Ultrasound-Targeted Microbubble Destruction Can Repeatedly Direct Highly Specific Plasmid Expression to the Heart

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Background—Noninvasive, tissue-specific delivery of therapeutic agents would be a valuable clinical tool. We have previously shown that ultrasound-targeted microbubble destruction can direct expression of an adenoviral reporter to the heart. The present study shows that this method can be applied to selectively deliver plasmid vectors to the heart.

Methods and Results—We used albumin and lipid microbubbles containing plasmids with a luciferase transgene to target the heart in rats. After 4 days, organs were harvested and analyzed for reporter gene expression. In a second set of experiments, the hearts of rats treated with plasmids were harvested at various time points during a 4-week period. Both luciferase activity and mRNA concentrations were measured. Luciferase transfection with plasmids showed highly specific gene expression in the heart, with hardly any activity in control organs. Time course evaluation showed high transgene expression in the first 4 days, with a rapid decline thereafter. Repeated treatment produced a second peak of transgene expression with similar decay.

Conclusions—Ultrasound-mediated destruction of microbubbles directs plasmid transgene expression to the heart with much greater specificity than viral vectors and can be regulated by repeated treatments. This noninvasive technique is a promising method for cardiac gene therapy. (Circulation. 2003;108:1022-1026.)

Key Words: echocardiography ■ gene therapy ■ ultrasonics ■ contrast media

The molecular understanding of disease has accelerated in recent years, producing many new potential therapeutic targets. A noninvasive delivery system that can target specific anatomical sites would be a great boon for many of these therapies, particularly those based on manipulation of gene expression. Viral vectors, which have been commonly used for gene therapy,1 are limited by hepatic toxicity, immunogenetic properties, inflammation, and low tissue specificity, as well as the difficulty and expense of producing large amounts of pure virus. The use of naked DNA or liposome carriers has the disadvantage of low transfection efficiency and the requirement for invasive delivery techniques. Many strategies have been developed for augmenting tissue specificity. These include decorating viral vectors with cell-specific ligands,2 the use of cell-specific3 or pathology-specific4 promoters in the transgene construction, and physical placement of the vector in the vicinity of the target by catheter-based methods5,6 or by direct injection.7-9

We have investigated ultrasound-targeted microbubble destruction (UTMD) as a method for delivering drugs or genes to specific tissues.10 This method involves the attachment of drugs or genes to gas-filled microbubbles, which are then circulated through the intravascular space and mechanically destroyed within the target organ by ultrasound.11-13 Theoretically, one should be able to target any anatomic site that is accessible to ultrasound, including selected diseased or damaged regions of an organ. Moreover, it should be possible to combine this approach with many of the other methods for enhancing tissue specificity.

We have previously shown that this method can deliver an adenoviral reporter gene to the heart.10 In the current study, we demonstrate that UTMD can deliver plasmid vectors to the heart with efficiency and far greater specificity than viral vectors. Moreover, the transient transgene expression of these vectors can be prolonged at will with repeated application of this noninvasive treatment.

Methods

Preparation of Microbubbles

Albumin microbubbles with a plasmid encoding a luciferase reporter (pGL3, Promega Corp) under the regulation of an inserted CMV promoter were produced by sonication of a 1-mL solution containing 4 mg plasmid, 10% dextrose, and 5% human serum albumin in perfluorocarbon gas for 8 to 10 seconds at 20 kHz (Heat System Inc, XL2020).14 One-half milliliter of this solution was then diluted with...
0.5 mL of PBS before injection. The parameters we used for generating plasmid microbubbles were recently determined, and the stability of inserted plasmids was verified. 15 Lipid stabilized microbubbles were produced by a modification of a previously described method. 16 One-half milliliter of a solution containing 2 mg CMV-luciferase plasmids, 1% DL-α-phosphatidylcholine, 0.25% DL-α-phosphatidylethanolamine, and 10% glycerol in PBS was incubated at 40°C for 30 min. It was then transferred to a tube containing 1 mL of perfluorocarbon gas and shaken vigorously for 20 sec. This solution was then diluted with 0.5 mL of PBS before injection. The amount of plasmid adherent to lipid microbubbles was determined by optical density at a wavelength of 260 nm after 3 washes in PBS and bubble destruction. Destroyed microbubbles without previous DNA loading were used for calibration before washes in PBS and bubble destruction. Destroyed microbubbles with 0.5 mL of PBS before injection.

