Optimization of Ultrasound Parameters for Cardiac Gene Delivery of Adenoviral or Plasmid Deoxyribonucleic Acid by Ultrasound-Targeted Microbubble Destruction

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OBJECTIVES
This study was undertaken to optimize echocardiographic parameters for successful gene delivery to the heart and to extend the method from adenoviral to plasmid deoxyribonucleic acid (DNA).

BACKGROUND
We have previously shown that ultrasound-targeted microbubble destruction can direct tissue expression of adenoviral transgenes to the heart. The optimal echocardiographic parameters for this technique have not been reported.

METHODS
Adenoviral or plasmid DNA encoding the luciferase reporter gene was incorporated into liposome microbubbles and infused intravenously into anesthetized rats. We systematically evaluated the effects of ultrasound parameters known to influence microbubble destruction, including electrocardiogram (ECG) triggering, ultrasound frequency, mode of ultrasound, and mechanical index, on gene expression in rat myocardium four days after treatment. In addition, gene expression in heart, liver, and skeletal muscle were compared between adenoviral and plasmid DNA.

RESULTS
Optimal ultrasound parameters for this technique include low-transmission frequency (1.3 MHz), maximal mechanical index, and ECG triggering to allow complete filling of the myocardial capillary bed by microbubbles. No difference was seen between ultraharmonics and power Doppler mode. Using adenoviral DNA, optimal ultrasound parameters yielded myocardial luciferase activity on the order of $10^4$ relative light units/mg protein/min but with even higher liver activity. Plasmid DNA was expressed in rat myocardium at similar levels but without detectable liver expression.

CONCLUSIONS
Ultrasound-targeted microbubble destruction can be used to deliver adenoviral or plasmid DNA to the myocardium. This technique holds great promise in applying the rapidly expanding repertoire of gene therapies being developed for cardiac disease. (J Am Coll Cardiol 2003;42:301–8) © 2003 by the American College of Cardiology Foundation

A number of techniques have been proposed for gene delivery to the heart, including intracoronary (1–4), intrapericardial (5,6), and direct myocardial injection (7–10). We have previously demonstrated successful transfection of rat myocardium in vivo by ultrasound-targeted microbubble destruction (UTMD) of microbubbles containing an adenovirus encoding a beta-galactosidase reporter gene (11). This study was undertaken to optimize echocardiographic parameters for successful gene delivery using adenoviral and plasmid deoxyribonucleic acid (DNA). We systematically evaluated the effects of four ultrasound parameters that are known to influence microbubble destruction, electrocardiogram (ECG) triggering, frequency, mode of ultrasound, and mechanical index, on luciferase expression in rat myocardium.

METHODS
Preparation of the adenovirus. AdCMV-luc is a recombinant, replication-defective adenovirus containing the firefly luciferase cDNA under control of the strong cytomegalovirus promoter. AdCMV-luc was prepared as previously described (12). Briefly, recombinant adenovirus was propagated in human 911 cells. Infected cells were lysed 48 h after infection with non-ionic detergent, and virus particles were precipitated with polyethylene glycol and further purified by CsCl density centrifugation and gel filtration. The concentrated virus was stored in Tris-buffered isotonic saline containing 10% glycerol at $-70^\circ$C.

Preparation of liposome microbubbles containing AdCMV-luc or plasmid DNA. Liposome microbubbles were prepared using a modification of a previously described method (13). A solution of 0.4% 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (Sigma Chemical Co., St. Louis, Missouri), 0.1% 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine, (Sigma Chemical Co.), and 10% glycerol was mixed with AdCMV-luc solution (1 ×

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10^{12} \text{ pfu/ml}) in a 2:1 ratio. Aliquots of 0.5 ml of this solution were placed in 1.5-ml clear vials; the remaining headspace was filled with the perfluoropropane gas (Air Products, Inc, Allentown, Pennsylvania). Each vial was then mechanically shaken for 20 s in a dental amalgamator (Vialmix, Bristol-Myers Squibb Medical Imaging, N. Billerica, Massachusetts). The lipid-coated microbubbles appear as a milky-white suspension floating on the top of a layer of liquid containing unattached AdCMV-luc. The subnatant was discarded, and the microbubbles were washed three times with phosphate-buffered saline to remove unattached DNA. Deoxyribonucleic acid concentration of the microbubbles was measured by optical density at a wavelength of 260, and integrity of the DNA was confirmed by gel electrophoresis. Confocal microscopy using fluorescent-labeled plasmid confirmed that the plasmid was incorporated into the phospholipid shell of the microbubbles. Figure 1 shows the amount of DNA per milliliter of washed microbubbles plotted against the initial amount of DNA in the solution. The mean concentration of six different preparations of microbubbles was $1.82 \pm 0.06 \times 10^9$ bubbles/ml. At the saturation dose of 4 mg/ml, the amount of plasmid DNA incorporated into the microbubble shell was calculated to be 0.33 pg/microbubble.

