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ORIGINAL ARTICLE

Efficacy and safety of myocardial gene transfer of adenovirus, adeno-associated virus and lentivirus vectors in the mouse heart

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Gene therapy is a promising new treatment option for cardiac diseases. For finding the most suitable and safe vector for cardiac gene transfer, we delivered adenovirus (AdV), adeno-associated virus (AAV) and lentivirus (LeV) vectors into the mouse heart with sophisticated closed-chest echocardiography-guided intramyocardial injection method for comparing them with regards to transduction efficiency, myocardial damage, effects on the left ventricular function and electrocardiography (ECG). AdV had the highest transduction efficiency in cardiomyocytes followed by AAV2 and AAV9, and the lowest efficiency was seen with LeV. The local myocardial inflammation and fibrosis in the left ventricle (LV) was proportional to transduction efficiency. AdV caused LV dilatation and systolic dysfunction. Neither of the locally injected AAV serotypes impaired the LV systolic function, but AAV9 caused diastolic dysfunction to some extent. LeV did not affect the cardiac function. We also studied systemic delivery of AAV9, which led to transduction of cardiomyocytes throughout the myocardium. However, also diffuse fibrosis was present leading to significantly impaired LV systolic and diastolic function and pathological ECG changes. Compared with widely used AdV vector, AAV2, AAV9 and LeV were less effective in transducing cardiomyocytes but also less harmful. Local administration of AAV9 was safer and more efficient compared with systemic administration.

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INTRODUCTION

The most common cause of death worldwide are cardiovascular diseases, including coronary artery disease and heart failure.[1] Current treatment strategies for ischemic diseases include prevention of disease progression with lifestyle changes and medication, and for patients with critical symptoms, revascularization procedures are applied. However, these conventional therapies cannot be performed for everyone, especially for elderly patients with several comorbidities. Therefore, there is an obvious need to develop efficient and minimally invasive treatment methods for these no-option patients.[2,3] Gene therapy is a promising novel treatment modality for cardiac diseases, and it has shown great potential and efficacy in pre-clinical trials.[2,4–6] Currently, there are five ongoing clinical gene therapy trials aiming to treat patients with coronary artery disease and four trials for developing heart failure treatment. Most commonly, adenovirus (AdV) and adeno-associated virus (AAV) vectors are used for delivering therapeutic genes into myocardium with intracoronary infusion, percutaneous or open-chest intramyocardial injection.[3] In previous cardiovascular clinical trials, the safety has been excellent even in the long-term follow-up.[7,8] However, none of the phase-II/III cardiovascular gene therapy trials have shown clinically relevant positive effects for several reasons, of which low gene transfer efficiency has been associated with several trials. In addition, a lack of a sophisticated efficient delivery method and lack of clinically relevant animal models have contributed to the problem.[2,3,6,9]

AdV vectors have thus far been the most widely used vectors in cardiac gene therapy. They are highly efficient in transducing cardiomyocytes,[4,10] but transgene expression is transient peaking a few days after the gene transfer and lasting for 2–4 weeks.[9,11] AAV vectors are promising gene delivery vectors that provide long-term transgene expression lasting for several months.[11–13] AAV serotype 2 (AAV2) has been commonly used for gene therapy studies, but it has only a moderate transduction efficiency in the heart.[14,15] AAV9 is the most efficient AAV vector of serotypes 1–9 for cardiac gene transfer and has fast onset of gene expression both by systemic route[16] and after direct injection to the left ventricle (LV) wall.[17] Lentivirus (LeV) vectors integrate transgenes into the host genome, thereby providing potential for lifelong expression of the therapeutic protein.[18] LeV has not been widely used for cardiac gene therapy, but it has shown some efficiency in transducing cardiomyocytes in murine models.[18–22] To our knowledge, there are currently no preclinical studies or ongoing clinical trials describing closed-chest/percutaneous intramyocardial gene transfer with AAV or LeV vectors.

In this study, we compared AdV, AAV2, AAV9 and LeV vectors for cardiac gene therapy with regards to transduction efficiency, myocardial damage and cardiac function by transthoracic

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echocardiography (TTE) and electrocardiography (ECG) in order to find the most efficient and safest vector for cardiac gene therapy. We used a sophisticated closed-chest intramyocardial injection method with ultrasound-guidance allowing minimally invasive local gene transfer with one injection through the skin straight into the LV wall. The gene transfer efficacy, safety and biodistribution after systemic AAV9 injections with three viral doses was studied. We show here that compared with widely used AdV vector, AAV2, AAV9 and LeV were not only less effective in transducing cardiomyocytes but also less harmful. Local administration of AAV9 was found to be safer and more efficient compared with systemic administration.

RESULTS

In vivo toxicity

During the follow-up of 28 days, two mice from the AAV9 LacZ intravenous (i.v.) 10^{12} dose group were found dead at day 26. The cause of death remains unknown, but on the previous day, one of the mice was unwilling to move and the body temperature was lower than normal. We did not notice any changes in the behavior of animals in the other treatment groups. There were no macroscopic abnormalities in the collected organs (lung, liver, spleen, kidneys, rectus femoris muscle or testis) at day 28. Changes in the weights of the mice in all groups were within 10% of the d0 weight during the follow-up, indicating that the mice did not have any serious welfare problems.

Transduction efficiencies

Intramyocardial gene transfer of AdV LacZ, AAV2 LacZ, AAV9 LacZ and LeV green fluorescent protein (GFP) led to local transgene expression close to the injection needle tract (Figures 1a–d, arrows). The transduction efficiency in the maximally transduced area near the needle tract in intramyocardially injected mice was quantified as the percentage of transgene-positive cardiomyocytes (Figure 1k). Gene transfer with AdV LacZ resulted in the highest transduction efficiency at d28 (Figures 1e and k). Earlier at d6, the transduction efficiency was slightly lower being 38 ± 9% (data not shown). The transduction efficiencies of AAV2 and AAV9 were at similar level compared with each other but about 50% lower than AdV transduction efficiency at d28 (Figures 1f, g and k). With LeV, the transduction efficiency was about 25% of AdV efficiency (Figures 1h and k). Corresponding to the localization of GFP immunostained cells near the needle tract, GFP-positive cells could be visualized also with native green fluorescence in LeV GFP-injected hearts (data not shown).

After systemic administration of AAV9 LacZ, the transgene expression could be seen throughout the myocardium (Figures 1i and j). For comparing the transduction efficiencies of intramyocardial injections with i.v. injections, the transgene-positive cell coverage was quantified as the size of the transduced area (percentage of the LV area). The transduced area was the largest 6 days after AdV delivery (19 ± 7% of the LV area, result not shown), decreasing toward d28 time point (Figure 1i). Intramyocardial gene transfer of AAV2 LacZ resulted in 10 ± 8%, AAV9 LacZ in 15 ± 3% and LeV GFP in 7 ± 3% transduced areas at d28. In the AAV9 LacZ i.v. group, the transduced area size was 8 ± 8% with the 10^{12} dose and decreased markedly with decreasing doses (Figure 1l).

