Am J Physiol Heart Circ Physiol 287: H172–H177, 2004.
First published March 4, 2004; 10.1152/ajpheart.01009.2003.

Indirect intracoronary delivery of adenovirus encoding adenylyl cyclase increases left ventricular contractile function in mice

David M. Roth,[1,2] N. Chin Lai,[3] Mei Hua Gao,[3] Jeffery D. Drumm,[3] Jesus Jimenez,[1] James R. Feramisco,[3] and H. Kirk Hammond[1,3]

[1]*Veterans Affairs San Diego Healthcare System, San Diego 92161; and Departments of* [2]*Anesthesiology and* [3]*Medicine,* [4]*University of California, San Diego, La Jolla, California 92093*

Submitted 27 October 2003; accepted in final form 25 February 2004

Roth, David M., N. Chin Lai, Mei Hua Gao, Jeffery D. Drumm, Jesus Jimenez, James R. Feramisco, and H. Kirk Hammond. Indirect intracoronary delivery of adenovirus encoding adenylyl cyclase increases left ventricular contractile function in mice. *Am J Physiol Heart Circ Physiol* 287: H172–H177, 2004. First published March 4, 2004; 10.1152/ajpheart.01009.2003.—We performed indirect intracoronary delivery of adenovirus vectors in mice and explored techniques including hypothermia and pharmacological means to increase cardiac gene transfer. Mice were maintained in a normothermic state or cooled to 25°C. The aorta or both the pulmonary artery and aorta were clamped while a needle was advanced into the left ventricular cavity to deliver adenovirus vectors encoding enhanced green fluorescent protein (EGFP) or murine adenylyl cyclase type VI (AC_{VI}) with saline, sodium nitroprusside, acetylcholine, or serotonin. Clamping was maintained for 30 s (normothermia) or 2 min (25°C) after adenovirus administration. Mice were killed 7 or 21 days later, and hearts were examined for EGFP expression. Compared with clamping the aorta alone and with no cooling, gene transfer was increased as follows: *1*) 1.3-fold with hypothermia to extend dwell time; *2*) 4.5-fold by clamping the aorta and the pulmonary artery; *3*) 11.4-fold with nitroprusside administration; *4*) 11.8-fold with serotonin addition, and *5*) 14.3-fold with acetylcholine delivery. Gene expression remained substantial at 21 days, and no significant inflammatory response was seen. Efficacy of the method was tested by performing gene transfer of adenovirus encoding AC_{VI}. Fourteen days after gene transfer, hearts isolated from mice that received adenovirus encoding AC_{VI} showed increased contractile function. Indirect intracoronary delivery of adenovirus vectors in mice is associated with efficient cardiac gene transfer and increased left ventricular function after AC_{VI} gene transfer.

left ventricular; contractility; serotonin; acetylcholine

INDIRECT INTRACORONARY ADENOVIRUS vector delivery to the heart has been described in rodents (12, 16). In general, these techniques involve cross-clamping the aorta and pulmonary artery (PA) and delivering adenovirus vectors at the aortic root proximal to the aortic clamp, which effectively provides access to the myocardium through the coronary arteries. Investigators have used hypothermia to allow prolonged cross-clamping time and to increase the dwell time of the adenovirus. Others have used pharmacological agents in attempts to increase cardiac gene transfer (2, 5, 7, 15–17, 25). However, no previous study has compared gene transfer with and without hypothermia or pharmacological agents in intact mice and examined the ability of indirect intracoronary delivery of an adenovirus vector to alter cardiac function. A goal of our study was to perform indirect intracoronary delivery of adenovirus in mice with and without pharmacological agents and hypothermia (to prolong aortic and pulmonary arterial cross-clamp time) and establish a method for maximizing cardiac gene transfer. A second goal was to use a potentially therapeutic gene, adenylyl cyclase type VI (AC_{VI}), to determine whether gene transfer using this method would be associated with altered cardiac function.

MATERIALS AND METHODS

Animals. All animals were treated in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, Revised 1996) and animal use protocols approved by the Veterans Affairs San Diego Healthcare System Institutional Animal Care and Use Committee (institutional assurance no. A365901). Male or female C57Bl/6 mice (n = 115; 16–25 g body wt) underwent thoracotomy and indirect intracoronary gene delivery. Of these, 82 survived the procedure and were enrolled in the study (overall survival, 71%). Sixty-two mice were used to assess gene transfer efficiency using adenovirus encoding enhanced green fluorescent protein (EGFP) at 7 and 21 days. Of the 62 mice, 8 were injected with phosphate-buffered saline (PBS) to use as controls for background fluorescence. Twenty mice were enrolled in the AC_{VI} study.

Intracoronary delivery of adenovirus. Mice were anesthetized (with 1.25% isoflurane inhalation admixed with 100% oxygen) and mechanically ventilated (pressure controlled). Electrocardiogram readings and rectal temperatures were monitored throughout the procedure. Surgery was performed with or without cooling of the mouse to 25°C using a pad through which cold water was circulated. The chest was opened with a midline thoracotomy between the third and sixth ribs, and an occluding ligature (5-0 silk suture) was placed around the proximal aorta or the proximal aorta and PA while a 27-gauge needle (on a tuberculin syringe) was advanced into the left ventricular (LV) cavity to deliver 2.5×10^{10} virus particles (vp) of adenovirus vector encoding either EGFP or murine AC_{VI} (volume, 100 μl). We used a first-generation, serotype 5, E1-deleted adenovirus purified by HPLC. The ratio of vp to plaque-forming units (pfu) was 26 for adenovirus encoding EGFP (Ad5.EGFP) and 50 for adenovirus encoding AC_{VI} (Ad5.AC_{VI}) as determined by HPLC for vp and HEK-293 cell assay for pfu.

