Cationic versus Neutral Microbubbles for Ultrasound-mediated Gene Delivery in Cancer¹

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Purpose:

To test whether plasmid-binding cationic microbubbles (MBs) enhance ultrasound-mediated gene delivery efficiency relative to control neutral MBs in cell culture and in vivo tumors in mice.

Materials and Methods:

Animal studies were approved by the institutional animal care committee. Cationic and neutral MBs were characterized in terms of size, charge, circulation time, and DNA binding. Click beetle luciferase (CBLuc) reporter plasmids were mixed with cationic or neutral MBs. The ability of cationic MBs to protect bound plasmids from nuclease degradation was tested by means of a deoxyribonuclease (DNase) protection assay. Relative efficiencies of ultrasound-mediated transfection (ultrasound parameters: 1 MHz, 1 W/cm², 20% duty cycle, 1 minute) of CBLuc to endothelial cells by using cationic, neutral, or no MBs were compared in cell culture. Ultrasound-mediated gene delivery to mouse hind limb tumors was performed in vivo ($n = 24$) with insonation (1 MHz, 2 W/cm², 50% duty cycle, 5 minutes) after intravenous administration of CBLuc with cationic, neutral, or no MBs. Tumor luciferase activity was assessed by means of serial in vivo bioluminescence imaging and ex vivo analysis. Results were compared by using analysis of variance.

Results:

Cationic MBs (+15.8 mV; DNA binding capacity, 0.03 pg per MB) partially protected bound DNA from DNase degradation. Mean CBLuc expression of treated endothelial cells in culture was 20-fold higher with cationic than with neutral MBs (219.0 relative light units [RLUs]/μg protein ± 92.5 [standard deviation] vs 10.9 RLUs/μg protein ± 2.7, $P = .001$) and was significantly higher ($P < .001$) than that in the no MB and no ultrasound control groups. Serial in vivo bioluminescence of mouse tumors was significantly higher with cationic than with neutral MBs ($[5.9 \pm 2.2]$ to $[9.3 \pm 5.2]$ vs $[2.4 \pm 0.8]$ to $[2.9 \pm 1.1] \times 10^4$ photons/sec/cm²/steradian, $P < .0001$) and versus no MB and no ultrasound controls ($P < .0001$). Results of ex vivo analysis confirmed these results ($\rho = 0.88$, $P < .0001$).

Conclusion:

Plasmid-binding cationic MBs enhance ultrasound-mediated gene delivery efficiency relative to neutral MBs in both cell culture and mouse hind limb tumors.

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Supplemental material: <http://radiology.rsna.org/lookup/suppl/doi:10.1148/radiol.12112368/-/DC1>

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Ultrasound-mediated gene delivery is a promising noninvasive and nonviral strategy for targeted gene therapy. Through a process termed sonoporation, selective insonation of tissue actuates local formation of transient cell membrane microperforations, enhancing blood vessel wall permeability and facilitating the ingress of therapeutic agents into cells (1–4). The putative primary mechanism for sonoporation is acoustic cavitation, whereby gas bodies oscillate and eventually collapse, releasing the energy necessary to induce transient cell membrane permeabilization (1,5,6). Secondary ultrasound bioeffects, such as microstreaming and other convective phenomena, are also thought to contribute (7,8). Ultrasound-mediated gene delivery has been shown to be markedly potentiated in the presence of microbubbles (MBs), which serve as exogenous cavitation nuclei and reduce the ultrasound energy threshold necessary for sonoporation to occur (1,9).

Although the therapeutic potential of ultrasound-mediated gene delivery with and without MBs has been demonstrated in cell culture (10–12) and in vivo studies (13–18), the degree of successful gene transfer achieved has been variable. Future clinical translation of this technology requires further improvements in in vivo gene delivery efficiency. This is determined by several parameters, including those related to the ultrasound beam applied (eg, frequency, intensity, duty cycle,

and duration), the MBs used (eg, concentration, size, and composition), and the target tissue (19,20). A key factor in gene delivery efficiency is the concentration of genetic material at the site of sonoporation (10–12,21). Although most published in vivo studies involved systemically administered mixtures of uncoupled plasmid DNA and MBs, efforts to improve ultrasound-mediated gene delivery efficiency have centered on functionalizing MBs to become carrier vehicles that directly bind and transport genetic cargo (14,15,22,23). This direct coupling strategy theoretically potentiates ultrasound-mediated gene delivery by protecting the circulating genetic material from premature clearance and degradation by endogenous nucleases while en route to the target treatment location, thereby increasing local gene concentration at the site of sonoporation (22). Furthermore, as acoustic destruction of the carrier MBs simultaneously triggers sonoporation and releases the genetic payload, the freed plasmid DNA is localized to the immediate vicinity of cell permeabilization, improving both delivery efficiency and site specificity (24,25).