**Animal preparation.** The study conformed to the “Position of the American Heart Association on Research Animal Use.” Wild-type lean Zucker rats (250 to 350 g) were anesthetized with ketamine and xylazine. A polyethylene tube (P10) was inserted into the right jugular vein by cutdown for intravenous infusion of microbubbles or control solutions. A 20-min infusion of 1 ml of either AdCMV-luc-microbubbles, microbubbles without AdCMV-luc, or AdCMV-luc without microbubbles was administered. The anterior chest was shaved, and echocardiography was performed using an S12 transducer (Sonos 5500, Philips Ultrasound, Andover, Massachusetts) to locate the heart and record left ventricle (LV) function in a short-axis view. During the infusion, an S3, S8, or S12 transducer was used to target microbubble destruction to the heart using a clamp to maintain its position and 1 to 2 cm of acoustic coupling.
gel between the transducer and the chest wall. After the infusion, LV function was reassessed with the S12 transducer; the jugular vein was then tied off, and the skin was closed. The rats were sacrificed four days later, and the liver and hindlimb skeletal muscle were harvested as positive and negative controls, respectively. The LV was isolated by careful dissection then divided into anterior and posterior sections, which were snap frozen with liquid nitrogen and stored at −70°C until assayed.

Luciferase assay. To detect expression of the transgene, the anterior and posterior LV sections, liver, and a section of hindlimb skeletal muscles were pulverized with a mortar and pestle and then disrupted with a Polytron in luciferase lysis buffer (0.1% NP-40, 0.5% deoxycholate and proteinase inhibitors, Promega Corporation, Madison, Wisconsin). The resulting homogenate was centrifuged at 10,000 × g for 10 min, and 100 μl of luciferase reaction buffer (Promega) was added to 20 μl of the clear supernatant. Light emission was measured by a luminometer (TD-20/20, Turner Designs Inc., Sunnyvale, California) in relative light units (RLU)/min (14). Total protein content was determined by a modification of the Lowry method (BCA protein assay reagent, Pierce Biotechnology, Inc., Rockford, Illinois)(15).

Experimental protocols. The first set of experiments was conducted to systemically evaluate the effects of several ultrasound parameters that are known to effect microbubble destruction on adenoviral gene expression using UTMD. Four different parameters were tested in the following order: ECG triggering, transducer frequency, mode of ultrasound, and acoustic power. For each experiment, a minimum of three rats for each group was tested.

To evaluate the effect of ECG triggering, continuous ultrasound, which is highly destructive of microbubbles, was compared with end-systolic–triggered ultrasound. The hypothesis underlying this experiment was that triggered ultrasound, by allowing complete replenishment of the myocardial capillary bed between ultrasound bursts, would allow greater gene expression than continuous ultrasound, which would destroy microbubbles within the LV cavity before they could enter the microcirculation. Triggered ultrasound was performed with an S3 transducer operating in ultraharmonic mode (transmit 1.3 MHz, receive 3.6 MHz) using a burst of four frames gated to every fourth heartbeat at end-systole. This sequence produced the highest microbubble signal in the myocardium on the imaging frame, with complete clearance of microbubbles at the end of the burst. This is the same triggering sequence used in the previous report of successful delivery of the beta-galactosidase reporter gene to rat myocardium (11). Continuous ultrasound was performed at a frame rate of 33 ms using otherwise-identical settings. For both groups, the mechanical index was set at 1.6, depth at 3 cm, gain at 50, and compression at 70, with a linear post-processing curve.

The effect of different transducer frequencies on gene delivery was assessed using three different transducers (S3, S8, and S12) to deliver ultrasound at 1.3 MHz, 5 MHz, and 12 MHz, respectively. The hypothesis was that the lowest frequency would provide superior gene expression because it is more destructive of microbubbles. Other ultrasound parameters were held constant, including the mechanical index 1.6, depth 3 cm, gain 50, compression 70, and a linear post-processing curve. Based on the results of the first experiment, ECG triggering was used with four bursts of ultrasound every fourth beat.