The adenovirus vector with or without agent (see below) was delivered as a bolus 10–15 s after cross-clamping, and the clamp was maintained for 30 s (for normothermic mice) or 2 min (for mice cooled to 25°C) after vector delivery. Mice that were cooled were rewarmed to 37°C immediately after the vascular clamps were removed by circulating warm water through the pad. Epinephrine was administered topically to the heart (1 μg in 0.1 ml volume), the

Address for reprint requests and other correspondence: D. M. Roth, VA San Diego Healthcare System, 3350 La Jolla Village Dr., San Diego, CA 92161 (E-mail: droth@ucsd.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

thoracotomy was closed, and mice recovered in their cages. Once it was established that hypothermia in combination with aortic and pulmonary arterial clamping provided the greatest extent of gene transfer, the following agents were added to the injectate in subsequent animals: nitroprusside (0.1 μg of sodium nitroprusside; Gensia Sicor Pharmaceuticals; Irvine, CA), acetylcholine (0.3 μg of acetylcholine chloride; Sigma; St. Louis, MO), and serotonin (0.2 μg of 5-hydroxytryptamine hydrochloride; Sigma). Control mice underwent identical surgical procedures including cross-clamping of the aorta and PA, cooling to 25°C, and injection of PBS instead of adenovirus vector with or without pharmacological agents.

Assessment of gene transfer by fluorescence. Mice were killed 7 days after gene transfer, and hearts were examined with a fluorescence microscope (×40 magnification; Nikon) for evidence of EGFP expression under fluorescent light with an excitation peak of 490 nm. Investigators assessing gene transfer were blinded as to what treatment the mice received. The LV free wall was flattened between two glass slides. Both subepicardium and subendocardium were viewed under the microscope, and images were acquired using a CoolSNAP-Pro digital camera (Media Cybernetics; Silver Spring, MD) with an exposure time of 15 s for each image. The LV free wall was divided into four quadrants, and a grayscale image was acquired for each quadrant (four subepicardial and four subendocardial images). The extent of green fluorescence in the LV free wall was quantified using Image-Pro Plus planimetry software (Media Cybernetics). Tissue from control animals (that did not receive Ad5.EGFP) was used to determine background fluorescence. The areas of all regions within a quadrant that had fluorescence readings higher than background levels were summed and then divided by the total area of the quadrant to calculate a percentage of fluorescence for that quadrant. The values for each quadrant of the heart were averaged to obtain a percentage of green fluorescence for the entire LV free wall. Images of lungs and livers also were acquired.

Assessment of gene transfer by real-time PCR. LV samples were obtained from 33 animals, and DNA was isolated using a DNeasy tissue kit (Qiagen; Valencia, CA). DNA was used as a template, and primers homologous to the Ad5 E2 domain were used for detection of recombinant adenovirus using real-time PCR. Primers for GAPDH were used as an internal control for each sample. Real-time PCR was performed using a TaqMan PCR machine for the real-time detection of amplification. Data were corrected with the GAPDH control and are presented as copy numbers per cell.

Assessment of gene transfer by deconvolution microscopy. Immunofluorescence imaging of EGFP was performed on the left ventricles of seven mice (4 EGFP treated and 3 control) after fixation in 4% paraformaldehyde, paraffin embedding, and sectioning (10 μm). Samples were deembedded using Microclear (Micron Environmental Industries; Fairfax, VA) and a graded series of alcohols. To improve specific staining, samples were treated sequentially with sodium borohydride and glycine and then subjected to antigen retrieval (DAKO target-retrieval solution; DAKO; Carpinteria, CA). Because the tissue processing diminished the intrinsic fluorescence of EGFP, we used indirect immunostaining to detect the expression of this transgene. Sections were incubated in blocking buffer (10% donkey serum or 10 mg/ml BSA in PBS) and then incubated with primary rabbit polyclonal antibodies to EGFP (Novus Biologicals; Littleton, CO). Samples then were incubated with immunofluorescence affinity-purified donkey anti-rabbit secondary antibodies conjugated to rhodamine (Jackson Immuno Research; West Grove, PA). Sections were incubated with Hoechst dye to stain the nuclei and mounted in gelvatol mounting medium. For controls, adjacent serial sections incubated with only the secondary antibody or with no antibodies were also examined. Images were captured with a DeltaVision deconvolution microscope system (Applied Precision; Issaquah, WA). The system includes a Photometrics charge-coupled device and a CoolSNAP camera mounted on a Nikon TE-200 microscope. Several optical sections spaced by ~1 μm were captured using a ×20 Plan-Apochromat lens and filter sets specific for Hoechst dye, EGFP, and rhodamine. Exposure times were kept constant for each color in the different samples within comparative experiments and were within the linear range of the camera. Data sets were deconvolved and analyzed using SoftWorx software (Applied Precision) on a Silicon Graphics Octane workstation.