Myocardial damage

To evaluate the myocardial damage associated with the gene transfers, the amount of fibrosis and inflammation was analyzed from the myocardium. From the intramyocardially injected hearts, the size of the fibrotic scar area was quantified (percentage of the LV area) and amount of inflammation scored with the scale of 0–3. Intramyocardial gene transfer produced a local fibrotic scar area with lymphocyte-intensive inflammatory reaction at the injection site in the LV wall 28 days after the gene transfer (Figures 2c–f, arrows). Apart from the local injury site and its instant vicinity, the morphology of the myocardium was normal in all the study groups. Size of the scar area was the largest after both 'empty' AdV cytomegalovirus (CMV) carrying no transgene and AdV LacZ injection, followed by AAV9 and AAV2 (Figure 2a). LeV led to the smallest scar in the LV wall. Only a minimal scar area (about 1% of the LV area) representing the needle tract was seen in the Needle and NaCl control groups. Similar trend between the groups was seen in the amount of inflammatory cells within the scar area (Figure 2b); both AdV gene transfers led to a severe inflammation followed by AAV9 gene transfer. After AAV2 and LeV injections, mild-to-moderate amount of inflammation was seen. In the Needle and NaCl groups, a few lymphocytes were present in the needle tract, and in addition, a few hypertrophic and/or degenerated myocytes were seen near the needle tract in 50% of the mice in the NaCl group (data not shown). Also, after AdV, AAV2 and AAV9 gene transfers, some degenerated and hypertrophic cardiomyocytes were present around the scar area in all the samples. Instead, in the LeV group a few hypertrophic cardiomyocytes were seen around the scar only in about 30% of the samples. Small amounts of lipofuscin pigment, which is an intralysosomal undegradable waste product accumulating as a response to oxidative damage,[23] was found within few cardiomyocytes in the scar area of intramyocardially treated groups apart from AdV LacZ (data not shown).

With systemic AAV9 injection, both fibrosis and inflammation were assessed by scoring with the scale of 0–3. I.v. administration of AAV9 LacZ with 10^{12} dose led to diffuse fibrosis throughout the myocardium, the amount of which was scored to be moderate (Figures 2g, i and j). In the AAV9 10^{11} and 10^{10} groups, there was only minor fibrosis, if any, seen as small focal spots of the scar tissue and morphology appeared to be mainly normal (Figures 2g, k and l). Similarly, the amount of inflammatory cells, mainly lymphocytes, was moderate in myocardium after AAV9 LacZ 10^{12} treatment and was decreased to modest to none in the 10^{11} and 10^{10} groups (Figure 2h). In the 10^{12} dose group, a few degenerated and hypertrophic cardiomyocytes were seen around the fibrotic areas; instead, in the 10^{11} and 10^{10} dose groups, morphology was mainly normal (data not shown). No lipofuscin pigment was seen in the AAV9 LacZ i.v. injected groups (data not shown).

LV size and function

The effects of the gene transfers to the LV size and function were quantified with echocardiography. In the intramyocardially injected hearts, acute edema at the injection site was seen as an increase in LV anterior wall (LVAW) thickness in diastole 6 days after gene transfers especially in the AdV CMV and AdV LacZ groups, and also to a smaller extent in the NaCl, AAV2 and LeV groups (Figure 3a). In addition, AdV LacZ gene transfers led to thinning of the LVAW at d28 owing to fibrosis at the injection sites (Figures 3a, g and j). In the AdV LacZ group, the LV end-diastolic diameter (LVEDD, Figure 3b) and LV volume (data not shown) were increased at d28, indicating LV dilatation. Also systolic LV function measured as Teicholz ejection fraction (EF) was significantly decreased in the AdV LacZ group at d28 owing to almost akinetic LVAW at the site of the injection scar, instead the LV posterior wall (LVPW) was contracting normally (Figures 3c, g and j).

Intramyocardial injection of AAV2 or AAV9 did not change the LVEDD (Figure 3b) or the systolic function (Figure 3c). AAV9 caused diastolic dysfunction to some extent, as the left atrium (LA) area (Figure 3d) and mitral valve (MV) *E/A* ratio was somewhat increased (Figure 3e). LeV did not change the LVEDD (Figure 3b) and it did not affect the cardiac function (Figures 3c–e). Also, in the AAV2, AAV9 and LeV groups, a local hypokinetic spot was seen at the injection site in the LVAW corresponding to the size of the

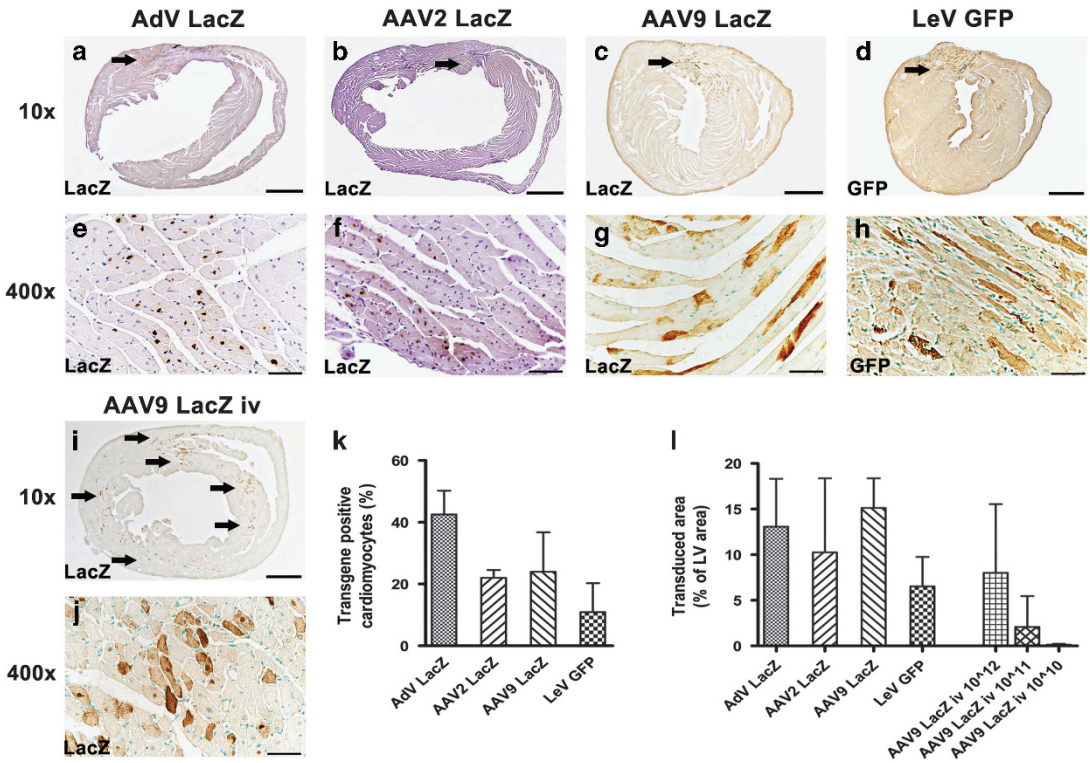

Figure 1. Gene transduction efficiencies of the viral vectors in the myocardium 28 days after gene transfer. Representative images of immunohistological stainings of the transgene expression after intramyocardial delivery of AdV LacZ (**a**, **e**), AAV2 LacZ (**b**, **f**), AAV9 LacZ (**c**, **g**) and LeV GFP (**d**, **h**) with the local transgene expression close to the needle track in the LVAW (arrows). LacZ stainings from the LacZ-transduced groups (**a**–**c**, **e**–**g**, **i**, **j**) and GFP stainings of LeV GFP-transduced group (**d**, **h**) seen with ×10 (**a**–**d**, **i**, scale bar 1000 μm) and ×400 (**e**–**h**, **j**, scale bar 50 μm) magnifications. Quantification of transgene-positive cardiomyocytes in the maximally transduced area near the needle tract (**k**). Representative images of LacZ-stained histological sections after systemic administration of AAV9 LacZ (10^{12} dose) with global diffuse transgene expression in the whole myocardium (**i**, **j**). The quantified size of the transduced area (percentage of LV area) after intramyocardial and systemic gene transfer (**l**). Results are represented as mean ± s.d., $n = 5$ per group, except in AdV LacZ and LeV GFP $n = 7$.

scar, leaving other parts of the LV non-affected or even hyperkinetic.