Next, ultraharmonic imaging, which transmits a single pulse per scan line, was compared with power Doppler imaging, which transmits multiple pulses per scan line. Because multiple pulses should be more destructive than a single pulse, we hypothesized that power Doppler imaging would result in more effective gene delivery. Ultrasound parameters were identical to those in the first experiment, with end-systolic triggering of four bursts of ultrasound every fourth heartbeat using the S3 transducer. The only difference between groups was the use of triggered ultraharmonics versus triggered harmonic power Doppler.

The effect of acoustic power on gene delivery was compared at mechanical index settings of 1.6 and 1.2 using the S3 transducer. It is known that liposome microbubbles disappear at both of these mechanical index settings. However, previous studies have shown that the mechanism of destruction involves violent cavitation of the microbubbles only at the maximal mechanical index. Therefore, we hypothesized that gene expression would be higher at a mechanical index of 1.6 than at 1.2. All other settings were held constant as above. For all of the above experiments, there were three rats in each group.

Delivery of plasmid DNA at different doses ranging from 1 to 8 mg/ml of microbubbles was tested using the optimal ultrasound settings as determined from the above experiments. In addition, the effect of increasing the mechanical index from its usual maximal value of 1.6 to 2.0 on expression of plasmid DNA was assessed. This was accomplished by manipulating the software within the ultrasound system to drive the S3 transducer at a higher voltage than normally allowed.

Statistical analysis. Differences in luciferase activity between experimental groups were compared by two-way analysis of variance (ANOVA) using the experiment intervention as one group and the tissue type (anterior myocardium, posterior myocardium, liver, and skeletal muscle) as the other group. A p value ≤0.05 was considered statistically significant. Post hoc Scheffé tests were performed only when the ANOVA F values were statistically significant.

RESULTS

Triggered versus continuous ultrasound. The results are illustrated in Figure 2. The difference between triggered and continuous ultrasound was statistically significant (F = 23.9, p < 0.0001), as was the difference between anterior myocardium, posterior myocardium, liver, and skeletal mus-
Liver activity was high (nearly $10^5$ RLU/mg protein/min) for all infusions containing AdCMV-luc and was significantly greater than myocardial activity by Scheffé test ($p < 0.05$). Luciferase activity in the anterior wall ($4.4 \pm 2.1 \times 10^4$ RLU/mg protein/min) was approximately half that of liver activity and was twice that of the posterior wall ($2.0 \pm 0.7 \times 10^4$ RLU/mg protein/min) with triggered ultrasound. Myocardial luciferase activity was seen only with microbubbles containing AdCMV-luc in the presence of ultrasound. No luciferase activity was detected in control infusions or in skeletal muscle.

**Ultrasound frequency.** The results are shown in Figure 3. Differences between frequencies ($F = 4.3$, $p = 0.025$) and tissues ($F = 48.4$, $p < 0.0001$) were statistically significant. Luciferase activity in the myocardium was inversely related to ultrasound frequency. At the lowest frequency (1.3 MHz), luciferase activity in the anterior LV ($3.8 \pm 0.2 \times 10^4$ RLU/mg protein/min) was twice that of the posterior wall ($2.0 \pm 0.6 \times 10^4$ RLU/mg protein/min). At the middle frequency, anterior wall luciferase activity was $5.9 \pm 0.9 \times 10^3$ RLU/mg protein/min, with no significant activity in the posterior wall ($1.6 \pm 1.0 \times 10^3$ RLU/mg protein/min). Again, there was no detectable luciferase activity observed in skeletal muscle, and liver activity was high ($10^5$ RLU/mg protein/min) in all rats receiving AdCMV-luc.

**Ultraharmonics versus power Doppler.** As shown in Figure 4, the difference between ultraharmonic and power Doppler imaging modes was not statistically significant ($F = 1.36$, $p = 0.26$). Again, luciferase activity in the anterior LV is roughly half that of liver and twice that of the posterior wall. These findings are consistent for all experiments. The differences between tissues were statistically significant ($F = 30.0$, $p < 0.0001$), primarily because of the markedly high liver activity and absent skeletal muscle activity.