For immunoperoxidase staining, the samples were further treated with peroxidase block (DAKO) and then preincubated in 10% donkey serum in PBS. After incubation with rabbit anti-EGFP antibody and subsequent incubation with goat anti-rabbit antibody conjugated to horseradish peroxidase, the samples were incubated with diaminobenzidine-substrate chromagen solution (DAKO), quenched in ammonium hydroxide, and mounted in gelvatol. Transmitted light images (bright field) were captured with a Hamamatsu three-color cooled charge-coupled device camera mounted on a Zeiss Axiophot microscope. A ×20 magnification Plan-Apochromat lens was used, as were excitation and emission filters specific for the indicated fluorophores. Exposure times were constant between samples in the same experiment based on the sample showing the most staining.

Assessment of inflammation. After visual assessment of gene transfer, a portion of LV tissue was placed in 10% buffered formalin for histological analysis. Tissue was paraffin embedded and sectioned (thickness, 5 μm). Sections were mounted on slides, stained with hematoxylin and eosin, and examined for inflammatory cell infiltrates. The tissue was scored for inflammation using a scoring range from 0 (no inflammation) to 3+ (severe inflammation).

Assessment of cardiac function. Fourteen days after indirect intracoronary injection of Ad5.AC_{VI} or PBS, mice were anesthetized and their hearts were removed for study ex vivo as previously described (9). The investigators performing the ex vivo perfusion and data analysis were blinded as to the group of mice studied. Aortas were cannulated and placed on a Langendorff system for retrograde perfusion with warmed Krebs-Hensleit solution oxygenated with 95% O_2-5% CO_2. A small, fluid-filled balloon attached to a high-fidelity pressure transducer (Millar Instruments; Houston, TX) was placed in the left ventricle through the mitral valve. The balloon was inflated until an end-diastolic pressure of 10 mmHg was achieved. Hearts were allowed to equilibrate for 15 min before baseline pressures were recorded. Hearts were then administered bolus doses of dobutamine equal to final concentrations of 0.01–100 μM at 5-min intervals. Peak rate of LV pressure development (+dP/d*t*) was calculated after acquisition at a sampling rate of 2,000 per second with Windaq acquisition software (Dataq; Akron, OH).

Statistical analysis. Differences in gene expression between experimental groups were determined with a two- by four-way ANOVA using SPSS 11.0 statistical software (SPSS; Chicago, IL). A comparison of gene expression at 7 and 21 days was performed using unpaired Student's *t*-test, cardiac function was analyzed by two-way ANOVA, and correlation between DNA copy number and percentage of green fluorescence was performed using linear regression analysis (GraphPad Software; San Diego, CA).

RESULTS

Adenovirus-mediated cardiac gene transfer was studied in mice using protocols as described in Fig. 1. Three mice underwent adenovirus delivery at normothermia with aortic clamping for 90 s (without PA clamping); no pharmacological agents were used. The remaining mice underwent adenovirus or PBS delivery at 25°C with clamping of the aorta or aorta and PA with or without addition of pharmacological agents.

Figure 2 shows representative images of left ventricles obtained via fluorescence microscopy, and Fig. 3*A* shows quantitative data for the various gene-transfer strategies. Adenovirus vector delivery at normothermia with cross-clamping of the aorta (90 s total clamp; 30 s adenovirus dwell time) with

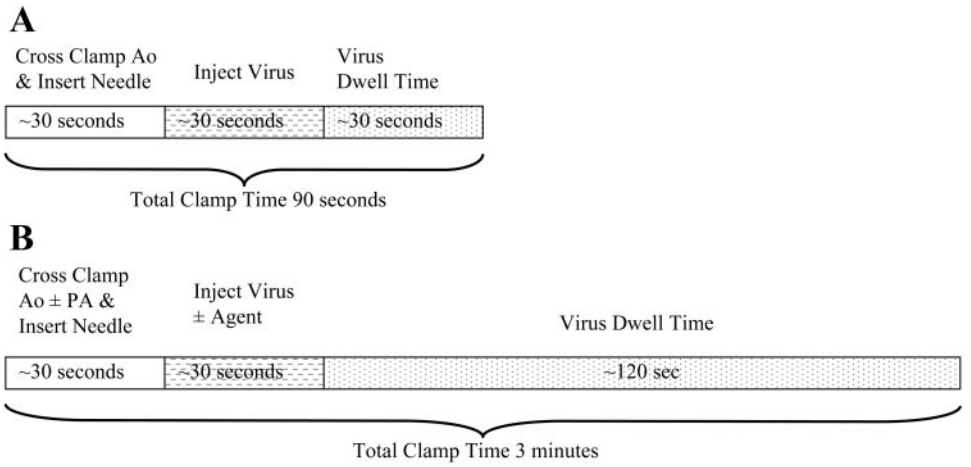


Fig. 1. Protocols for indirect intracoronary virus delivery to the mouse heart at normothermia (*A*) or hypothermia (cooled to 25°C; *B*). Ao, aorta; PA, pulmonary artery.

no pharmacological agents provided 3% gene transfer to the left ventricle. With the use of the same protocol with hypothermia (25°C) to permit 2 min of adenovirus dwell time, gene expression in the left ventricle was increased 1.3-fold. By adding pulmonary arterial clamping to this protocol, EGFP expression was increased 4.5-fold in the left ventricle. These experiments indicated that clamping both the aorta and PA and using hypothermia provided increased gene transfer.