Surprisingly, intramyocardial injection of NaCl led to dilatation of LV (Figure 3b) and decrease in systolic function (Figure 3c) seen in TTE as global hypokinesia (Figures 3f and i), although the myocardial damage was minimal at the histological level (Figures 2a–c). The injection site at the LVAW could be visually detected in TTE only in one mouse out of six as a small hypokinetic spot in the LV wall (data not shown).

After systemic gene transfer of AAV9 LacZ with 10^{12} dose, the systolic LV function was significantly decreased at d28 (Figures 3c, h and k). The decreased EF was a result of global hypokinesia of the LV owing to extensive fibrosis (Figures 2g, i and j). A significant increase in the LA area (Figures 3d, l and m) and an increase in MV *E/A* ratio (Figures 3e, n and o) was seen, indicating severely impaired diastolic function. The increased *E/A* ratio was explained by a marked decrease in MV *A* peak, whereas no significant changes were seen in MV *E* peak (Figures 3n and o, data not shown). No significant changes were seen in TTE results in the groups of AAV9 LacZ i.v. gene transfers with viral doses of 10^{11} and 10^{10} viral genomes (vg) during the follow-up (data not shown).

Electrocardiogram

To further characterize the function of the LV, the ECG signal (lead II) was analyzed. The heart rate of the mice varied between 420 and 520 b.p.m. during the isoflurane anesthesia (data not shown). There were no significant changes in the ECG parameters in the NaCl group at d28 apart from shortened PQ time and an increased S amplitude, evidently due to a higher heart rate and an increased LV volume, respectively (Figures 4a–c). There were no changes in QRS time in any of the groups, meaning that there were no marked defects in ventricular depolarization. However, there was significant increase in QRSp time in the AdV CMV group and also to some extent in the AAV9 LacZ i.v. 10^{12} group at d28 ($P = 0.056$ compared with d0), indicating changes in the beginning of repolarization. Decreased *R* amplitude was seen in AdV CMV, AdV LacZ, LeV GFP and most markedly in the AAV9 LacZ i.v. 10^{12} groups, which most likely can be explained by the scar tissue. The most extensive changes in ECG parameters were seen in AAV9 LacZ i.v. 10^{12} treated mice, which exhibited significantly increased *P* and *Q* wave durations and decreased *P*, *R* and *S* wave amplitudes at d28 compared with baseline (Figures 4a, d and e) as signs of increased LA size and fibrotic LV. In addition, some signs of disturbances in repolarization were seen as a disappearance of the *J* wave and JT depression in 50% of the mice at d28 in the same study group (Figures 4d and e). In all the other groups, the ECGs curves were normal in shape and no arrhythmias were detected at day 28 (data not shown).

Biodistribution

For comparing the biodistribution of the systemic AAV9 LacZ gene transfer to intramyocardial gene transfer, the expression of LacZ transgene was quantified from the safety tissues 28 days after local or systemic injection of AAV9 LacZ. Table 1 depicts that AAV9 LacZ i.v. gene transfer with 10^{12} dose led to LacZ transgene expression above the detection limit in all the tested kidney, liver and testis samples at d28. Transgene expression was also detected in 90% of the spleen samples and 30% of the lung samples but in none of the rectus femoris muscle samples of the 10^{12} dose group. By reducing the systemic viral dose by 10- and 100-fold, the number of transgene positive samples was markedly reduced

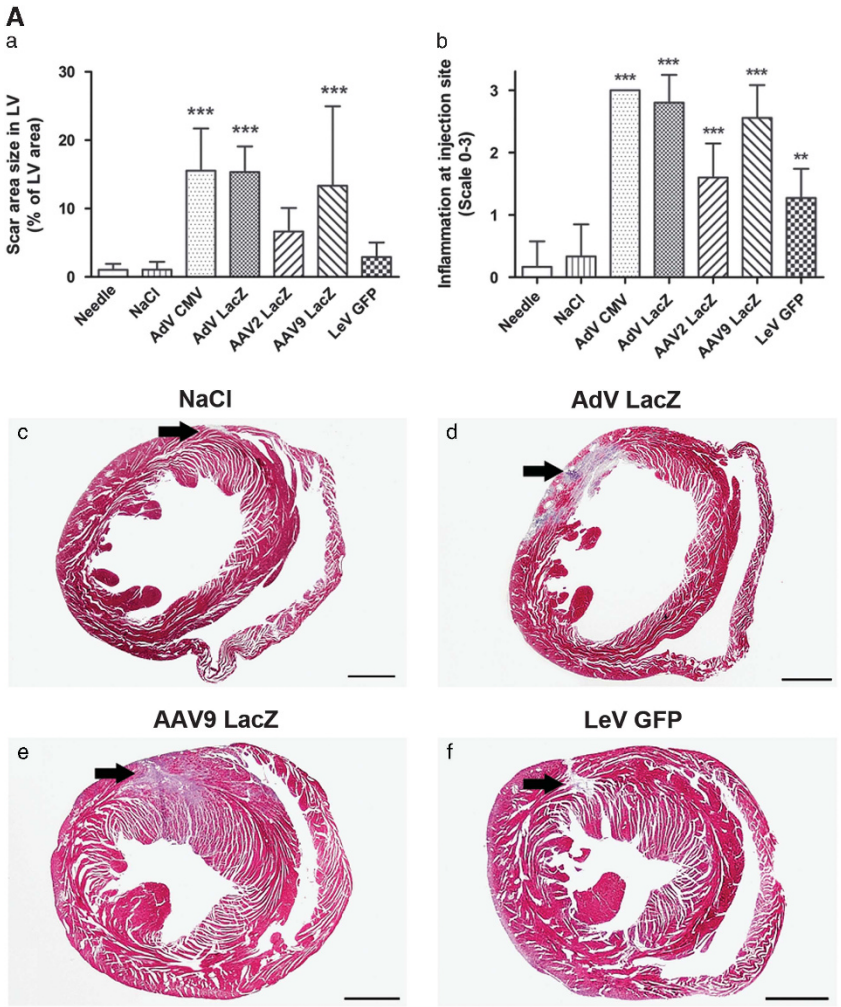

Figure 2. Myocardial damage 28 days after intramyocardial (**A**) and systemic (**B**) gene transfer. Local fibrotic scar with lymphocyte-intensive inflammatory reaction in the LV wall after intramyocardial injection of NaCl (**c**), AdV (**d**), AAV9 (**e**) and LeV (**f**) seen in the representative Masson trichrome-stained sections of hearts (magnification × 10, scale bar 1000 μm). The size of the local scar area in the LV wall (**a**, quantified from Masson trichrome-stained sections) and the amount of inflammation at the injection site (**b**, scored with the scale 0–3, where 0, no inflammation; 1, minor inflammation; 2, moderate inflammation; and 3, severe inflammation). Amount of diffuse fibrosis (**g**) and lymphocyte-intensive inflammation (**h**) after systemic AAV9 LacZ gene transfer with decreasing viral doses scored with the scale 0–3 (0, no fibrosis per inflammation; 1, minor fibrosis per inflammation; 2, moderate fibrosis per inflammation; and 3, severe fibrosis per inflammation). Representative Masson trichrome-stained sections of AAV9 LacZ i.v. 10^{12} vg dose (**i**, **j**) and 10^{11} vg dose (**k**, **l**) with × 10 (**i**, **k**; scale bar 1000 μm) and × 100 (**j**, **l**; scale bar 250 μm) magnifications. Results are shown as mean ± s.d., one-way ANOVA with Dunnet's *post hoc* test, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with NaCl-injected hearts (**a**) or 10^{12} dose group (**b**). $n = 6$ per group, except in AAV9 LacZ (**a**, **b**) $n = 9$, in LeV GFP $n = 10$ (**a**, **b**) and in AAV9 LacZ i.v. 10^{12} $n = 9$ (**g**, **h**).

in all other tissues except the liver, in which the transgene expression was detected in all the studied samples 28 days after i.v. injection. Although in the liver a clear dose–response was seen in the LacZ expression, the relative amount of LacZ mRNA expression was 5.4 in the 10^{12} group, 3.8 in the 10^{11} group and 1.0 on average in the 10^{10} group. After intramyocardial delivery of AAV9 LacZ, 80% of the liver samples were positive for transgene expression, but the level of expression was really low, 16-fold lower than in the AAV9 LacZ i.v. 10^{12} group with a relative value of 0.3 compared with the i.v. 10^{10} group. Also, low levels of transgene expression were detected in 10% of the lungs and testes, but no transgene expression was seen in the kidney, spleen or rectus femoris muscle after intramyocardial AAV9 LacZ injection.