In the first protocol, 12 rats were transfected with CMV-luciferase plasmids with the use of albumin or lipid microbubbles (6 rats in each group). Rats were killed, and organs harvested 4 days after UTMD treatment. For luciferase assays, heart, left lung, liver (midportion), muscle (M quadratus), brain, and pancreas were obtained. The entire anterior and posterior portions of the heart, determined in situ, were analyzed separately after detachment of the atria. The whole pancreas and 100-mg samples of the other organs were disrupted in a Polytron homogenizer in 1 mL luciferase lysis buffer. The solution was cleared by centrifugation and 20 μL of supernatant was analyzed in a TD20/20 luminometer (Turner Designs) with the use of a commercial substrate (Luciferase Assay System, Promega). Protein concentration was determined by the method of Lowry with a commercial kit (Pierce Endogen) to allow calculation of relative luminescence units per mg protein per minute (RLU/mg per minute). Hearts of another 3 rats (treated with lipid microbubbles) were harvested to evaluate spatial distribution of luciferase activity in the heart. Apex, mid-ventricles, basal ventricles, and atria were processed separately as described above. A control experiment was done with lipid microbubbles in 3 different groups of rats (2 rats each): The first group received microbubbles with plasmid without ultrasound application; the second group received plasmid and ultrasound without microbubbles; the third group received microbubbles and ultrasound without plasmid immediately followed by a plasmid infusion (2 mg in 20 min).

In the second protocol, all experiments were performed with the use of lipid microbubbles. For the time course experiment, 18 rats were transfected with CMV-luciferase plasmids, and hearts were harvested after 2, 4, 7, 14, 21, and 28 days (3 rats in each group). To evaluate the effect of repeated treatment on transgene expression, a separate set of 9 rats were transfected with luciferase plasmids. Three rats underwent a single transfection and were killed on day 8, 3 were treated again on day 4 and killed on day 8, and 3 were treated again on day 4 and killed on day 12.

Hearts were dissected into atria, anterior ventricles, and posterior ventricles. Total RNA was isolated from atria and anterior ventricles (Trizol, Invitrogen). To quantify luciferase-mRNA concentrations, cDNA was produced by reverse transcriptase after digesting plasmid contaminants with DNAse. For quantitative PCR, luciferase-specific primers were used to amplify product from cDNA representing 50 ng of total RNA. PCR was run in triplicate with SYBR green fluorophore (Molecular Probes) in an Opticon device (MJ Research). Expression level was interpolated from a standard curve generated from a series of dilutions at cycle times in which threshold intensity was clearly exceeded. For relative quantification of luciferase plasmid concentration in heart tissues, we took advantage of unavoidable plasmid contamination in total RNA. RNA was digested with the use of RNase H. It was then used for quantitative PCR with luciferase primers. Posterior ventricles were used for luciferase assays as described above.
Mean and standard deviation was calculated for all rats treated with CMV-luciferase cDNA containing vectors, and differences in luciferase activity between groups were compared by ANOVA for multiple group comparisons. For the time-course experiments, data were analyzed by repeated-measures ANOVA.

**Results**

**Microbubble Preparation**

Mean microbubble size was 2.33±0.66 μm for albumin bubbles and 1.9±0.2 μm for lipid bubbles. Concentrations were 1.9±0.3×10⁸ bubbles/mL for the albumin preparation and 1.5±0.3×10⁸ bubbles/mL for the lipid bubbles. The recovered plasmid amount after 3 washes was 400±20 μg/mL bubble suspension for lipid microbubbles and 340±15 μg/mL for albumin microbubbles. Successful binding of DNA on bubbles was confirmed by immunofluorescence microscopy after staining with propidium iodine.

**Luciferase Activity in Target and Control Organs**

Successful gene transfer was obtained with both microbubble preparations achieving high transgene activity and organ specificity.

Animals treated with microbubbles containing CMV-luciferase plasmids showed substantial activity in the heart and negligible activity in any control organ, including the liver. Only one rat receiving albumin microbubbles to the heart showed detectable activity in the liver (2.1 RLU/mg per minute), and one rat receiving lipid microbubbles showed activity in the pancreas (11.6 RLU/mg per minute). All other control organs showed no activity. No significant difference was seen between albumin- and lipid-treated animals for activity in the posterior ventricle (255±261 versus 569±540 RLU/mg per minute; P=0.15) (Figure 1). Percentage of total activity in the heart was higher in the anterior versus posterior portion with both formulations (albumin: 89.4±9.9% versus 10.6±9.9%, P<0.01; lipid: 77.2±18.1% versus 22.8±18.1%, P<0.01). When looking at spatial distribution within the heart, the mid and basal ventricle appeared to have higher activity (apex 933±755 versus mid-ventricle 2926±2600 versus basal-ventricle 2720±2016 versus atria 1061±788 RLU/mg per minute). In the negative control experiments, all hearts had undetectable luciferase activity.

**Time-Course Evaluation of Transgene Expression and the Effect of Repeated Treatment**

Time-course evaluation showed highest transgene expression in the first 4 days, with a rapid decline thereafter. Retransfection after 4 days achieved prolongation of transgene expression.

