Echocardiographic Destruction of Albumin Microbubbles Directs Gene Delivery to the Myocardium

Ralph V. Shohet, MD; Shuyuan Chen, MD; Yan-Ting Zhou, MD; Zhuowei Wang, MD; Robert S. Meidell, MD; Roger H. Unger, MD; Paul A. Grayburn, MD

Background—The noninvasive, tissue-specific delivery of therapeutic agents to the heart would be a valuable clinical tool. This study addressed the hypothesis that albumin-coated microbubbles could be used to effectively deliver an adenoviral transgene to rat myocardium by ultrasound-mediated microbubble destruction.

Methods and Results—Recombinant adenovirus containing β-galactosidase and driven by a constitutive promoter was attached to the surface of albumin-coated, perfluoropropane-filled microbubbles. These bubbles were infused into the jugular vein of rats with or without simultaneous echocardiography. Additional controls included ultrasound of microbubbles that did not contain virus, virus alone, and virus plus ultrasound. One group underwent ultrasound-mediated destruction of microbubbles followed by adenovirus infusion. Rats were killed after 4 days and examined for β-galactosidase expression. The hearts of all rats that underwent ultrasound-mediated destruction of microbubbles containing virus showed nuclear staining with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside substrate, indicating expression of the transgene. None of the control animals showed myocardial expression of the β-galactosidase transgene. By quantitative analysis, β-galactosidase activity was 10-fold higher in the treated group than in controls (P<0.0001).

Conclusions—Ultrasound-mediated destruction of albumin-coated microbubbles is a promising method for the delivery of bioactive agents to the heart. (Circulation. 2000;101:2554-2556.)

Key Words: echocardiography • gene therapy • microbubbles

The first approved clinical trial of gene therapy began in 1990,10 years ago. During this time, our understanding of the molecular mechanisms of disease has rapidly advanced, providing many new targets for specific genetic therapy. However, successful gene therapy remains an elusive and challenging goal, as summarized in several recent reviews.2–4 One of the most important obstacles is the lack of an effective delivery system that can be targeted to specific organs or tissues.

In tissues other than the liver, the endothelial barrier effectively prevents hematogenous delivery of adenovirus. This is a particularly important limitation for organs such as the heart, which can only be reached through invasive procedures. Ultrasound-mediated microbubble destruction has been proposed as a method for delivering drugs or genes to specific tissues, including the heart.6–11 In an exteriorized spinotrapezius model,6 it was demonstrated that ultrasonic destruction of gas-filled microbubbles caused microvessel rupture, with local extravasation of red blood cells, and that polymer microspheres could be driven as much as 200 μm into the parenchyma with this method.7 Here we describe a novel use of ultrasound-mediated microbubble destruction to effectively deliver a reporter gene to rat hearts.

Methods

Preparation of Microbubbles
Perfluoropropane-filled albumin microbubbles were made as previously described in our laboratory.12 A 10 mL solution containing 1% human serum albumin and 5% fructose along with 5 mL of perfluoropropane gas (Air Products, Inc) was sonicated using a 20-kHz probe (Heat System Inc, XL2020). The upper foam layer was discarded, and the lower suspension was frozen at −20°C overnight and then lyophilized for 28 to 30 hours. Mean microbubble size and concentration were 3.0±1.2 μm and 1.6±0.2×10⁶ bubbles/mL, respectively.

Preparation of the Adenovirus β-Galactosidase Gene
Recombinant adenovirus containing the cDNA encoding the Escherichia coli β-galactosidase gene and a nuclear localizing signal peptide (AdCMV-β-Gal) was prepared as previously described.13 Briefly, recombinant adenovirus was propagated in human embryonic kidney 293 cells. Infected cells were lysed with detergent overnight and then centrifuged at 3×10¹⁰ plaque forming units/mL until used.

Received March 10, 2000; revision received April 7, 2000; accepted April 13, 2000.

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Circulation is available at http://www.circulationaha.org

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Attachment of AdCMV-β-Gal to the Microbubble

A 1 mL solution of AdCMV-β-Gal (1.0 x 10^10 pfu/mL) was added to a 1 mL microbubble suspension and mixed for 2 hours at 4°C. The mixture separated into 2 distinct layers. The upper layer consisted of microbubbles with attached virus; the bottom layer, which contained unattached virus, was discarded.

The concentration of microbubbles with attached AdCMV-β-Gal was 1.2 x 10^10 bubbles/mL; the mean diameter was 3.5 ± 1.4 μm. The viral titer of these microbubbles was determined by standard techniques as previously described. The microbubble suspension contained ~5 x 10^9 β-Gal transgene units per milliliter.

Animal Preparation

Wild-type lean Zucker rats (300 to 400 g) were anesthetized with sodium pentobarbital (50 mg/kg IP), and a thin polyethylene tube was inserted into the right jugular vein by cutdown. Echocardiography was performed using a Sonos 5500 (Agilent Technologies) with an S3 transducer operating in second harmonic mode (transmit 1.3 MHz/receive 2.6 MHz) that had a mechanical index of 1.5 and a depth of 4 cm. Images were ECG-triggered to deliver a burst of 3 frames of ultrasound every 4 to 6 cardiac cycles. This burst eliminated all the microbubbles visible in the myocardium, and the triggering interval allowed complete replenishment of the microbubbles before the next burst. After infusion of microbubbles or control, the jugular vein was tied off and the skin was closed.