Because the phosphate backbone of DNA and RNA render them highly anionic, MBs with cationic lipid shells have thus been utilized as a simple strategy to engineer MBs that spontaneously complex with genetic cargo by means of charge interactions (14,22,26–30). To determine whether systemic administration of genes directly coupled onto MBs confers a transfection efficiency advantage in ultrasound-mediated gene delivery versus traditional coadministration of uncoupled genes and MBs, a direct comparison between the two approaches is necessary. To the best of our knowledge, such a direct comparison has not been previously

reported in an in vivo cancer model following systemic MB administration.

Therefore, the purpose of this study was to test whether plasmid-binding cationic MBs enhance ultrasound-mediated gene delivery efficiency relative to control neutral MBs in cell culture and in vivo tumors in mice.

Materials and Methods

One author (M.S.) is an employee of Bracco Research (Geneva, Switzerland). Authors who were not affiliated with commercial entities (D.S.W., C.P., M.A.P., R.P., J.R., S.S.G., J.K.W.) had control of the data and information submitted for publication.

Click Beetle Luciferase and β -Galactosidase Reporter Plasmid Preparation

A reporter plasmid encoding for red-shifted click beetle luciferase (CBLuc) driven by a cytomegalovirus promoter was used to evaluate ultrasound-mediated transfection efficiency in cell culture and in a

Advances in Knowledge

- The use of plasmid-binding cationic microbubbles (MBs) in ultrasound-mediated gene delivery enhances transfection efficiency relative to neutral MBs in both cell culture (20 fold) and tumors in mice (2.4–3.2 fold).
- Cationic MBs are able to directly bind, transport, and protect anionic plasmid DNA from degradation by endonucleases, thereby increasing local DNA concentration at the site of ultrasound-mediated gene delivery in the in vivo setting.

Implication for Patient Care

- The transfection efficiency advantages afforded by using plasmid-binding cationic MBs in ultrasound-mediated gene delivery may facilitate the future application of ultrasound and MB-mediated gene therapy in patients.

Published online before print

10.1148/radiol.12112368 Content code: OT

Radiology 2012; 264:721–732

Abbreviations:

ANOVA = analysis of variance
b-gal = β -galactosidase
CBLuc = click beetle luciferase
DNase = deoxyribonuclease
EDTA = ethylenediaminetetraacetic acid
MB = microbubble
PBS = phosphate-buffered saline
RLU = relative light unit

Author contributions:

Guarantors of integrity of entire study, D.S.W., M.A.P., J.K.W.; study concepts/study design or data acquisition or data analysis/interpretation, all authors; manuscript drafting or manuscript revision for important intellectual content, all authors; manuscript final version approval, all authors; literature research, D.S.W., C.P., J.K.W.; experimental studies, D.S.W., C.P., M.A.P., R.P., J.K.W.; statistical analysis, D.S.W., J.R., J.K.W.; and manuscript editing, D.S.W., C.P., M.A.P., S.S.G., M.S., J.K.W.

Funding:

This research was supported by the National Institutes of Health (grants R25 CA11868, R21 CA13929, and P50 CA114747).

Potential conflicts of interest are listed at the end of this article.

mouse tumor model. The reporter plasmid with the *lacZ* gene (β -gal), which encodes for β -galactosidase (b-gal), was used to assess the spatial distribution of in vivo ultrasound-mediated gene delivery. Both plasmids were constructed in the pcDNA 3.1-puromycin (+) vector backbone (modified pcDNA 3.1 (+); Invitrogen, Carlsbad, Calif) by polymerase chain reaction (PCR) cloning. By using the pC-BRLuc vector (Promega, Madison, Wis) as a template, the CBLuc gene fragment was amplified by means of PCR (Triple Master PCR System; Eppendorf, Hauppauge, NY) using the forward primer CCGGC-TAGCATGGTAAAGCGTGAGAAAAATG and the reverse primer CGCCTCGAGC-TAACCGCCGGCCTTCACCAAC, which contain *Nhe* I and *Xho* I restriction enzyme sites, respectively. For β -gal, the pSV- β -gal control vector (Promega) was used as template and the *lacZ* gene fragment was amplified by using the forward primer CTAGCTAGCATGATCGACCCCGTGGTGCTG and the reverse primer CC-GCTCGAGTTAACCGGTCTTCTGGCAC-CAC, which also contain *Nhe* I and *Xho* I restriction enzyme sites, respectively. The amplified gene fragments were then ligated into the pcDNA 3.1-puromycin (+) vector at the corresponding *Nhe* I and *Xho* I enzyme digestion sites. Both clones were sequence confirmed, were amplified by using the Endotoxin Free Maxiprep kit (Qiagen, Valencia, Calif) according to manufacturer instructions, and were resuspended in Tris-ethylenediaminetetraacetic acid (EDTA) buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0; EMD Chemicals, Gibbstown, NJ). DNA concentrations were quantified by absorbance measurement (260 nm) with a spectrophotometer (Agilent, Santa Clara, Calif).

