Preservation of myocardial function after adenoviral gene transfer in isolated myocardium

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Lehnart, S. E., P. M. L. Janssen, W. M. Franz, J. K. Donahue, J. H. Lawrence, E. Marban, J. Prestle, and G. Hasenfuss. Preservation of myocardial function after adenoviral gene transfer in isolated myocardium. Am J Physiol Heart Circ Physiol 279: H986–H991, 2000.—Adenoviral gene transfer to the heart represents a promising model for structure-function analyses. Rabbit hearts were subjected to an ex vivo perfusion protocol that achieves gene transfer in >90% of cardiac myocytes. Contractile function of isolated myocardial preparations of these hearts was then observed for 2 days in a recently developed trabecula culture system. In sham-infected hearts, the initial developed force (Finit) (15.6 ± 3.7 mN/mm2; n = 12) did not change significantly after 48 h (17.0 ± 1.9 mN/mm2; P = 0.46). In adenovirus-infected preparations, Finit (14.3 ± 1.8 mN/mm2; n = 21) did not significantly differ from the control (P = 0.75) and was unchanged after 48 h (15.3 ± 2.5 mN/mm2; P = 0.93). After 2 days of continuous contractions, we observed homogenous and high-level expression of the reporter genes β-galactosidase and luciferase coding for β-galactosidase and luciferase coding for β-galactosidase and Luc coding for firefly luciferase. Luciferase activity increased more than 2,500-fold from background levels of 8.7 × 103 ± 5.0 × 103 relative light units (RLU)/mg protein (from hearts transfected with promotorless adenovirus with luciferase transgene construct AdNullLuc, n = 5) to 23.4 × 106 ± 11.1 × 106 RLU/mg protein (from hearts transfected with adenovirus with Rous sarcoma virus promoter and luciferase transgene construct AdRSVLuc, n = 5) in infected myocardial preparations (P < 0.005). Our results demonstrate a new ex vivo approach to achieve homogenous and high-level expression of recombinant adenoviral genes in contracting myocardium without adverse functional effects.

adenovirus; trabecula; rabbit

Gene transfer to the heart for the replacement of missing cellular proteins or for the expression of therapeutic gene products represents a promising approach for the treatment of myocardial disorders (5, 17, 22, 24, 26). Previous studies (1, 12, 16, 27) have demonstrated the feasibility of recombinant adenoviruses as gene delivery systems to improve cardiac function on a cellular level by expression of gene products considered to be relevant to the failing heart, e.g., adenoviral transfer of a potassium channel gene increases K+ currents and shortens action potential duration in failing cardiac myocytes (27), and overexpression of the sarcoplasmic (endo)reticulum Ca2+-ATPase in cardiac myocytes enhances sarcoplasmic reticulum Ca2+-uptake and shortens prolonged intracellular Ca2+ transients (12).

The intact heart muscle represents a much higher burden in terms of effective gene transfer to tissue-bound cardiac myocytes and in the study of consecutive functional effects. Few studies have been able to show effective expression of adenovirally introduced proteins in the working heart (16, 23), and functional studies of the heart after adenoviral infection under in vivo conditions have been limited by strong immunogenicity and unknown intrinsic toxicity of the vector system against the host cell (11, 31). Therefore, it might be difficult to study functional implications of adenoviral gene transfer under in vivo conditions. On the other hand, the functional study of adenovirus-infected cultured cardiac myocytes, thereby avoiding an immunogenic response, is limited by their progressive dedifferentiation over the expression time (6) and by technical difficulties to qualitatively evaluate and to extrapolate the contractile function of isolated cells to the more physiological multicellular myocardial architecture accomplishing loaded contractions. Therefore, multiday observation of adenoviral-infected isolated myocardial preparations would allow us to identify adverse functional effects caused by intrinsic toxicity of one of the most efficient gene transfer vehicles. To achieve this goal, we established a new technique to study consequences of adenoviral transgene expression on myocardial function in a multicellular muscle preparation under well-defined physiological conditions. The technique is based on a recently developed trabecula culture system (20) and a highly efficient adenoviral gene transfer protocol (8, 9) and shows that...
tractile function is not altered by adenoviral gene transfer per se.