Histology of the safety tissues after AAV9 LacZ gene transfer

To study the histological outcome of the biodistribution of AAV9 LacZ transgene, the morphology of the safety tissues after systemic and intramyocardial gene transfer of AAV9 LacZ was examined. Histological morphology was essentially normal in the safety tissues in all the study groups apart from the liver, in which some minor changes were seen (data not shown). There were mild-to-moderate amounts of 'dropout' necrosis in the liver samples in all the AAV9 LacZ injected samples, that is, single hepatocytes near the central veins were replaced by a few inflammatory cells, mostly lymphocytes. In addition, in some liver samples of AAV9 LacZ i.v. injected mice, there were lighter stained hepatocytes seen in zone three near the central vein, but no actual ballooning degeneration was present. In one AAV9 LacZ i.v. 10^{11} dose liver sample a minor amount of microvesicular steatosis was seen, and in one AAV9 LacZ i.v. 10^{12} dose liver sample some mitoses were present. Bile ducts were normal in all the samples. No fibrosis was seen in the Masson trichrome-stained sections in any of the safety tissues.

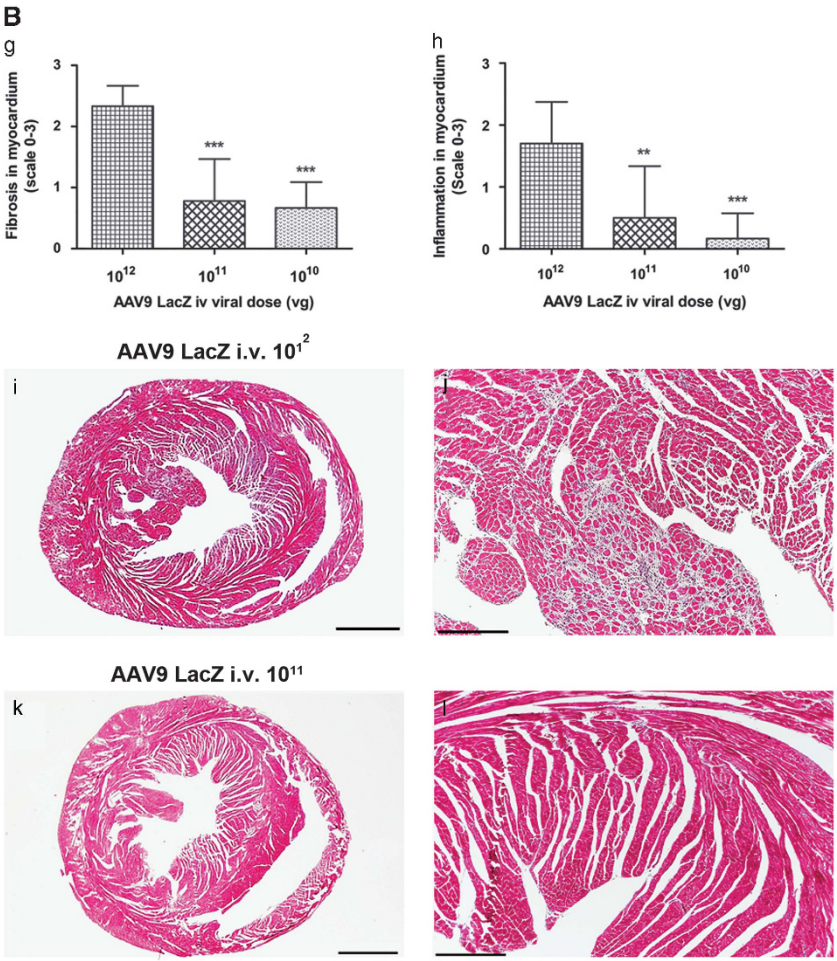

Figure 2. Continued.

DISCUSSION

Gene therapy has great potential for the treatment of cardiac diseases, such as coronary artery disease and heart failure. However, the optimal delivery of transgenes to the heart remains a challenge and the efficiency of the gene transfer vectors and delivery methods needs to be improved without forgetting the safety aspects. Local transfer of the gene drug straight into the target tissue is considered more efficient than systemic administration, and in local administration unnecessary side effects in non-target tissues caused by systemic administration can be minimized.[6,11] In addition, optimal gene transfer method should cause as little trauma as possible. In clinical intramyocardial gene therapy studies, the focus has shifted from the intramyocardial injections, which require thoracotomy, to using more sophisticated minimally invasive percutaneous catheter injections allowing more precise and local intramyocardial gene transfer.[6,9,24] For these reasons, we wanted to exploit sophisticated closed-chest intramyocardial gene delivery setting, in which the injections are carried out straight to LV wall through the chest with ultrasound guidance. With the minimally invasive injection method utilized here, it is possible to avoid the traumatic open-chest procedures, which are often used in preclinical studies, and also mimic more closely the human percutaneous approach. To our knowledge, this is the first time that the three most widely used viral vectors in cardiac gene therapy have been compared side by side with the same method and, furthermore, the first time that AAV and LeV gene transfers have been studied with this gene transfer method. In addition, we wanted to compare the effects of systemic AAV9 injection to intramyocardial injection.

AdV vectors are known to be efficient in delivering transgenes into cardiomyocytes.[11] Accordingly, we demonstrated that transduction efficiency was the best after AdV gene delivery compared with AAV and LeV and the transduced area was similar in size as seen with previous study using the same injection method.[25] The transduction efficiency of AAV2 and AAV9 was somewhat lower compared with AdV gene transfer, which is consistent with earlier study in mouse cardiomyocytes, although in titer-matched comparison AAV transduction efficiency reached the efficiency of AdV vector.[26] AAV9 has been reported to have high cardiac tropism and superior efficiency in transducing cardiac cells compared with other AAVs. Instead, the transduction efficiency of AAV2 has been shown to be poorer compared with AAV9.[15] In our study, AAV2 was as efficient in transducing cardiomyocytes as AAV9, but AAV2 produced a smaller scar and less inflammation, suggesting that AAV2 would be a better choice for intramyocardial gene transfer from the safety perspective. In our study, the systemic AAV9 injection led to transduction of cardiomyocytes throughout the myocardium, but the efficiency did not reach the levels reported by others.[27,28] Neither of the studies reported any myocardial damage after i.v. injection with the same dose of 1×10^{12}.