Using this protocol (aortic and pulmonary arterial clamping at 25°C with 2 min dwell time), we then addressed whether the use of pharmacological agents could further the extent of gene transfer. We tested sodium nitroprusside, acetylcholine, and serotonin. These agents were associated with an 11.4- to 14.3-fold increase in gene transfer compared with aortic clamping and normothermia and a 2.5- to 3.2-fold increase compared with the same protocol without the pharmacological agents (Figs. 2 and 3*A*). In mice injected with PBS, there was no evidence of LV fluorescence above background levels ($n = 8$). Figure 4 shows that EGFP gene expression in the left ventricle, using these optimal protocols, was sustained for at least 21 days. Similar amounts of gene transfer were observed in the lungs and livers of all animals under all conditions. Extensive gene transfer was noted in the liver, whereas gene transfer was minimal or undetectable in lungs of all mice that received adenovirus encoding EGFP.

To determine the relationship between visual assessment of fluorescence and adenovirus DNA copy number, we examined the relationship of these two methods by linear regression analysis. There was a correlation between the amount of green fluorescence in the hearts and the adenovirus DNA copy number present in the cells ($P < 0.0001$; Fig. 3*B*).

Immunostaining and deconvolution microscopy were used as a third method to confirm gene transfer to the left ventricle. Figure 5 shows representative images of left ventricle from a mouse that received PBS (Fig. 5, *A* and *B*) or adenovirus encoding EGFP (Fig. 5, *C* and *D*). This figure demonstrates extensive gene transfer to cardiac myocytes.

We next prospectively tested whether the optimal gene-transfer strategy would result in a physiological effect when used with a potentially therapeutic gene, AC_{VI}. Ad5.AC_{VI} was delivered during aortic and pulmonary arterial clamping and hypothermia (2 min dwell time) in combination with serotonin. Control animals received identical treatment except PBS was delivered. Hearts were removed 14 days after the procedure, and LV contractile function ($+dP/dt$) was measured ex vivo in a blinded manner. Figure 6 shows mice that received adenovirus AC_{VI} had increased LV dP/dt values in response to dobutamine in a wide range of doses ($P < 0.0001$, two-way ANOVA).

A separate group of six mice were studied 21 days after intracoronary injection of PBS ($n = 2$), adenovirus EGFP and acetylcholine ($n = 2$), or serotonin ($n = 2$) to determine whether there was evidence of myocardial inflammation. Hearts showed no inflammation or very mild inflammation, and there were no differences in animals receiving adenovirus or PBS. No evidence of inflammation was found in the lung and mild inflammation was noted in the liver of one mouse that received Ad5.EGFP and serotonin.

DISCUSSION

The most important finding of this study is that indirect intracoronary delivery of adenovirus vectors can be used to obtain high yield and physiologically relevant gene transfer to murine heart. Three components appear to be required for substantial gene transfer. First, aorta clamping, which is a

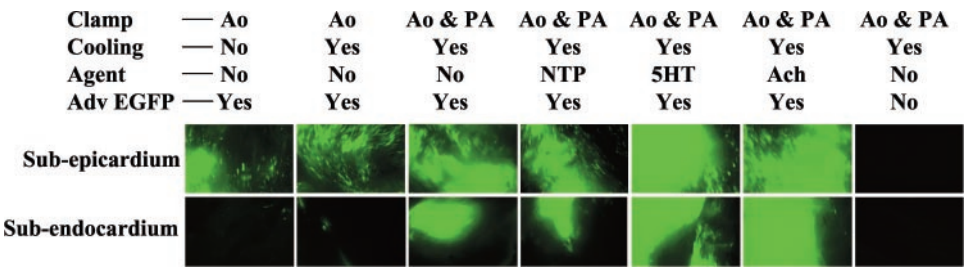
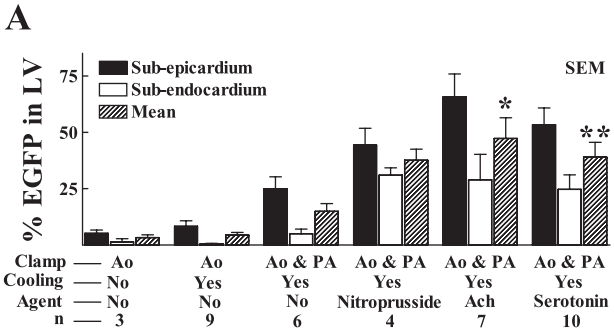


Fig. 2. Representative immunofluorescence images of left ventricles (LVs) from mice after indirect intracoronary delivery of either adenovirus enhanced green fluorescent protein (EGFP) or phosphate-buffered saline (*far right*). Images viewed and photographed under fluorescent light (490 nm) at ×40 magnification with 15-s exposure. NTP, sodium nitroprusside; 5HT, serotonin; ACh, acetylcholine.