MATERIALS AND METHODS

Preparation and ex vivo transfection of rabbit hearts. Female New Zealand White rabbits (1.5–2.5 kg) received heparin anticoagulation (1,000 U iv) before pentobarbital sodium anesthesia (Nembutal, 60 mg/kg iv). Under sterile conditions, the hearts were rapidly excised and rinsed twice in ice-cold, HEPES-modified Krebs-Henseleit (K-H) buffer containing (in mM) 138.2 Na\(^{+}\), 5.0 K\(^{+}\), 1.2 Mg\(^{2+}\), 1.0 Ca\(^{2+}\), 144.4 Cl\(^{-}\), 1.2 SO\(_4^{2-}\), 1.2 H\(_2\)PO\(_4^{2-}\), 20.0 HEPES, and 15.0 glucose, saturated with O\(_2\) at pH 7.4. Hearts were mounted and suspended in an insulated chamber at 35–37°C and were retrogradely perfused via the ascending aorta by a modified Langendorff-perfusion technique (8) with oxygenated K-H buffer for 5 min at 30–40 ml/min; this was followed by 25 ml of oxygenated K-H buffer containing 1 mg/ml albumin and 1.6 × 10\(^8\) plaque-forming units (pfu)/ml of recombinant adenovirus. Higher virus concentrations could not be used without compromising the myocardial viability during the perfusion protocol, which would occur due to the altered composition of solutions used in our experiments if more virus was added. The virus-containing perfusate was recirculated for 60 min at 37°C with a controlled flow rate of 30 ml/min. After the infection protocol, the hearts were perfused with virus-free K-H buffer to wash out the virus (for at least 5 min). The high transfection efficiency of this perfusion protocol (over 90% transfected cardiac myocytes) was tested at regular intervals by quantifying the percentage of cells expressing β-galactosidase (β-Gal) by evaluation of primary cultures of cardiac myocytes of AdCMVLacZ (adenovirus-transfected human cytomegalovirus-promoted LacZ transgene construct)-infected hearts (8, 9). As a control, hearts were sham infected using the same protocol without viral vectors or with AdNULLLuc (adenovirus vector with promoterless luciferase gene) for 60 min. All procedures and protocols were approved by the local Animal Care and Use Committee in accordance with institutional guidelines.

Adenovirus vectors. Three types of first-generation human type 5 recombinant adenoviruses (Ad) were used: AdCMVLacZ, encoding Escherichia coli β-Gal under control of the human cytomegalovirus (CMV) immediate early promoter, and two different adenoviral luciferase constructs containing the firefly luciferase gene in the presence or the absence of the Rous sarcoma virus (RSV) long terminal repeat promoter (AdRSVLuc and AdNULLLuc, respectively) (10). High-titer adenovirus stocks were prepared and tested for replication-competent adenoviruses as described (7, 13). Adenoviral titers were determined by averaging two plaque titration assays on HEK 293 cells as described previously (8). Although the current adenoviral vectors might have limitations in effectiveness in “long-term” expression (months/years), the fast and robust expression makes them especially useful in these “short-term” expression experiments (days/weeks).

Myocardial trabecula dissection. To dissect thin, uniform myocardial trabeculae and small papillary muscles from the free wall and septum of the right ventricle, hearts were additionally perfused with K-H solution containing (in mM) 141.2 Na\(^{+}\), 5.0 K\(^{+}\), 2.0 Mg\(^{2+}\), 1.0 Ca\(^{2+}\), 127 Cl\(^{-}\), 2.0 SO\(_4^{2-}\), 1.2 H\(_2\)PO\(_4^{2-}\), 20.0 HCO\(_3^{-}\), 10.0 glucose, and 20.0 2,3-butanediene monoxime (BDM) as a cardioprotective ingredient (25) in equilibrium with 95% O\(_2\)-5% CO\(_2\). After spontaneous beating of the suspended heart had stopped, preparations were carefully dissected under a binocular dissecting microscope (19, 28).