The main limitation of using LeV in cardiovascular approaches has been low transduction efficiency and low titers of virus preparations,[11,29] which is consistent with our findings and explains why a smaller viral dose was used with LeV vector compared with AdV and AAV. In addition, with the current LeV production methods, the LeV preparations with LacZ transgene had even lower titers owing to larger size of the LacZ gene in comparison to the GFP gene. Therefore, we were forced to use

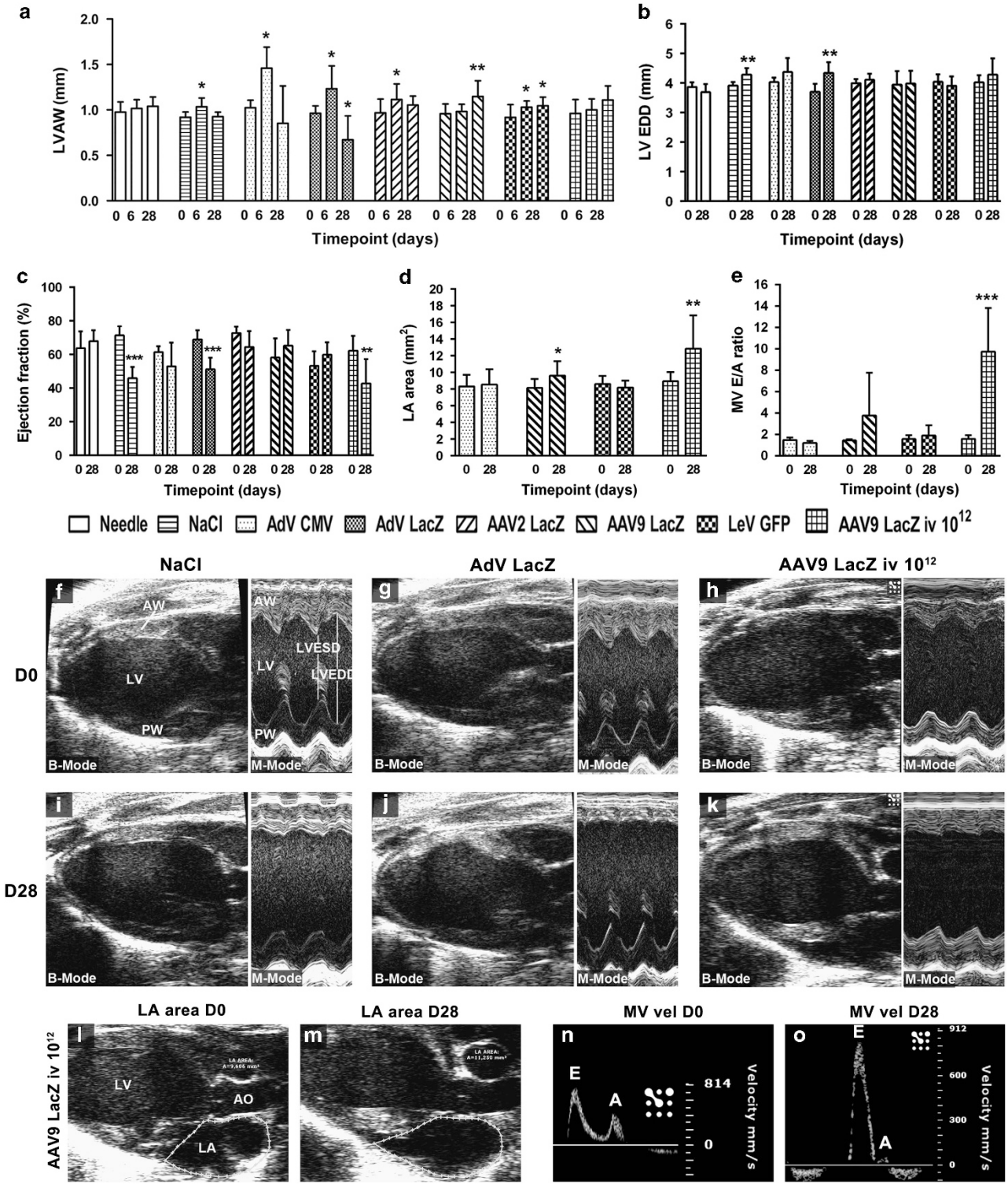

Figure 3. Echocardiography measurements of LV dimensions and function. LVAW thickness in diastole (**a**), LVEDD (**b**), ejection fraction (**c**), LA area (**d**) and MV *E/A* ratio (**e**) at d28. Representative B-Mode (long axis view) and M-Mode (short axis view) images of intramyocardially injected NaCl (**f**, **i**), AdV LacZ (**g**, **j**) and i.v. injected AAV9 LacZ 10^{12} dose (**h**, **k**) before the gene transfers at day 0 (D0, **f**, **g**, **h**) and 28 days after the gene transfer (D28, **i**–**k**). Representative long axis view B-Mode images of LA area at D0 (**l**) and D28 (**m**) and Doppler images of mitral valve flow velocities at D0 (**n**) and D28 (**o**) of the AV9 LacZ i.v. 10^{12} group. Results are expressed as mean ± s.d., $n = 6$ per group, except $n = 9$ in AdV LacZ and AAV9 LacZ i.v. 10^{12}, $n = 10$ in AAV9 LacZ and $n = 11$ in LeV GFP. One-way ANOVA with Dunnet's *post hoc* test (**a**) or Student's *t*-test (**b**–**e**) was used, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with day 0 value within each group. MV vel, mitral valve flow velocity; *E*, *E* peak representing passive diastolic filling; *A*, *A* peak representing atrial contraction.

a

	Time (days)	NaCl (n=6)	Ad CMV (n=6)	Ad LacZ (n=4)	AAV2 LacZ (n=6)	AAV9 LacZ (n=10)	LV GFP (n=14)	AAV9 LacZ iv 10^{12} (n=9)
P dur (ms)	0	16,7 ± 2,0	13,2 ± 1,3	15,1 ± 0,5	17,3 ± 2,4	17,0 ± 2,4	14,6 ± 1,7	15,9 ± 0,8
	28	16,3 ± 1,8	13,9 ± 1,1	14,9 ± 1,2	17,1 ± 2,2	16,9 ± 2,3	14,5 ± 2,1	17,3 ± 1,7*
PQ (ms)	0	44,4 ± 2,3	42,1 ± 3,3	44,0 ± 4,1	44,2 ± 4,2	45,1 ± 2,7	43,8 ± 2,4	43,3 ± 4,1
	28	40,9 ± 2,7*	44,4 ± 1,6	39,5 ± 1,9	44,5 ± 3,6	44,7 ± 5,1	43,1 ± 3,2	46,0 ± 6,0
Q dur (ms)	0	0,4 ± 0,6	0,7 ± 0,8	0,6 ± 0,7	1,0 ± 0,8	1,1 ± 1,4	0,6 ± 1,0	1,0 ± 1,3
	28	0,5 ± 0,8	0,4 ± 0,7	0,6 ± 0,7	0,6 ± 0,7	1,0 ± 1,4	0,5 ± 0,9	2,9 ± 1,4**
QRS (ms)	0	10,5 ± 0,5	10,0 ± 0,8	10,5 ± 0,7	10,4 ± 1,6	11,1 ± 1,8	11,2 ± 0,9	10,8 ± 0,7
	28	11,3 ± 1,4	9,8 ± 0,3	10,5 ± 0,5	10,6 ± 1,1	10,8 ± 1,4	10,5 ± 0,8	12,3 ± 2,6
QRSp (ms)	0	16,8 ± 1,2	14,0 ± 0,9	15,4 ± 1,6	15,5 ± 1,9	16,3 ± 2,1	15,3 ± 1,2	15,0 ± 1,0
	28	16,2 ± 1,4	15,5 ± 1,1*	17,3 ± 1,1	16,6 ± 1,2	16,5 ± 2,2	15,2 ± 1,0	18,2 ± 4,9
QTc (ms)	0	41,2 ± 5,0	39,2 ± 1,6	41,4 ± 2,5	41,0 ± 1,4	42,1 ± 5,6	40,7 ± 2,9	40,3 ± 3,8
	28	42,0 ± 1,3	40,1 ± 5,0	40,8 ± 1,8	38,3 ± 3,7	41,5 ± 3,9	40,2 ± 2,7	38,3 ± 6,0
Amp P (mV)	0	1,0 ± 0,3	1,1 ± 0,2	1,3 ± 0,2	0,9 ± 0,2	1,4 ± 0,4	1,3 ± 0,3	1,3 ± 0,2
	28	1,1 ± 0,3	1,1 ± 0,2	1,1 ± 0,2	0,9 ± 0,2	1,2 ± 0,5	1,3 ± 0,3	1,0 ± 0,2**
Amp R (mV)	0	7,1 ± 1,3	8,5 ± 1,9	9,5 ± 1,5	7,7 ± 1,4	9,3 ± 3,0	11,6 ± 2,3	11,9 ± 2,7
	28	8,1 ± 1,6	5,7 ± 2,2*	5,7 ± 0,8**	8,2 ± 1,3	8,3 ± 2,3	9,7 ± 1,6*	5,5 ± 2,4***
Amp S (mV)	0	-2,9 ± 1,1	-3,6 ± 2,0	-4,7 ± 2,2	-3,5 ± 1,1	-3,2 ± 1,3	-5,3 ± 2,0	-4,5 ± 1,4
	28	-4,8 ± 1,3*	-2,5 ± 1,2	-3,7 ± 1,9	-2,7 ± 1,3	-2,8 ± 1,2	-4,5 ± 1,9	-1,6 ± 0,4***