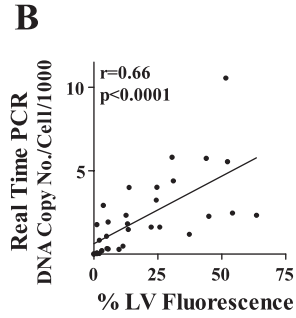


Fig. 3. Percentages of EGFP expression in LVs after indirect intracoronary virus delivery are indicated for the protocols studied (*A*). Overall, $P = 0.03$ from two- by four-way ANOVA for all aortic and pulmonary arterial clamp experiments; *$P = 0.005$ vs. aortic and pulmonary arterial clamp alone; **$P = 0.02$ vs. aortic and pulmonary arterial clamp alone. Correlation exists between the amount of green fluorescence in the tissues and the adenovirus DNA copy number in the cells (*B*).

prerequisite for indirect intracoronary delivery, should be accompanied by simultaneous clamping of the PA. Second, increased dwell time, which is made possible by cooling the animals to 25°C, appears also to increase gene transfer. Third, the use of pharmacological agents (sodium nitroprusside, serotonin, and acetylcholine) increases gene transfer to the heart and reduces the transmural gradient seen with indirect intracoronary delivery.

We showed that indirect intracoronary delivery of adenovirus encoding AC_{VI} produced a significant change in LV contractile function. Adenylyl cyclase is the effector molecule linked to the β-adrenergic receptor and the stimulatory GTP-binding protein (G_s) in cardiac myocytes (26). We have shown previously that transgenic mice with cardiac-directed AC_{VI} expression (9) and pigs after intracoronary delivery of adenovirus encoding AC_{VI} (18) have increased cardiac contractile responses to β-adrenergic receptor stimulation. Thus we have outlined an experimental method that will allow investigators to exploit a variety of transgenic mouse lines by allowing efficient exogenous gene transfer in pathophysiological settings of interest.

Hajjar et al. (12) described clamping the aorta and PA of rats to perform indirect intracoronary delivery of adenovirus. Ikeda et al. (16) described using an indirect intracoronary adenovirus delivery technique in hamsters. This group clamped both the aorta and PA during hypothermia to prolong cross-clamp time to 4–5 min and added cardioplegic solution and histamine to increase adenovirus-mediated gene transfer to the heart. We modified these techniques and systematically studied components of the adenovirus delivery protocol to produce efficient cardiac gene transfer to mouse heart.

Cooling the mice to 25°C allowed us to increase the aortic and pulmonary arterial cross-clamping times to prolong the time the adenovirus was in contact with the coronary circulation without coronary reflow. Dwell time with or without coronary reflow appears to affect the extent of adenovirus gene transfer to the heart (6, 20). Cooling the animal to 25°C alone did not adversely affect the efficiency of cardiac gene transfer. Donahue et al. (6) proposed that cooling inhibited the rate of infection of isolated cardiac myocytes with adenovirus. However, at 24°C (near the temperature achieved in our studies), the percentage of infected cells was not changed. We showed that clamping both the aorta and PA after cooling the mice produced a 4.5-fold increase in adenovirus-mediated gene transfer compared with aortic clamping alone. This may result from prevention of LV distension that is observed with aortic clamping alone, thus improving coronary perfusion and adenovirus delivery.

We also showed that addition of pharmacological agents further increases overall gene-transfer efficiency and improves gene transfer to the subendocardium. Interestingly, the agents increased gene transfer when injected simultaneously with the adenovirus and did not require prior exposure for 3–5 min as described for hamsters when histamine was used to increase gene transfer (16). Various agents have been used to increase adenovirus gene transfer to heart including serotonin (7, 30), acetylcholine (2), histamine (16, 18, 19), adenosine (28, 31), low calcium concentrations (7, 20), bradykinin (19), substance P (17), vascular endothelial growth factor (19, 25), nitroglycerin, and sildenafil (25). The molecular mechanisms by which these agents may increase gene transfer remain to be elucidated. However, these pharmacologically diverse agents share the ability to alter vascular permeability or coronary blood flow. Given the size of an adenovirus particle (90 nm; Ref. 29) and the maximal estimated size of capillary endothelial junctions (10–15 nm; Ref. 23), the portal of entry of adenovirus particles from the vessel lumen to the cardiac interstitium does not appear to be the endothelial cell junctions. We speculate that endothelial cell transcytosis is the probable entry portal (22, 23). Serotonin has been shown to increase transcytosis across venular endothelial cells (8). Nitroprusside, serotonin, and acetylcholine all may produce coronary vasodilation and

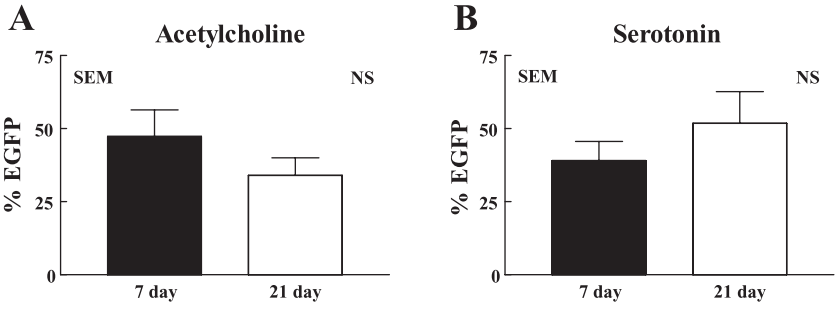


Fig. 4. Percentage of EGFP expression in LVs after indirect intracoronary virus delivery at 7 and 21 days in mice that received acetylcholine (*A*) and serotonin (*B*) with adenovirus during aortic and pulmonary arterial clamping with cooling to 25°C. NS, not significant.