**Trabecula culture system.** Preparations were mounted in a closed sterile chamber (Scientific Instruments, Heidelberg, Germany) between the basket-shaped extension of a force transducer and the hooklike extension of a micromanipulator screw (20). The BDM-containing solution was exchanged under sterile conditions for BDM-free K-H solution, and the extracellular Ca\(^{2+}\) concentration was increased stepwise to 2.0 mM/l. The solution was then exchanged for 199 cell culture medium (Sigma) equilibrated with 95% O\(_2\)-5% CO\(_2\) containing the following modifications (in mM): 2.0 L-carnitine, 5.0 creatine, 5.0 taursine, and 2.0 L-glutamine as well as 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 20 IU/l human insulin (Opti-Pen, Hoechst, Germany). The muscles were stimulated at ~30% over a threshold voltage (2–4 V) through 5-ms asymmetric pulses at a frequency of 0.5 Hz. The muscles were carefully stretched to the length at which passive force development was ~2–10% of active developed force at 1.75 mM Ca\(^{2+}\), reflecting a sarcomere length between 2.0 and 2.2 μm (28). After force had stabilized, online data collection was started. All experiments were performed under isometric conditions for 48 h at a 0.5-Hz stimulation frequency at 37°C with a pH of 7.4. For more detailed description of the setup and procedures, see Janssen et al. (20). After 48 h of continuous contractions, 1 μl isoproterenol was added to test for β-adrenergic response and the presence of contractile reserve of these preparations.

**Reporter gene assays.** Forty-eight hours after adenoviral infection and continuous monitoring of contractile function in the trabecula culture system, the myocardial preparations were evaluated qualitatively for β-Gal expression by histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or quantitatively for luciferase activity by chemoluminescent assay. Myocardial preparations that were infected with AdCMVLacZ were fixed in 0.1% glutaraldehyde in PBS for 20 min at room temperature, washed with PBS, and then stained with (in mM) 15.0 K\(_4\)Fe(CN)\(_6\), 15.0 K\(_4\)Fe(CN)\(_6\) and 1.0 MgCl\(_2\), and 1.0 mM X-Gal in PBS at 37°C. The staining solution was aspirated, and the trabeculae were permanently fixed in 1% glutaraldehyde in PBS. Even though we did not detect any endogenous β-Gal activity in control experiments with nontransfected hearts, staining of the myocardial preparations was performed at alkaline conditions (pH 8.5) to avoid false-positive detection of endogenous mammalian β-Gal activity (30). Thicker preparations (>350 μm) were sometimes divided and restained to overcome the diffusion limitations of X-Gal. After 12 h, the X-Gal solution was removed, and the trabeculae were evaluated microscopically for blue staining, embedded in paraffin, sectioned at 15 μm, mounted on glass slides, and then counterstained with hematoxylin and eosin. Histological sections were evaluated for diffuse β-Gal expression at ×40 magnification. Because the intense blue staining diminished individual cell borders, we were not able to quantitatively give the average percentage of stained cardiac myocytes in the multicellular preparation.

Alternatively, myocardial trabeculae were infected with replication-defective adenoviruses encoding firefly luciferase. The promoterless construct AdNULLLuc served as a negative control for background activity determination. Alternatively, the Luc transgene was under the control of the RSV promoter for maximal Luc expression (10). After 48 h of continuous contractions, myocardial tissue was frozen in liquid nitrogen. Myocardial preparations were homogenized in lysis buffer (1% vol/vol Triton X-100, 1 mM 1,4-dithiothreitol, and 100 mM potassium phosphate, pH 7.8) and centri-
fuged, and the supernatant was used to perform luciferase assays (10). All tissue luciferase recordings were normalized for protein concentration as determined by the Bradford assay. Luciferase activity is given in relative light units (RLU) per milligram protein after correction for background activity (10).

