AAV-treated groups, $n = 3$ for saline control group; $^{\#}P = 0.05$ AAV-6-Luc vs AAV-2-Luc; $^{*}P = 0.0079$ AAV-2-Luc+rhVEGF vs AAV-2-Luc.

probably due to a limited ability of the viral vector to cross the endothelial barrier. In contrast, AAV-6 vectors resulted in significantly higher transmural transduction levels in the LAD target area, suggesting that—like in mice—AAV-6 vectors are suitable for a cardiac gene transfer via a transvascular route.[22,23,36]

Detection of vector genomes in tissue samples revealed that the increased luciferase reporter activities in the target area were associated with presence of more vector genomes in the LAD area, suggesting a predominant gene transfer into the target region. Although significant amounts of vector genomes were detected in liver and lung, absence of luciferase expression in these organs underline the specificity of the CMV-enhanced MLC-promoter sequence.

In summary, our study for the first time demonstrates the ability of retroinfusion to efficiently transfer AAV vectors. Upon approval in preclinical studies this system may be applicable in clinical gene transfer protocols of cardiac diseases.

Materials and methods

Generation of adeno-associated viral vectors

All AAV vectors used in this study were produced using a two-plasmid system.[35,37] The plasmid providing the reporter construct to be encapsidated was described previously[22] and harbors the firefly luciferase reporter gene under control of the 1.5 kb rat MLC 2v promoter[38] fused to a CMV enhancer.[22]

For production of AAV-2 vectors with detargeted capsids we used Pdg (R484E; R585E) containing mutations of two amino acids involved in heparin binding of AAV-2 (R484E; R585E).[35] Cross packaging of AAV-6-pseudotyped vectors was accomplished with pDP6.[37] After 2 days, cells were harvested and viruses were purified using iodixanol gradients.[39] Gradient fractions were concentrated using VIVASPIN columns (Sartorius, Göttingen, Germany) thereby exchanging iodixanol against phosphate-buffered saline. The AAV genomic, capsid and replicative titers were determined as described previously.[40]

Gene transfer using selective pressure-regulated retroinfusion

The present investigation was carried out according to the *Guide for the Care and Use of Laboratory Animals* and was approved by the Bavarian Animal Care and Use Committee.

Fifteen German farm pigs (mean body weight 22 ± 3 kg) were anesthetized and monitored as described previously.[14,19,41,42] Catheter introducer sheaths were placed in the right carotid artery and right external jugular vein. Full anticoagulation was achieved by bolus injection of heparin 10 000 IU followed by continuous application of 5000 IU h^{-1}. For selective retroinfusion, the anterior interventricular vein was catheterized using a 6F retroinfusion catheter.[13–15,17,19,41–43] In all animals a 7F guiding catheter was placed in the left coronary artery and the LAD was wired. During retrograde delivery of the AAV vectors, the LAD artery was occluded by a PTCA balloon distal to the first diagonal branch in all groups.

For regional application of adeno-associated viral vectors a modified technique of continuous pressure-regulated retroinfusion[13,17,19] was used with inactivation of the suction device.[14] Briefly, the high-pressure reservoir (2.5 atm) was filled with saline solution (0.9%) which was kept at 37 °C. The adeno-associated vector solution (3.5×10^{10} vg diluted in 20 ml saline) was delivered during 2×10 min of continuous retroinfusion. Between the retroinfusion periods, 5 min of reperfusion were allowed to avoid tissue damage due to myocardial infarction. Animals were treated with AAV-2/CMV_{enh}-MLC1.5-Luc ($n = 5$) or AAV-6/CMV_{enh}-MLC1.5-Luc ($n = 5$). Since coadministration of VEGF has shown a positive effect on transduction efficiencies most probably due to increased vascular permeability,[23] we additionally compared the effect of 100 μg rhVEGF (Prospec Technogene, Rehovot, Israel) on transduction with the modified AAV-2 vectors ($n = 5$). Saline-treated animals were used as negative control group ($n = 3$).

At the end of the experiment all catheters and introducer sheaths were removed. After 4 weeks the animal was anesthetized again, intubated and catheter introducer sheaths were placed as described above. The patency of the LAD artery was confirmed by coronary angiography. Upon a lethal dose of potassium, the heart was excised and the left ventricle was cut into slices of 1 cm thickness from the apex to the basis as described previously.[14] Each slice was divided into eight transmural segments and each segment was separated equally into an epimyocardial, a midmyocardial and an endomyocardial sample.

Analysis of gene transfer

Transduction of the myocardium as well as peripheral tissues was determined using PCR or Luciferase assays.

PCR amplification of vector DNA was performed for selected myocardial samples from the target (LAD territory) and a control region (RCX territory) as well as from selected systemic organs (aorta, lung, liver, muscle, brain, gonads). Genomic DNA was extracted from tissue samples using the Qiamp Tissue Kit (Qiagen, Hilden, Germany). A total of 800 ng genomic DNA was used for PCR amplification (40 cycles) of a 677 bp fragment of the luciferase reporter gene using the primers 5′-GACGCCAAAAACATAAAGAAAG-3′ and 5′-CCAAAAATAGGATCTCTGGC-3′. PCR products were analyzed by gel electrophoresis (1.5%; w/v).

Reporter gene activity (luciferase activity) in tissue homogenates from myocardial samples and selected systemic organs was quantified as described previously.[13] Luciferase activities in RLU are related to the protein concentration in each sample determined with a protein assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Statistics

All values are presented as mean ± s.e.m. Measurements of luciferase expression were analyzed by a nonparametric Kruskal–Wallis test. Whenever a statistically significant effect was obtained, we performed multiple comparison tests between the individual groups using the Mann–Whitney *U*-test. A *P*-value below 0.05 was considered to be statistically significant.

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