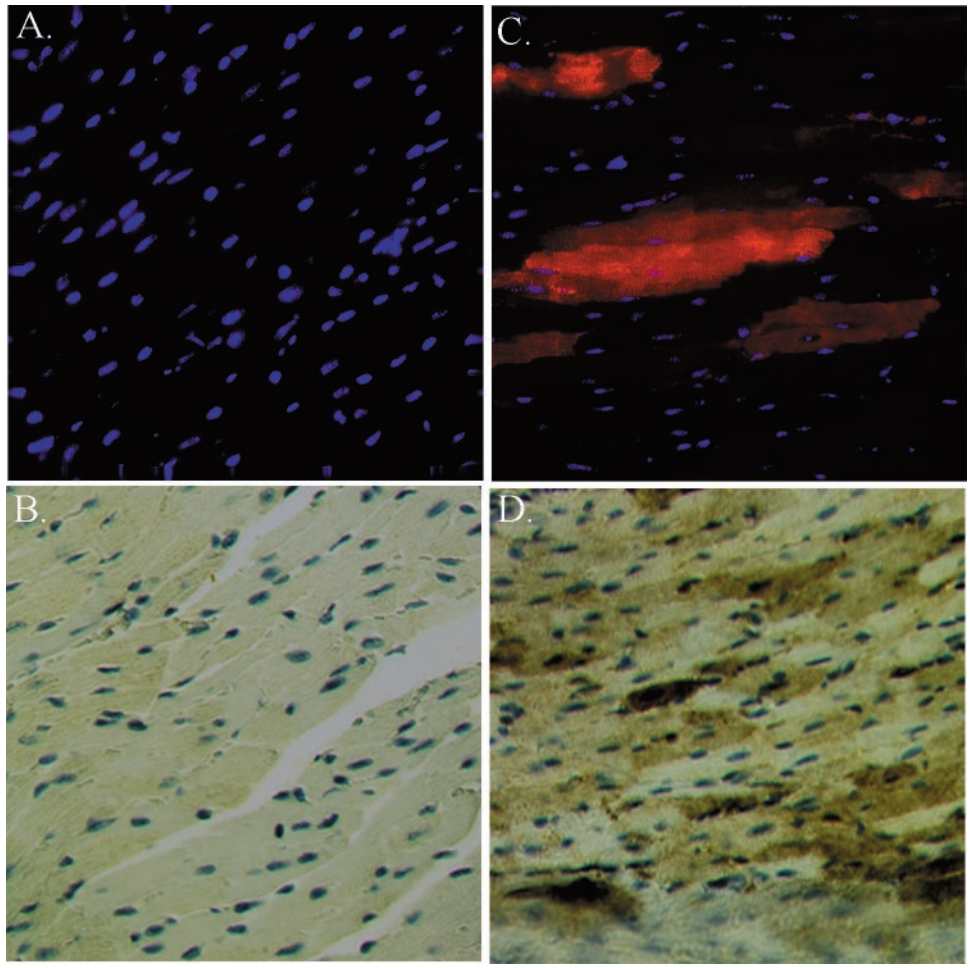


Fig. 5. Individual mice received either aortic and pulmonary arterial cross-clamp, cooling, and indirect intracoronary PBS and serotonin (*A* and *B*) or aortic and pulmonary arterial cross-clamp, cooling, and indirect intracoronary adenovirus (Adv) encoding EGFP and serotonin (*C* and *D*). EGFP detection is shown in an optical section by immunofluorescence staining (red) using fluorescence deconvolution microscopy (*A* and *C*). Other images (*B* and *D*) indicate immunohistochemical staining for EGFP by horseradish peroxidase using bright-field microscopy. All images acquired at ×20 magnification.

can release nitric oxide either by direct conversion to nitric oxide in the blood (nitroprusside; Ref. 14) or by receptor interaction on endothelial cells (acetylcholine and serotonin; Refs. 4 and 21). Nitric oxide produces coronary vasodilation (24), can increase vascular permeability (32), may be involved in formation of vesicles necessary for endothelial cell transcytosis (1), and augments adenovirus-mediated gene transfer to the heart in vivo (25). Additionally, serotonin, through stimulation of the Bezold-Jarisch reflex (27), or acetylcholine, through activation of muscarinic receptors (3), can produce transient heart block and bradycardia that theoretically may increase diastolic perfusion and increase gene transfer.

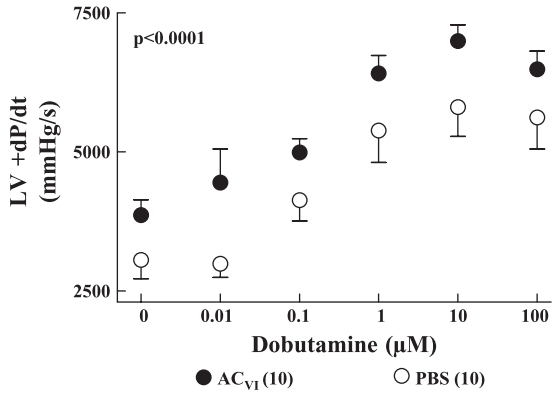


Fig. 6. Cardiac inotropic response to dobutamine in isolated, perfused hearts of mice received adenovirus-encoding murine adenylyl cyclase VI (AC_{VI}) with serotonin or PBS with serotonin during indirect intracoronary delivery with clamping of aorta and PA and cooling to 25°C. *P* value was obtained with two-way ANOVA; nos. of animals are shown in parentheses.