Data analysis and statistics. For a period of at least 48 h, twitch contractions of myocardial trabeculae were monitored online by a data acquisition and analysis program written in LabView (National Instruments; 1-kHz sample frequency). The following contractile parameters were analyzed: peak developed force ($F_{\text{dev}}$; mN/mm²), diastolic force ($F_{\text{diast}}$; mN/mm²), time from stimulation to peak force (TTP; ms), maximum relative rate of force development ($\frac{\text{df}}{\text{d}t_{\text{max}}}/F$; s⁻¹), maximum relative rate of force decline ($\frac{\text{df}}{\text{d}t_{\text{min}}}/F$; s⁻¹), time from peak force to 50% relaxation (RT₅₀; in ms), and times from stimulation to 50% and 90% relaxation (TT₅₀ and TT₉₀, respectively; in ms). The initial developed force ($F_{\text{init}}$; mN/mm²) was calculated as the average developed force during the first hour of stimulation. Preparations that displayed rapid rundown (>40%) of $F_{\text{dev}}$ during the first 3 h or in which $F_{\text{init}}$ was <5 mN/mm² were discarded from further study. Unless otherwise stated, all experiments are presented as means ± SE.

Force and twitch timing data were analyzed using two-factor repeated measures ANOVA: we tested 33 subjects (muscles), with (virus) factors (control, LacZ, and Luc) and time (0 and 48 h). This test design detects any significant differences between 0 and 48 h, within each group, among the viral factors, and in the interaction between virus and time. Statistical significance was determined by Student’s t-test for paired or unpaired data where applicable. $P < 0.05$ was accepted as significant.

RESULTS

Basic characteristics of contraction and relaxation. Myocardial preparations were subdivided into three groups according to the infection protocols: sham-infected preparations ($n = 12$) served as the control group and were compared with the intervention groups LacZ ($n = 11$) and Luc ($n = 10$). The average dimensions of the preparations were the following: length, 3.85 ± 0.19 mm; thickness, 363 ± 27 μm; width, 412 ± 23 μm; and cross-sectional area, 0.128 ± 0.013 mm². Average size and distribution of the preparation dimensions were similar in all groups. In pilot experiments we found no effect of the perfusion protocol per se on contractile function. The contracture parameters behaved similarly over a 48-h period under identical conditions with this study and without Langendorff perfusion (20). At 0.5 Hz stimulation frequency and 37°C, the $F_{\text{init}}$ was 15.6 ± 3.7 mN/mm² in the control group, 14.2 ± 2.3 mN/mm² in the LacZ group (unpaired t-test, $P = 0.75$), and 14.3 ± 2.8 mN/mm² in the Luc group ($P = 0.77$). Also, there were no significant differences in the diastolic force ($F_{\text{diast}}$) at $t = 0$ h for the control group (1.64 ± 0.45 mN/mm²), the LacZ group (1.05 ± 0.21 mN/mm², $P = 0.26$), and the Luc group (1.21 ± 0.24 mN/mm², $P = 0.41$) or between the LacZ group and the Luc group ($P = 0.62$). ANOVA indicated no changes in contractile parameters $F_{\text{dev}}$ and $F_{\text{diast}}$ with respect to the variables time (0 and 48 h; $F_{\text{dev}}; P = 0.97$ and $F_{\text{diast}}; P = 0.55$) and virus (control, LacZ, and Luc groups; $F_{\text{dev}}; P = 0.90$ and $F_{\text{diast}}; P = 0.52$) or between the interaction of these factors (time × virus; $F_{\text{dev}}; P = 0.99$ and $F_{\text{diast}}; P = 0.94$). Furthermore, there was no significant difference in the number of preparations discarded for further study between the control and either of the gene transfer groups. In total, 7 (4 Luc, 2 sham, and 1 LacZ) of 40 preparations failed to qualify for further study. Both sham- and LacZ- or Luc-transfected preparations did not show contractile abnormalities over the time course of 48 h.