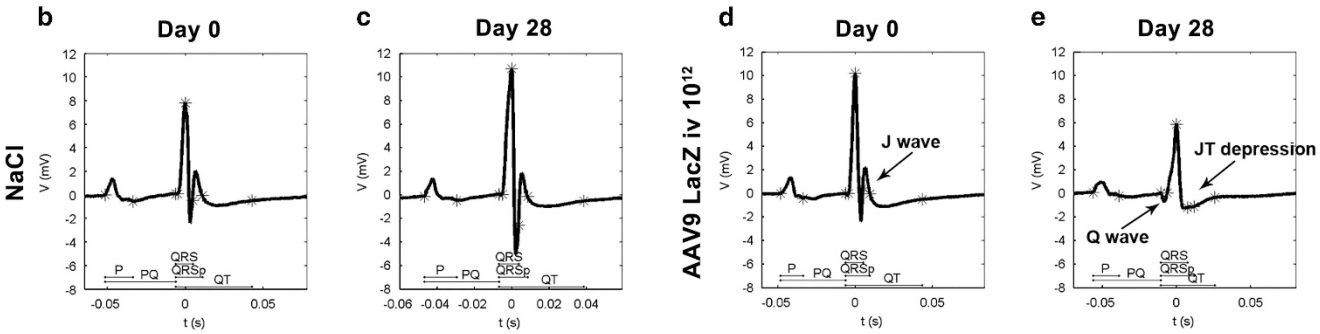

Figure 4. ECG measurements at d0 and d28 (**a**). Representative ECG of NaCl injected at baseline (**b**) and at d28 (**c**) and of systemic AAV9 injected with 10^{12} dose at baseline (**d**) and at d28 (**e**). Results are shown as mean ± s.d. Student's *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with day 0 value within each group. Number of mice in each group is indicated in the upper row of table **a**. P dur, duration of P wave; Q dur, duration of Q wave; Amp, amplitude.

Table 1. Biodistribution of AAV9 LacZ transgene expression in AAV9 LacZ i.v. groups compared with AAV9 LacZ intramyocardial (i.m.) gene transfer group at d28 measured by quantitative RT-PCR

Group	*Kidney*	*Liver*	*Lungs*	*Spleen*	*Rectus femoris muscle*	*Testis*
AAV9 LacZ i.v. 10^{12}	9/9	10/10	3/10	9/10	0/10	10/10
AAV9 LacZ i.v. 10^{11}	2/6	6/6	1/6	1/6	1/6	5/6
AAV9 LacZ i.v. 10^{10}	1/6	6/6	0/6	2/6	0/6	1/6
AAV9-LacZ i.m.	0/10	8/10	1/10	0/10	0/10	1/10

Abbreviations: AAV, adeno-associated virus; i.v., intravenous; RT-PCR, reverse transcriptase-PCR. The results are shown as tissue samples expressing the transgene/all analyzed samples in the treatment group.

different marker gene in LeV injections making it more challenging to compare the LeV group to AdV and AAV groups, in which the transgene, promoter and viral dose were the same. In the LeV construct, the GFP gene was driven by phosphoglycerate kinase-1 (PGK) promoter. With a stronger promoter, transduction efficiency could be possibly improved, namely, the CMV promoter has shown to drive higher enhanced GFP expression levels than the PGK promoter with LeV vectors in adult rat cardiomyocytes.[20] Taking these into consideration, transduction efficiency of about 11% after LeV gene transfer in the immediate proximity of the needle track with the highest possible dose of $1–4 \times 10^7$ transducing units was found to be good, even though it was the lowest compared with AdV and AAVs. Approximately, the same transduction efficiency[20] and also transduction efficiencies of even up to 40% have been reported in rat models.[19,21]

Intramyocardial viral gene transfer resulted in inflammation and fibrosis at the injection site, which were the largest after intramyocardial AdV gene transfer and the smallest after LeV gene transfer. The immune reactions associated with AdV vectors are generally known, and the inflammatory effects in the mouse myocardium after intramyocardial gene transfer have been previously reported by us[10] and others.[25] However, optimal low doses of AdVs produced according to good manufacturing practice protocols cause only little inflammation in myocardium, and AdVs have proven to be safe in preclinical and clinical trials.[7,8,11,30,31] In contrast to AdVs, AAV vectors are associated with low immunogenicity in murine models,[12,13,32,33] and in general, no adverse cardiac effects have been reported with intramyocardial AAV2[34] and AAV9[17,35] injections. However, the immunogenicity of AAV has recently become apparent in clinical trials, and the

immune responses are currently profiled more thoroughly in preclinical models.[32,33] Consistently with our findings, Fleury *et al*.[20] reported that after intramyocardial injection with LeV the tissue inflammation was significantly milder compared with AdV vectors in rats. They suggest that immune responses to the VSV-G envelope protein, enhanced GFP, and contaminants that copurify with vector particles may account for tissue inflammation.[20] In our study, GMP grade viral vectors were used, which should reduce adverse effects caused by potential contaminants.

Acute inflammatory responses secondary to needle injury are thought to be a drawback of direct needle injections.[24] But only minor myocardial damage was seen herein and also previously with intramyocardial NaCl/phosphate-buffered saline (PBS) injections with this injection method.[10,25] Despite the really small tissue damage that NaCl injection produced, it surprisingly led to LV dilatation and a decrease in EF owing to global hypokinesia. Instead, in the needle group in which no liquid was injected into the myocardium, no change in LV function was seen, although the tissue damage was similar as in the NaCl group. Also, intramyocardial gene transfers of AAV2, AAV9 and LeV did not impair the systolic function of the LV, even though larger fibrotic areas were present in the LV wall, suggesting that the closed-chest intramyocardial gene transfer method itself does not explain the worsening of the EF in the NaCl group. The mechanism behind this phenomenon remains unknown, but this should be taken into consideration in the future studies when using saline injections as controls.