**Mechanical index.** By ANOVA, there was a statistically significant difference between a mechanical index of 1.6 and 1.2 ($F = 11.1$, $p = 0.0049$). In the anterior wall, luciferase activity was sixfold higher at a mechanical index of 1.6 than at 1.2 ($2.4 \pm 1.6 \times 10^4$ vs. $0.4 \pm 0.3 \times 10^4$ RLU/mg protein/min). In the posterior wall, luciferase activity was $1.6 \pm 0.7 \times 10^4$ RLU/mg protein/min at a mechanical index of 1.6 but was barely detectable at a mechanical index of 1.2.

**Delivery of plasmid DNA.** Experiments using plasmid DNA were performed using the optimal echocardiographic parameters determined from the previous experiments. Specifically, ECG triggering was performed at every fourth end-systole using a transmission frequency of 1.3 MHz with ultraharmonic mode and a mechanical index of 1.6. Figure 5 shows luciferase activity in the heart, liver, and skeletal muscle. In the anterior wall, luciferase activity was $5.3 \pm 2.4 \times 10^3$ RLU/mg/min at the highest dose compared with $3.8 \pm 1.8 \times 10^3$ RLU/mg/min in the posterior wall with
virtually no expression in liver or skeletal muscle (F = 19.7, p < 0.0001). In addition, the increase in luciferase activity for the different doses of DNA per milliliter of microbubbles was statistically significant (F = 6.5, p = 0.0015). Figure 6 shows the effects of using a mechanical index setting of 2.0 with plasmid DNA. At this mechanical index, anterior wall luciferase activity was $1.2 \pm 0.2 \times 10^4$ RLU/mg/min compared with $5.2 \pm 1.4 \times 10^3$ RLU/mg/min at a mechanical index of 1.6. Posterior wall activity was roughly half that seen in the anterior wall ($5.0 \pm 3.8 \times 10^3$ RLU/mg/min and $2.0 \pm 0.9 \times 10^3$ RLU/mg/min, respectively) with no detectable liver or skeletal muscle activity. At this ultrahigh mechanical index setting, plasmid luciferase activity was on the same order of magnitude as that seen with adenovirus at a mechanical index of 1.6. Importantly, there were no significant ventricular arrhythmias or decreases in fractional area shortening at any of the mechanical index settings in this study.
DISCUSSION

These experiments demonstrate high levels of myocardial luciferase activity after delivery of viral and plasmid transgenes using UTMD. Optimal expression was observed using triggered ultrasound at a transmission frequency of 1.3 MHz and a high mechanical index. There was no significant difference between ultraharmonic and power Doppler modes. Using these optimal ultrasound settings, plasmid DNA could be successfully delivered to rat myocardium without the liver expression that limits the use of adenoviral vectors (16). We consistently found higher expression in the anterior than in the posterior wall, a finding most likely explained by attenuation due to microbubbles in the LV cavity.

Triggered versus continuous ultrasound. Because destruction of microbubbles is required for successful gene delivery using UTMD, one might think that continuous ultrasound (30 frames/s) would be superior to ECG-
triggered ultrasound. However, continuous ultrasound destroys microbubbles within the LV cavity and myocardial arterioles before they ever reach the capillary bed. In addition, because myocardial capillary blood flow velocity is slow (<1 mm/s) (17), the capillary bed can never completely fill with microbubbles during continuous ultrasound at destructive acoustic pressure. This phenomenon has been mathematically modeled by Wei et al. (18), who showed that the ultrasound signal intensity rapidly rises and then reaches a plateau when plotted against pulsing interval during triggered ultrasound. The rate of rise corresponds to blood flow velocity, and the plateau corresponds to capillary blood volume. Thus, by using triggered ultrasound at relatively long pulsing intervals, one can wait until the entire myocardial capillary volume is filled with microbubbles before destroying them. The present study confirms that ECG triggering is superior to continuous ultrasound for optimizing gene expression by UTMD.

**Effect of frequency and ultrasound mode.** Higher ultrasound frequencies have a higher axial resolution and yield much better images, particularly in small animals such as rodents. However, it is well known that lower frequencies are more destructive of microbubbles (19,20). This study confirms that much lower frequencies are required to successfully deliver and express genes in the myocardium. However, we were not able to control pulse duration, line density, and beam width for the three different transducers. For example, the pulse duration is longer for the S3 transducer, which would tend to increase microbubble destruction. However, peak negative pressure and line density are higher on the S8 and S12 transducers, which would tend to offset the difference in pulse duration. Despite these considerations, the differences in gene expression with the S3 transducer were so large that we believe the conclusion that lower frequencies are superior for UTMD remains justified.