Influence of transgene expression on myocardial performance. Sham-transfected myocardial trabeculae contracted continuously over 48 h and did not show alterations of contractile parameters, e.g., $F_{\text{dev}}$ at $t = 48$ h was 17.0 ± 1.9 mN/mm² compared with 15.6 ± 3.7 mN/mm² at $t = 0$ h ($P = 0.46$; Fig. 1). After a 2-day time span, neither LacZ-transfected ($P = 0.31$) nor Luc-transfected ($P = 0.06$) myocardial preparations displayed a significant change from $t = 0$ h (ANOVA; time, $P = 0.55$; virus, $P = 0.09$; time × virus, $P = 0.94$) in the change in RT₅₀, despite robust expression of the reporter genes (Fig. 2). Similarly, there were no significant differences between the groups for other twitch timing parameters (TT₅₀, TT₉₀). Additionally, there were no significant differences within the control group, the LacZ group, or the Luc group regarding ($\frac{\text{df}}{\text{d}t_{\text{max}}}/F$ or $\frac{\text{df}}{\text{d}t_{\text{min}}}/F$) over 48 h, and there were no significant differences between the groups (Fig. 3).

Effects of reporter gene expression on β-adrenergic signaling. Inotropic stimulation of the heart via β-adrenergic receptors represents the most powerful way to

![Fig. 1. Average time courses of peak force ($F_{\text{dev}}$; top lines) and diastolic force development ($F_{\text{diast}}$; bottom lines) over 48 h of the control group (promotorless adenovirus with luciferase transgene construct (AdNULLLuc)) (solid lines), the LacZ [adenovirus with human cytomegalovirus promoter and Luc transgene (AdCMVLuc)]-transfected group (dotted lines), and the Luc [adenovirus with Rous sarcoma virus promoter and luciferase transgene construct (AdRSLuc)]-transfected group (dashed lines). The Luc transgene codes for luciferase and the LacZ transgene codes for β-galactosidase (β-Gal). $F_{\text{dev}}$ and $F_{\text{diast}}$ were calculated as fractions of initial developed force; average normalized data were later multiplied by average force per cross-sectional area for display purposes. Changes in these parameters due to adenoviral gene expression were not significant over 48 h (control group, n = 11; LacZ group, n = 11; and Luc group, n = 10).]
increase cardiac contractility. Global adenoviral infection followed by 48 h of sustained contractions did not significantly affect the \(\beta\)-adrenergic pathway. Figure 4 shows a representative experiment with 1 \(\mu\)M isoproterenol to stimulate a \(\beta\)-adrenergic response after 48 h of continuous contractions in a Luc-infected preparation. On average, maximal developed force in these myocardial preparations after exposure to isoproterenol increased to 351 \(\pm\) 6114\% in LacZ-transfected (\(n = 3\)) and to 326 \(\pm\) 791\% in Luc-transfected preparations (\(n = 3\)) compared with 340 \(\pm\) 104\% in the control group (\(n = 3\)). Relaxation as reflected by RT50 was accelerated to 69 \(\pm\) 2\% in the LacZ group (\(n = 3\)), to 66 \(\pm\) 6\% in the Luc group (\(n = 3\)), and to 63 \(\pm\) 4\% in the control group (\(n = 3\)).

Expression pattern of LacZ in myocardial trabeculae. Myocardial preparations that were subjected to LacZ transfection and kept contracting continuously for 48 h were fixed and stained for \(\beta\)-Gal expression (\(n = 11\)). As shown in Fig. 5, there is widespread and robust expression of the LacZ transgene, with nearly all cardiac myocytes staining positive. Sham-infected preparations or those infected with the promotorless Luc transgene adenovirus demonstrated no X-Gal staining.

Myocardial expression of luciferase. Forty-eight hours after infection with 1.6 \(\times\) 10⁹ pfu/ml of AdRSVLuc and continuous stimulation, myocardial preparations showed high levels of luciferase activity. Myocardial preparations that were infected with AdNULLLuc served as background levels and had an average luciferase activity of 8.7 \(\pm\) 5.0 \(\times\) 10³ RLU/mg protein. In sharp contrast, the constitutive RSV promoter served for a significant increase in luciferase activity to 23.4 \(\pm\) 11.1 \(\times\) 10⁶ RLU/mg protein in transfected myocardial preparations, being more than 2,500-fold higher than control levels (\(n = 5\); \(P < 0.005\)).