Efficient transduction of myocardial cells has been achieved in small animals with systemic AAV9 delivery,[16,27,35] but no major adverse effects have been previously reported. Therefore, the severe cardiac fibrosis leading to impaired LV function and even to deaths as a result of systemic AAV9 gene transfer with 10^{12} vg dose, which is generally used in preclinical studies, was an unpleasant surprise. Previously, it has been reported that after AAV9-CMV-LacZ i.v. gene transfer of 1×10^{12} viral particles (vp) there was no inflammation, no changes in LV function and the survival rate of mice was 100% after 12 weeks follow-up.[34] Consistently with our findings, significant inflammatory cell infiltration in the heart was seen after AAV6-CMV-LacZ i.v. delivery of 1×10^{12} vg dose, but it was thought to be due to widespread expression of a bacterial protein (β-gal) and not associated with viral components.[36] Instead, in our study it seems that LacZ transgene did not induce the tissue inflammation and damage, at least in the case of AdV as the amount of fibrosis and inflammation was similar in the AdV CMV group carrying no transgene compared with the AdV LacZ group. According to our results, AAV9 i.v. dose of 10^{12} vg is too high to be used safely in mouse cardiac gene transfers. Decreasing the viral dose 10- and 100-fold not only significantly decreased the tissue damage but also reduced the transgene expression to barely detectable levels. Although not feasible gene transfer method for human studies,[11] systemic AAV9 delivery is generally used in preclinical trials with small animals and the harmful effects should be carefully addressed when assessing the effects of the gene transfer in the future.

Mouse ECG has not been widely studied and as far as is known, there are no reports of preclinical cardiac gene transfer studies with ECG data. Previously, we have reported that AdV gene transfers and NaCl injections do not to have any effects on surface ECG 6 days after the gene transfer.[10] Also, in this study there were no marked changes in the surface ECG (lead II) of intramyocardially injected mice. The most dramatic changes were seen after systemic AAV9 LacZ 10^{12} gene transfer. *P* wave duration was increased most likely as a consequence to an increased LA area, as seen in humans.[37] Formation of deeper *Q* waves, an increase in *Q* duration together with marked decreases in *P*, *R* and *S* amplitudes together with repolarization disturbances are probably caused by myocardial fibrosis. QRSp time, which measures the depolarization and early repolarization of ventricles, was somewhat increased in both AdV groups and AAV9 LacZ i.v. 10^{12} groups, possibly indicating that repolarization is somewhat disturbed. This could also be explained by fibrosis, which was the most abundant in these groups.

Reducing the harmful side effects of AdV or improving the transduction efficiency of the safer AAV and LeV vectors will be needed in the future. Modifications of the AdV vector have improved the safety in mouse studies.[25,38] Also, third-generation AdV might be less immunogenic with longer-lasting transgene expression, but their potential in human trials has not been tested.[9] As AAV and LeV intramyocardial injections were well tolerated, several injections could be carried out to increase the transduction efficiency.

The immune responses to the vector can limit the vector transduction, duration of gene expression or result in immune clearance of transduced cells. There are differences in the development of immune response against viral vectors, transgene products and the gene-modified cells between animal models and humans, which sets challenges in translating the results into clinical studies.[29,32,33] Although mouse models are invaluable in understanding the biological function of transferred genes and the viral vectors, information derived from small animal models should be extrapolated with caution to human therapies.

In summary, compared with AdV, which is widely used, AAV2, AAV9 and LeV were less effective in transducing cardiomyocytes but also less harmful. Local administration of AAV9 was safer and more efficient compared with the systemic administration. Our findings emphasize the importance of the careful evaluation of the possible adverse effects of viral vectors and delivery methods in gene therapy trials. We also encourage to further study the potential of LeV vectors in cardiac gene therapy.

MATERIALS AND METHODS

Experimental animals

Altogether 112 C57Bl/6J male mice (Harlan Laboratories, Indianapolis, IN, USA), 8–15 weeks of age, were used for the experiments. The number of mice in each group is indicated in the figure legends. The sample size was mainly six at minimum to ensure high enough statistical power. More mice per group was taken initially for the experiments, and experiments were repeated, if necessary, to get at least 5–6 mice per group successfully analyzed at the end. The preestablished exclusion criterion was that the mice into which the gene transfer was not successfully performed were excluded (that is, if no needle mark was seen in the histology). Mice were randomized for the study groups, and the analyses were performed in a blinded manner. All animal procedures were approved by The National Animal Experiment Board of Finland (reference number for the license was ESAVI-2011-003264) and carried out in accordance with the guidelines of The Finnish Act on Animal Experimentation. The animals were kept in standard housing conditions in The National Laboratory Animal Center of The University of Eastern Finland, Kuopio, Finland. Diet and water were provided *ad libitum*.

Viral constructs

AdV vectors were the first-generation serotype five replication-deficient, E1a/b- and E3-deleted preparations, in which the LacZ transgene with a nuclear localization signal (NLS) was driven by the CMV promoter. For evaluating possible harmful effects of the LacZ transgene itself, an 'empty' AdV CMV construct carrying only the CMV promoter without an actual transgene was used. AdV were produced as previously described.[10]

AAV2 vectors were produced according to a previously described protocol[39] with some modifications. Briefly, 293T cells were transfected with AAV2 vector plasmid with calcium phosphate precipitation. Cells were harvested 48–72 h after transfection. Viral vector was released from cells by three freeze-thaw cycles and the vector-containing media was purified by iodixanol-gradient centrifugation and heparin-affinity chromatography. Fractions containing the purified vector were collected and dialyzed against PBS. The purified vector was stored in PBS at −70 °C until use. AAV9 production was carried out as previously described.[40] In AAV2 and AAV9 vectors, the LacZ was under the CMV promoter and in AAV2 it carried a NLS, whereas in AAV9 expression was cytoplasmic.

Third-generation HIV-1-based LeVs were prepared with the standard calcium phosphate transfection method of 293T cells as previously described.[41] In the LeV, the GFP transgene was under the human PGK promoter.

Gene transfer

The gene transfer procedures were performed for normal non-diseased mice with healthy hearts for assessing the role of plain therapy in the myocardium. Mice were anesthetized with isoflurane inhalation (induction: 4.5% isoflurane, 450 ml min^{-1} air, maintenance: 2.0% isoflurane, 200 ml min^{-1} air; Baxter International, Deerfield, IL, USA). TTE-guided intramyocardial gene transfer of AdV CMV, Ad LacZ, AAV2 LacZ, AAV9 LacZ and LeV GFP to the anterior wall of LV in a closed-chest manner were carried out as previously described by us.[10] Briefly, injections were carried out with a 30-gauge disposable needle in a 50-µl Hamilton syringe connected to a micromanipulator system (Fujifilm VisualSonics Inc., Toronto, Ontario, Canada). The needle was penetrated through the chest in between the ribs and inserted intramyocardially into the LV wall without entering the lumen of LV. Injections of viral constructs in 10 µl volume were carried out and visualized in TTE images. For postoperative analgesic, carprofen (50 mg ml^{-1}, Rimadyl, Pfizer Inc., NY, USA) was given.

In AdV CMV and AdV LacZ gene transfers viral dose of 1×10^{10} vp and in AAV2 LacZ and AAV9 LacZ gene transfers 1×10^{10} vg diluted with sterile 0.9% NaCl in 10 µl volume were used. In LeV GFP gene transfers, 10 µl of non-diluted viral preparations with the highest possible titers were used corresponding to a viral dose of $1.3–4\times10^{7}$ transducing units. For controlling the effect of intramyocardial injection itself, a group of mice received 10 µl sterile 0.9% NaCl. The mechanical effect of a plain needle was controlled by performing the injection otherwise similarly but without any injected solution. For i.v. delivery of AAV9 LacZ, three decreasing doses were used: 1×10^{12}, 1×10^{11}, and 1×10^{10} vg in 200 µl volume injected via tail vein.