We also compared two imaging modalities: ultraharmonics and power Doppler. We anticipated that power Doppler, because it transmits multiple pulses per scan line, would be superior to ultraharmonics. However, no significant difference was found, perhaps because both techniques resulted in complete clearance of microbubbles from the myocardium during each burst of ultrasound. This suggests that microbubble destruction by both techniques was virtually complete at a triggering interval every fourth cardiac cycle.

**Effect of mechanical index.** The finding that luciferase expression was sixfold higher in the anterior myocardium at a mechanical index of 1.6 than at 1.2 has important implications regarding the mechanism of gene delivery and expression by UTMD. At both settings, microbubbles were destroyed, and visual clearance of microbubbles was complete. However, recent studies from Ferrara’s laboratory have shown that the mechanism of microbubble destruction varies with the delivered acoustic pressure as well as microbubble physical properties (21–23). At lower acoustic pressures, gas diffusion out of the microbubble is enhanced, and the microbubble dissolves over a period of milliseconds (24). At maximal acoustic pressure, the microbubbles undergo violent fragmentation over a period of nanoseconds due to Raleigh-Taylor instability (24). Such rapid microbubble collapse is known to generate a secondary shock wave (25,26). Whether the fragmentation of the microbubble, the secondary shock wave, or both, contribute to the transmission of adenoviral particles from the microbubble into the surrounding tissues is not currently known. However, the finding that maximal acoustic pressure results in higher myocardial gene expression provides indirect evidence that rapid microbubble fragmentation is necessary for efficient gene delivery by UTMD. This finding is relevant not only for establishing the appropriate ultrasound settings for UTMD but also for engineering microbubbles specifically designed for gene or drug delivery.

**Comparison with other methods of adenoviral gene delivery.** Simple intracoronary injection of adenovirus does not produce high levels of transgene expression (2–4). However, temporary coronary occlusion or administration of substances that increase vascular permeability have been shown to increase the effectiveness of intracoronary infusion of adenovirus. For example, Logeart et al. (4) demonstrated myocardial luciferase activity with such interventions that was roughly 10-fold lower than direct LV injection of adenovirus in rabbits. The present study demonstrates high levels of luciferase gene expression using systemic intravenous infusion of AdCMV–luc-microbubbles during echocardiography. Ultrasound–targeted microbubble destruction directs gene delivery to specific organs with what we anticipate will be substantial improvement in the therapeutic ratio for a broad range of genetic manipulations. We did not directly compare UTMD to more invasive methods of gene delivery, such as direct LV injection or coronary infusion, but the advantages of this much less invasive approach in cardiac gene therapy are readily apparent.

**Delivery of plasmid DNA.** UTMD can successfully deliver plasmid DNA to myocardium. In these optimization experiments, the levels of luciferase expression were similar to that obtained using adenovirus but without the profound liver uptake associated with adenovirus. This is the first study to actually demonstrate successful expression of a plasmid transgene using UTMD. Vannan et al. (27) reported the expression of chloramphenicol acetyltransferase using plasmid attached to the surface of cationic liposomes. However, they did not achieve organ specificity, because the levels of chloramphenicol acetyltransferase were similar in the heart to organs that were not exposed to ultrasound. In our study, there was no liver or skeletal muscle expression of luciferase, indicating that UTMD can be used to achieve organ specificity with plasmid DNA. Further studies are needed to determine whether plasmid DNA expression in the heart by UTMD can be enhanced by combining it with other tissue-targeting strategies, such as manipulation of the gene promoter or adding cell-specific ligands to the shell, development of ultrasound transducers specifically designed...
for gene delivery, or improvements in microbubble shell characteristics that allow more DNA to be loaded onto the microbubbles.

SafetY of UTMD. We have previously reported that UTMD in rat hearts is associated with a small troponin T leak without histologic evidence of inflammation or myonecrosis or echocardiographic evidence of LV dysfunction (28). In this study, there was no evidence of LV dysfunction by two-dimensional echocardiography; we did not measure troponin T. However, we routinely use a 20-min infusion of the microbubbles because in our early experience with the technique, rapid bolus injections of contrast agent resulted in profound LV dysfunction.

Conclusions. High levels of transgene expression can be achieved in the heart using UTMD containing adenoviral or plasmid DNA. Optimal ultrasound parameters for this technique include a low-transmission frequency (1.3 MHz), maximal mechanical index, and ECG triggering to allow complete filling of the myocardial capillary bed by microbubbles.

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