The rats were killed 4 days later, and the liver and a hindlimb skeletal muscle were harvested as positive and negative controls, respectively. The heart was removed and cut into 3 short-axis slices at the apex, mid-left ventricle, and base. To detect expression of the transgene, the mid-left ventricle and apical sections were briefly fixed in 2% paraformaldehyde, immersed in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining solution for 15 hours at 37°C, and then post-fixed. Sections were counterstained with fast red. The basal section was snap-frozen in liquid nitrogen, and quantitative assessment of galactosidase activity was performed according to standard methods. Differences in galactosidase activity between experimental groups were compared by ANOVA.

Experimental Protocol

This protocol was designed to determine the effectiveness of in vivo delivery of AdCMV-β-Gal to the myocardium using ultrasound-mediated microbubble destruction. The rats were divided into 6 groups of 6 rats each as follows:

- Group 1: Echocardiographic destruction of microbubbles without AdCMV-β-Gal
- Group 2: Echocardiographic destruction of microbubbles containing AdCMV-β-Gal (experimental group)
- Group 3: Microbubbles containing AdCMV-β-Gal without echocardiography
- Group 4: AdCMV-β-Gal (no microbubbles) without echocardiography
- Group 5: Echocardiography during infusion of AdCMV-β-Gal (no microbubbles)
- Group 6: Echocardiographic destruction of microbubbles followed by AdCMV-β-Gal infusion

Group 2 was the experimental target; the remaining groups served as controls. Because of the risk of volume overload, we administered the infusions (2 mL) over 30 minutes.

Results

The livers of all rats that received the virus showed extensive β-galactosidase activity, whereas none of the skeletal muscle samples demonstrated staining. The hearts of all 6 rats in the experimental group (group 2) showed blue staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, indicating expression of β-galactosidase (Figure 1A). Microscopic examination revealed nuclear localization of β-galactosidase (Figure 1B). None of the control rats showed myocardial staining (Figures 1C and 1D), which confirmed that the destruction of the microbubbles containing the virus was responsible for the observed β-galactosidase expression in the rat myocardium. Figure 2 shows that galactosidase activity was 10-fold higher in the group treated with echocardiographic destruction of microbubbles (group 2) containing AdCMV-β-Gal than in the controls (P<0.001). The group that underwent microbubble destruction followed by an infusion of AdCMV-β-Gal (group 6) had a 2-fold increase in β-galactosidase activity, suggesting that disruption of the endothelial barrier is an important factor in viral transduction. None of the treated animals demonstrated abnormal left ventricular systolic function during or immediately after ultrasound-mediated destruction of the microbubbles.

![Figure 1](image_url)  
**Figure 1.** A, Short-axis section of rat heart reveals obvious galactosidase staining 4 days after echocardiographic destruction of microbubbles containing AdCMV-β-Gal. B, Fast red stain of a 7-μm section from heart in A shows nuclear localization of galactosidase staining (×20). C, Representative section from a control rat showing no evidence of galactosidase staining. D, Microscopic section from same control rat showing no galactosidase staining.

![Figure 2](image_url)  
**Figure 2.** Graph of β-galactosidase activity in the various experimental groups in this study. A 10-fold increase in activity occurred in the rats that underwent echocardiographic disruption of microbubbles containing AdCMV-β-Gal (P<0.0001 by ANOVA).
Discussion
This report describes the first successful demonstration that the ultrasound-mediated disruption of gas-filled microbubbles can be used to direct transgene expression to a specific organ, in this case, the heart. Previous investigators successfully delivered labeled red blood cells and polymer microspheres to rat skeletal muscle or oligonucleotides to the dog kidney. This study demonstrates the usefulness of this technique for the delivery of a common gene-therapy vector that is capable of high-level expression of a transgene in the heart. We used β-galactosidase in these experiments to allow for histological localization of viral transduction and quantitation of transgene expression. However, this indicator shows some nonspecific activity in control hearts. It is likely that a more specific indicator would show far greater tissue-specific expression than the 10-fold effect demonstrated here.

There are many potential applications of this method to specific cardiovascular diseases, including ischemic heart disease, congestive heart failure, and arrhythmias. Previous attempts at gene therapy in the myocardium have used direct injection of plasmid DNA or adenoviral vectors, an approach that is invasive and potentially hazardous. Intracoronary infusion of a virus can also infect cardiomyocytes. The technique described here has the advantages of a noninvasive approach and efficient, tissue-specific targeting. Moreover, it is reasonable to consider this method for other tissues that are accessible to ultrasound.

Further work is needed to optimize the echocardiographic parameters for microbubble destruction, to maximize the amount of adenovirus that can be attached to the microbubbles, and to determine the range of reagents amenable to ultrasound-mediated microbubble delivery. For example, it seems likely that other gene therapy vectors could be delivered by this route. There is no obvious reason why this technique should be limited to viruses. It may provide sufficient increases in tissue-specific targeting to be useful for the delivery of a range of bioactive molecules, including plasmid DNA, enzymes, signaling molecules, or drugs. Further research is needed to develop this technique into a clinically useful tool.

Acknowledgments
This work was supported in part by National Institutes of Health grant 1 K24 HL03980-01 (to Dr Grayburn), the Dallas Veterans Administration Research Corporation, the Center for Biomedical Inventions, and the Reynolds Center at the University of Texas Southwestern Medical Center (to Dr Shohet).

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_Circulation_. 2000;101:2554-2556
doi: 10.1161/01.CIR.101.22.2554

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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