Luciferase mRNA was most abundant on day 2 and day 4, both for anterior ventricles and atria (Figure 2A). After day 4, mRNA concentration declined rapidly but was still detectable after 28 days at a low level. When looking at relative plasmid concentration, the highest amount was seen on day 2 with a decline on day 4 (Figure 2B). Corresponding luciferase activity in the posterior heart showed a peak at day 4 and a substantial decline thereafter (Figure 2C). Minimal residual activity was still detectable on day 28.

In the retransfection experiment transgene expression was substantially higher in the group with retransfection harvested after 8 days (black bars) compared with the group without retransfection (gray bars) (Figure 3A and 3B), reaching a level of transgene expression similar to that obtained after the initial treatment. This difference was statistically significant for mRNA in anterior ventricles (231 000±69 000 versus 75 000±54 000 molecules, P<0.05) and in atria (173 000±48 000 versus 62 000±45 000 molecules, P<0.05) and showed a strong trend toward significance for luciferase activity in posterior ventricles (3665±2298 versus 872±320 RLU/mg per minute, P=0.06). Retransfected hearts harvested after 12 days (white bars) showed a significant decline for all 3 groups compared with retransfected hearts harvested after 8 days.
Discussion
This study was designed to explore the applicability of UTMD for plasmid delivery to the heart. We have shown that UTMD can deliver plasmids to the heart, achieving transient transgene expression with striking tissue specificity. Transgene expression can be prolonged by repeated UTMD. UTMD directed CMV-luciferase plasmids to the heart with very high organ specificity. Only 2 animals showed any activity in control organs. Taking into account the high sensitivity of luciferase assays, this result stands in clear contrast to luciferase activity in control organs when using adenovirus. Previous experiments with adenovirus had shown that luciferase activity was detectable in all organs and was often 10-fold higher in the liver when compared with target organs (data not shown). We anticipate that organ specificity with naked DNA will be primarily limited by the spatial precision of the ultrasound beam, which has to be investigated with animal models that permit greater anatomic resolution. We further expect that DNA released into the circulation by microbubble destruction within the cardiac chambers is rapidly degraded by plasma DNases, reducing risk of toxicity at nontargeted sites.

The plasmid amount used for microbubble production is high. We have previously established the optimal concentration to achieve maximum DNA loading on bubbles. Considering that only 17% to 20% of DNA is bound on microbubbles, the actual amount available for transfection to the heart is 340 to 400 μg. This is still lower than used by some other groups for direct myocardial injection into rat hearts. Furthermore, toxicity of naked DNA is relatively low, and plasmids are easy and inexpensive to produce.

The lipid composition we used in our study is not cationic. Therefore, spontaneous binding of DNA to the bubbles is reduced. However, plasmids were added before bubble production, thus being incorporated into the microbubble shell. In our experience, this technique allows larger amounts of plasmid DNA to adhere to the microbubbles. Lipid microbubbles have an advantage in being more easily produced and stored, at least with presently available formulations. Convenience in bubble production for lipid microbubbles was the reason for using these in the second part of the study. The
composition of the microbubble presents an opportunity for improvement of the technique. It may be possible to modify charge characteristics, lipid composition, or even add specific protein ligands to improve the performance of microbubbles as delivery vehicles for transgenes.

Luciferase activity was higher in the anterior portion of the heart with both microbubble preparations, possibly due to attenuation of the ultrasound energy delivered to the posterior wall by intracardiac microbubbles. In larger animals, specific gene transfer to the posterior heart probably could be obtained through a transsophageal approach.

The time-course experiments show the transient character of transgene expression in the heart when using this method. However, we have shown that the duration of transgene expression can be regulated by repeated transfections. A brief period of transgene expression may be useful for many clinical applications, such as cardiac angiogenesis, in which treatment should be stopped after new vessels have grown. Given the minimally invasive nature of UTMD, repetition of UTMD should have little adverse effect. The possibility of damage to the heart due to UTMD has been evaluated in a study performed to specifically address this question, in which we have shown a slight troponin leak without histological or functional changes.

The decline in luciferase activity after 4 days was concordant with a decline in luciferase mRNA molecules. This suggests that intracellular plasmids are degraded rapidly. The data for relative plasmid concentration in total RNA supports this conclusion. The mechanisms whereby the plasmid is inactivated or cleared are not well understood. Further investigation into this phenomenon would be useful to learn how to prolong the duration of transgene expression.

From these studies we conclude that UTMD of microbubbles containing plasmid DNA can generate high levels of transgene activity restricted to the heart. A remarkable aspect of using plasmids with this technique is that we obtain high transgene expression in a target organ without the liver expression, (and, we predict, without the toxicity) seen with viral vectors. Ultrasound-mediated microbubble destruction should improve the efficacy and reduce the dose and side effects of a broad range of bioactive agents, including but not limited to gene therapy vectors, and provides a useful new tool for manipulating gene expression in the living animal.

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