Indirect intracoronary delivery of adenovirus was not associated with increased inflammatory infiltrates on histological inspection compared with animals not receiving adenovirus. It appears that the procedure itself (the thoracotomy and cross-clamping), even when not associated with delivery of adenovirus, is associated with minor inflammation of the heart, as we observed this in nearly all animals in the present study. We have previously shown that direct intracoronary delivery of adenovirus vectors is not associated with myocardial inflammation (10, 11, 18). Indeed, a review of the literature regarding direct intracoronary delivery of adenovirus indicates that myocardial inflammation is rare and occurs only when very high titers of adenovirus per gram of perfused heart are delivered. Modest amounts of adenovirus per gram of perfused heart ($<5 \times 10^{11}$ vp/g) are well tolerated, and inflammation is rarely seen (13). In contrast, in the rat, using the indirect intracoronary route and doses of 2.5×10^{11} (D. M. Roth and H. K. Hammond's laboratories, unpublished observations) and 5×10^{12} vp/g of left ventricle, inflammation is moderate to severe (12). This may be due to our having used HPLC-purified adenovirus in the present study, whereas in the rat studies

(unpublished), we used CsCl-purified adenovirus (no HPLC). These observations suggest that impurities in the adenovirus preparation that are removed by HPLC or species differences may be an important element in the inflammatory response.

In conclusion, we have shown the feasibility of indirect intracoronary adenovirus-mediated gene transfer to mouse heart. Hypothermia to allow extended clamping of the great vessels and the use of pharmacological agents that may promote transcytosis greatly enhanced the extent of gene transfer. The amount of gene transfer is sufficiently high to produce physiologically relevant changes in cardiac performance and will allow testing of potentially therapeutic transgenes in murine models of cardiovascular disease.

ACKNOWLEDGMENTS

The authors thank Matthew Spellman (technical assistance), Paul Clopton (statistical analysis), Joseph Voland (histopathology), and Julie Sherman (immunofluorescence and deconvolution microscopy). Deconvolution microscopy was performed in the University of California, San Diego Cancer Center Digital Imaging Core.

GRANTS

This work was supported by Merit Awards (to D. M. Roth and H. K. Hammond) from the Department of Veterans Affairs and National Heart, Lung, and Blood Institute Grants 1PO1 HL-66941-01A1 (to H. K. Hammond, D. M. Roth, and J. R. Feramisco) and 2P50 HL-53773-06 (to H. K. Hammond). J. Jimenez is supported by a National Heart, Lung, and Blood Institute research supplement for under-represented minorities.