DISCUSSION

In light of upcoming gene therapy protocols in the future, we set out to directly demonstrate a lack of adverse effects on myocardial function despite robust overexpression of adenoviral transgenes. The present study demonstrates highly effective gene transfer to multicellular myocardial preparations without adverse influences on contractile function. This was accomplished by combining a recently developed myocardial trabecula culture system (20) with an ex vivo gene transfer technique (8, 9). Under physiological conditions and continuous monitoring, contractile performance was stable in infected preparations and was not significantly different from control preparations throughout this period. Neither the perfusion protocol...
nor adenovirus infection and reporter gene expression adversely influence contractile function of these multicellular preparations. Thus, by use of an adenoviral perfusion protocol in intact hearts, it is possible to induce high levels of transgene expression into myocardial preparations without inducing adverse effects by toxicity of the adenoviral vector system on myocardial function.

Previous studies investigating the influence of transgenes on myocardial function have used cardiac myocytes under primary culture conditions as a model to study adenoviral gene transfer and subsequent effects of recombinant proteins on contractility (1, 12, 22, 27). The culture of myocardial preparations used in this study has several advantages over the culture of isolated cardiac myocytes, because it represents 1) the more complex situation of gene delivery into a differentiated multicellular architecture like the intact heart muscle (2, 8, 9, 11, 14, 16, 23, 29); 2) the more physiological situation of loaded conditions in the study of contractile function (4, 20); and 3) the stable contractile behavior, protein synthesis, and nondifferentiating cellular integrity of electrically stimulated myocardial preparations (3, 4, 20). In addition, the recent demonstration of the long-term culture of human trabeculae for up to 6 days suggests the possibility of using this gene transfer protocol to investigate pathophysiological alterations in the failing human heart (19).

The functionally intact myocardial preparations from transfected hearts showed high-level expression of different reporter genes after 2 days. β-Gal expression was homogenous and robust within thin preparations, and myocardial luciferase activity was more than 2,500-fold higher than control levels. The functional effects of adenovirus infection and of reporter gene expression have been evaluated in a controlled preparation of intact myocardium. The absence of toxic effects of the adenoviral vector and the expressed reporter genes LacZ and Luc on contractile function is in close agreement with studies on isolated cardiac myocytes (15, 22).

The trabecula culture method as a technique for assessment of recombinant transgene expression on myocardial function may be an alternative to conventional transgenic animal models. Developmental adaptation in transgenic animals to overexpression or knock-out of a given protein often results in a new phenotype. This can be difficult to differentiate for the specific effects of the given transgene on myocardial function from secondary effects, e.g., transgenic animals overexpressing sarcoplasmic (endo)reticulum Ca^{2+}-ATPase (18) or calsequestrin (21) also exhibit increased transcription of other physiologically important proteins like phospholamban. Similarly, functionally meaningful overexpression in transgenic mice downregulates gene expression of Ca^{2+}-regulatory proteins like the ryanozine receptor, triadin, and junctin by 50% or more, whereas phospholamban and Ca^{2+}-ATPase are upregulated. Adenoviral gene transfer techniques may not only bypass developmental adaption, but they may also be used to knock out a target gene by programming antisense strategies that block gene expression, and they represent a future therapeutic approach under investigation.

In conclusion, adenoviral transfection of the heart by a well-defined and effective perfusion protocol does not affect myocardial function under physiological conditions. The technique used in this study achieves homogenous and high-level expression of the reporter genes LacZ and Luc. Continuous multiday monitoring of myocardial function under ex vivo conditions in the trabecula culture system during establishment of transgene expression showed no adverse functional effects due to possible intrinsic toxicity of the adenoviral vector system in cardiac myocytes. Adenoviral perfusion and infection of the myocardium therefore seems to be safe in terms of functional side effects. This
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approach may help to investigate transgene effects in the heart by a structure-function analysis of recombinant proteins on the level of the contracting myocardium.

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