Transthoracic echocardiography and electrocardiography

TTE measurements were carried out under isoflurane anesthesia, as described earlier,[10] with Vevo 2100 Ultrasound Systems (Fujifilm VisualSonics Inc.) before the gene transfer (d0) and 6 (d6) and 28 days (d28) after the gene transfer. The MS-400 high-frequency ultrasound probe operating at 18–38 MHz was used. Briefly, for TTE and ECG measurements, mice were placed in supine position on a heated platform (THM100, Indus Instruments, Houston, TX, USA) for maintaining the body temperature at 36–37 °C (monitored via rectal probe). To obtain the ECG signal, the paws of the mice were connected to the electrode pads on the platform by using ECG gel and fixed with a skin tape. The recorded ECG represents the standard limb lead II. Heart rate and respiration were monitored during anesthesia via ECG pads.

LV dimensions (LVAW, LVPW, LVEDD) and EF, were determined from parasternal short-axis *M*-mode measurements of TTE. EF was calculated by the Vevo software with the Teicholz formula and LV mass with the formula: $1053\times((\text{LVEDD;d+LVPW;d+LVAW;d})^3-\text{LVEDD}^3)$. The area of the LA was determined with the 2D area tool from parasternal long-axis view *B*-mode image taken more laterally than the normal long-axis view to visualize the LA at its largest point. The MV flow velocities were measured with the aid of the color Doppler signal from the apical four-chamber view.

The raw data of ECG was analyzed with a Matlab-based ECG analysis program (Kubios HRV, version 2.0 beta 4, Department of Physics, University of Eastern Finland, Kuopio, Finland), which was modified specially for analyzing mouse ECG.[42] The time intervals *P* wave duration, PQ time, *Q* wave duration, QRS and QRSp width, QTc time (mean QT/(mean RR/100)^{½}) and amplitudes of *P*, *R* and *S* wave were analyzed from the mean curve generated from a 30-s ECG recording. In mouse ECG, there is an additional wave in the early repolarization right after the QRS complex called J wave,[43] and QRSp time is measured from the beginning of the QRS complex to the point where J wave returns to the isoelectric line.

Histology

Mice were killed 28 days after the gene transfer and tissue samples of the heart, lungs, liver, spleen, kidneys, quadriceps femoris muscle and testis were collected. In addition, 6 days after AdV LacZ gene transfer heart tissue was harvested for histology to study the time point of the highest transgene expression. After PBS perfusion, tissues were fixed with 4% paraformaldehyde in 7.5% sucrose for 4 h and kept in 15% sucrose overnight. Histological stainings were carried out from 5-µm thick paraffin-embedded sections apart from the LeV GFP heart samples, which were frozen to OCT (Optimal Cutting Temperature, Tissue-Tek, Sakura Finetek, Torrance, CA, USA) and cut to 8-µm thick frozen sections. Hematoxylin/eosin (HE) stainings were used to find the injection site from the intramyocardially injected hearts and for studying general histology in all the samples. The immunohistological and Masson trichrome stainings were performed on sections next to the section with the needle tract. Myocardial scar area/fibrosis was analyzed from Masson trichrome (Accustain trichrome stains; Sigma-Aldrich, St Louis, MO, USA) stained sections. Transduction efficiency was evaluated from LacZ-stained (rabbit anti-beta-galactosidase polyclonal antibody, dilution 1:2500, Merck Millipore, Darmstadt, Germany) sections of AdV LacZ- and AAV2/9 LacZ-transduced hearts and from GFP-stained (rabbit Anti-GFP antibody, ab 290, dilution 1:1500, Abcam, Cambridge, UK) sections of LeV GFP-transduced hearts.

The number of transgene-positive cardiomyocytes in intramyocardially injected hearts was quantified from LacZ- or GFP-stained sections from five microscopic fields at ×400 magnifications from each animal. The size of the transduced area (percentage of the LV area) was quantified from ×12.5 magnifications. All quantifications were carried out in a blinded manner by using the AnalySIS software (Soft Imaging System, Muenster, Germany). The amount of transduced cardiomyocytes is presented as a mean percentage of all cardiomyocytes in a field in maximally transduced area, that is, right next to the needle track in intramyocardially injected hearts. Green fluorescence from LeV GFP-transduced hearts was evaluated with a fluorescence microscope from frozen sections mounted in Vectashield Hard-Set Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA), which counterstains the DNA allowing the visualization of nucleai with the blue fluorescence.

Myocardial damage associated with the intramyocardial and systemic gene transfer was evaluated by an experienced pathologist from the HE-stained heart sections with largest amount of damage. The general histological morphology was analyzed, and the samples were scored with the following scale: 0–3 for inflammation with the following scoring criteria: 0, no inflammation; 1, minor amount of inflammatory cells (few cells); 2, moderate amount of inflammatory cells (tens of cells); and 3, large amount of inflammatory cells (several tens to hundreds of cells). The given scores were proportioned within the data. The scar area size in the LV wall (percentage of the LV area) of intramyocardially injected mice was quantified from ×12.5 magnified Masson-stained sections in a blinded manner with the AnalySIS software (Soft Imaging System). Myocardial fibrosis in the i.v. injected mouse hearts was analyzed in a blinded manner by three independent researchers from Masson trichrome-stained microscopic sections on a scale 0–3 using the following scoring criteria: 0, no fibrosis; 1, minor fibrosis (just the needle tract); 2, moderate fibrosis; and 3, severe fibrosis. The given scores were proportioned within the data. The results are shown as a mean ± s.d. of all observations.

The general histological morphology of safety tissues of AAV9 LacZ intramyocardially and i.v. injected mice was evaluated from HE-stained paraffin-embedded sections. The liver samples of AAV9 LacZ-injected mice were scored by the pathologist in terms of dropout necrosis, amount of lymphocytes/eosinophilic granulocytes in the dropout necrotic area, ballooning degeneration, microvesicular steatosis and morphology of bile ducts on the scale 0–3 (none–minor–moderate–large amount). In addition Masson trichrome stainings were carried out for detecting possible fibrosis.

Biodistribution

Biodistribution of AAV9 LacZ transgene expression was determined from the safety tissues of AAV9 LacZ i.v. injected mice and compared with the group that received AAV9 LacZ intramyocardially. The studied organs were kidney, liver, lungs, spleen, rectus femoris muscle and testis. Total RNA from the tissues was isolated with TRI-Reagent (Sigma-Aldrich). RNA samples were DNAse treated by the DNA Free Kit (Ambion by Life Technologies, Carlsbad, CA, USA) and reverse transcribed using Revertaid (Thermo Fischer Scientific, Waltham, MA, USA). Quantitative real-time PCR was performed with a StepOnePlus Real Time PCR system (Applied Biosystems, Foster City, CA, USA). LacZ mRNA expression levels in the tissues were determined using specific TaqMan Gene Expression Assay for LacZ (Mr03987581_mr; Applied Biosystems) and related to PPIA housekeeping gene expression levels (Mm03302254_g1; Applied Biosystems).

Statistical analyses

Statistical analyses were carried out with Student's paired *t*-test in the Excel Software 2010 (Microsoft Corporation, Redmond, WA, USA) when comparing two groups/time points and with one-way analysis of variance with Dunnet's *post hoc* test in the GraphPadPrism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) when comparing three or more groups/

time points. The tests used are indicated in the figure legends. *P*-value < 0.05 was considered statistically significant, and the following symbols were used for *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Results are expressed as mean ± s.d.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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