REFERENCES

1. **Bates DO, Hillman NJ, Williams B, Neal CR, and Pocock TM.** Regulation of microvascular permeability by vascular endothelial growth factors. *J Anat* 200: 581–597, 2002.
2. **Beeri R, Guerrero JL, Supple G, Sullivan S, Levine RA, and Hajjar RJ.** New efficient catheter-based system for myocardial gene delivery. *Circulation* 106: 1756–1759, 2002.
3. **Brown JH and Taylor P.** Muscarinic receptor agonists and antagonists. In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, edited by Hardman J and Limbird L. New York: McGraw-Hill, 2001, p. 155–158.
4. **Caulfield MP.** Muscarinic receptors—characterization, coupling and function. *Pharmacol Ther* 58: 319–379, 1993.
5. **Champion HC, Georgakopoulos D, Haldar S, Wang L, Wang Y, and Kass DA.** Robust adenoviral and adeno-associated viral gene transfer to the in vivo murine heart: application to study of phospholamban physiology. *Circulation* 108: 2790–2797, 2003.
6. **Donahue JK, Kikkawa K, Johns DC, Marban E, and Lawrence JH.** Ultrarapid, highly efficient viral gene transfer to the heart. *Proc Natl Acad Sci USA* 94: 4664–4668, 1997.
7. **Donahue JK, Kikkawa K, Thomas AD, Marban E, and Lawrence JH.** Acceleration of widespread adenoviral gene transfer to intact rabbit hearts by coronary perfusion with low calcium and serotonin. *Gene Ther* 5: 630–634, 1998.
8. **Feng D, Nagy JA, Hipp J, Dvorak HF, and Dvorak AM.** Vesiculo-vacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine and serotonin. *J Exp Med* 183: 1981–1986, 1996.
9. **Gao M, Lai N, Roth D, Zhou J, Zhu J, Anzai T, Dalton N, and Hammond H.** Adenylylcyclase increases responsiveness to catecholamine stimulation in transgenic mice. *Circulation* 99: 1618–1622, 1999.
10. **Giordano FJ, Ping P, McKirnan MD, Nozaki S, DeMaria AN, Dillmann WH, Mathieu-Costello O, and Hammond HK.** Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. *Nat Med* 2: 534–539, 1996.
11. **Grines C, Watkins M, Helmer G, Penny W, Brinker J, Marmur J, West A, Rade J, Marott P, Hammond H, and Engler R.** Angiogenic gene therapy (AGENT) trial in patients with stable angina pectoris. *Circulation* 105: 1291–1297, 2002.
12. **Hajjar RJ, Schmidt U, Matsui T, Guerrero JL, Lee KH, Gwathmey JK, Dec GW, Semigran MJ, and Rosenzweig A.** Modulation of ventricular function through gene transfer in vivo. *Proc Natl Acad Sci USA* 95: 5251–5256, 1998.
13. **Hammond HK.** Intracoronary gene transfer of fibroblast growth factor in experimental and clinical myocardial ischemia. *Gene Ther* 1: 325–342, 2002.
14. **Harrison DG and Bates JN.** The nitrovasodilators: new ideas about old drugs. *Circulation* 87: 1461–1467, 1993.
15. **Hoshijima M, Ikeda Y, Iwanaga Y, Minamisawa S, Date MO, Gu Y, Iwatate M, Li M, Wang L, Wilson JM, Wang Y, Ross J Jr, and Chien K.** Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat Med* 8: 864–871, 2002.
16. **Ikeda Y, Gu Y, Iwanaga Y, Hoshijima M, Oh SS, Giordano FJ, Chen J, Nigro V, Peterson KL, Chien KR, and Ross J Jr.** Restoration of deficient membrane proteins in the cardiomyopathic hamster by in vivo cardiac gene transfer. *Circulation* 105: 502–508, 2002.
17. **Iwatate M, Gu Y, Dieterle T, Iwanaga Y, Peterson KL, Hoshijima M, Chien K, and Ross J Jr.** In vivo high-efficiency transcoronary gene delivery and Cre-LoxP gene switching in the adult mouse heart. *Gene Ther* 10: 1814–1820, 2003.
18. **Lai N, Roth D, Gao M, Fine S, Head B, Zhu J, McKirnan D, Kwong C, Dalton N, Urasawa K, Roth D, and Hammond H.** Intracoronary delivery of adenovirus encoding adenylyl cyclase VI increases left ventricular function and cAMP-generating capacity. *Circulation* 102: 2396–2401, 2000.
19. **Logeart D, Hatem SN, Heimburger M, Le Roux A, Michel JB, and Mercadier JJ.** How to optimize in vivo gene transfer to cardiac myocytes: mechanical or pharmacological procedures? *Hum Gene Ther* 12: 1601–1610, 2001.
20. **Logeart D, Hatem SN, Rucker-Martin C, Chossat N, Nevo N, Haddada J, Heimburger M, Perricaudet M, and Mercadier JJ.** Highly efficient adenovirus-mediated gene transfer to cardiac myocytes after single-pass coronary delivery. *Hum Gene Ther* 11: 1015–1022, 2000.
21. **Luscher TF and Barton M.** Biology of the endothelium. *Clin Cardiol* 20: II3–II10, 1997.
22. **Michel CC and Curry FE.** Microvascular permeability. *Physiol Rev* 79: 703–761, 1999.
23. **Minshall RD, Tiruppathi C, Vogel SM, and Malik AB.** Vesicle formation and trafficking in endothelial cells and regulation of endothelial barrier function. *Histochem Cell Biol* 117: 105–112, 2002.
24. **Moncada S, Palmer RMJ, and Higgs EA.** Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43: 109–142, 1991.
25. **Nagata K, Marban E, Lawrence JH, and Donahue JK.** Phosphodiesterase inhibitor-mediated potentiation of adenovirus delivery to myocardium. *J Mol Cell Cardiol* 33: 575–580, 2001.
26. **Post SR, Hilal-Dandan R, Urasawa K, Brunton LL, and Insel PA.** Quantification of signalling components and amplification in the β-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes. *Biochem J* 311: 75–80, 1995.
27. **Saxena PR and Villalon CM.** Cardiovascular effects of serotonin agonists and antagonists. *J Cardiovasc Pharmacol* 15: S17–S34, 1990.
28. **Shah A, Lilly R, Kypson A, Tai O, Hata J, Pippen A, Silvestry S, Lefkowitz R, Glower D, and Koch W.** Intracoronary adenovirus-mediated delivery and overexpression of the β_2-adrenergic receptor in the heart. Prospects for molecular ventricular assistance. *Circulation* 101: 408–414, 2000.
29. **Stewart PL, Fuller SD, and Burnett RM.** Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO J* 12: 2589–2599, 1993.
30. **Weig HJ, Laugwitz KL, Moretti A, Kronsbein K, Stadele C, Bruning S, Seyfarth M, Brill T, Schomig A, and Ungerer M.** Enhanced cardiac contractility after gene transfer of V2 vasopressin receptors in vivo by ultrasound-guided injection or transcoronary delivery. *Circulation* 101: 1578–1585, 2000.
31. **Wright MJ, Wightman LML, Latchman DS, and Marber MS.** In vivo myocardial gene transfer: optimization and evaluation of intracoronary gene delivery in vivo. *Gene Ther* 8: 1833–1839, 2001.
32. **Yuan Y, Granger HJ, Zawieja DC, DeFily DV, and Chilian WM.** Histamine increases venular permeability via a phospholipase C-NO synthase-guanylate cyclase cascade. *Am J Physiol Heart Circ Physiol* 264: H1734–H1739, 1993.