MB Preparation and Characterization

Methods for cationic and neutral MB preparation (Fig 1) and characterization for size, charge, in vivo blood half-life, capacity to bind plasmid DNA, and ability to protect DNA from nuclease degradation are detailed in Appendix E1 (online).

Ultrasound-mediated Transfection in Cell Culture

The relative transfection efficiencies of ultrasound-mediated gene delivery

Figure 1

Figure 1: Schematics of **(a)** neutral and **(b)** cationic MBs. The shells of both MBs consisted of the same lipid monolayer surrounding a perfluorobutane gas core, except that **(b)** cationic MBs also had **(c)** DSTAP incorporated into the shell to render the surface positively charged. Both MB types were coated with **(d)** DPPE-PEG₅₀₀₀ for MB stability. See Appendix E1 (online) for further details.

with cationic versus neutral MBs were first evaluated in cell culture by using SVR (ATCC, Manassas, Va), a murine vascular endothelial tumor cell line. Cell line preparation and maintenance methods are detailed in Appendix E1 (online). As gas-filled MBs are buoyant in liquid media, transfection experiments were performed with an inverted setup (Fig 2), with MB and CBLuc mixtures placed below SVR cells facing downward to maximize contact between cells and MBs.

First, a sterilized plastic ring was inserted (cap of a 50-mL conical centrifuge tube with a 24-mm-diameter opening) in the wells of six-well plates, and OptiMem serum-free medium (Invitrogen) was added to fill the well up to the top of the ring. Four micrograms of CBLuc were incubated with either 5×10^7 cationic, 5×10^7 neutral, or no MBs (100- μ L total mixture

volume in 0.9% saline; $n = 3$ per group per experiment) for 15 minutes at room temperature to allow for spontaneous binding between the anionic DNA and the positively charged surfaces of cationic MBs and were then added to the OptiMem medium. SVR cells grown to 70%–80% confluency on sterilized 25-mm-diameter glass coverslips (VWR Scientific, West Chester, Pa) were washed once with phosphate-buffered saline (PBS). The coverslips were then placed on the ring insert with the cells facing downward and toward the medium containing CBLuc-MB mixtures. Additional OptiMem medium was added on top of the coverslip to aid ultrasound transmission. To minimize the generation of standing waves, the six-well plates were placed on top of a platform and were partially submerged in a 37°C degassed water bath with an ultrasound-absorbing

rubber pad underneath (16). After MBs were allowed 5 minutes to float and contact the surfaces of the inverted cells, ultrasound was applied at 1 MHz, 1 W/cm², and 20% duty cycle for 1 minute by using the Sonitron 2000 ultrasound machine (Rich-Mar, Chattanooga, Tenn) and a 20-mm transducer submerged in the media above the coverslips. A negative control group consisted of SVR cells exposed to CBLuc-cationic MB mixtures in identical fashion but without application of ultrasound. All experiments were performed in triplicate.

Bioluminescence Assessment of Ultrasound-mediated Transfection in Cell Culture

Functional reporter gene expression in treated SVR cells was quantitatively assessed 24 hours after transfection by using a commercial luciferase assay (Promega) per manufacturer instructions, with light emission measured with a luminometer (20/20n Luminometer; Turner Biosystems, Sunnyvale, Calif) for 10 seconds. Measurements were normalized to total protein content in the measured sample (BCA Protein Assay-RAC; Pierce, Rockford, Ill). CBLuc activity was expressed as relative light units (RLUs) per microgram of protein.

Mouse Tumor Model for in Vivo Ultrasound-mediated Gene Delivery

All animal studies were approved by the institutional Administrative Panel on Laboratory Animal Care. All in vivo experiments were performed by using 6–8-week-old female nude mice (Charles River Laboratories, Wilmington, Mass) anesthetized with 2% isoflurane (Aerrane; Baxter, Deerfield, Ill) in oxygen administered at a rate of 2 L/min. SVR cell tumor xenografts were established by subcutaneous injection of 1.5×10^6 SVR cells mixed with 40 μ L of basement membrane matrix (Matrigel; BD Biosciences, San Jose, Calif) into the right dorsal hind limbs of mice positioned prone on a 37°C heating pad. Tumors were allowed to grow to a maximum diameter of 5 mm before experimentation.

Figure 2

Figure 2: Experimental setup for ultrasound (US)-mediated gene delivery in cell culture experiments. Glass coverslips bearing vascular endothelial cells were inverted, such that the cells were facing down, and were placed on a plastic ring platform in the well of a six-well plate. CBLuc-MB mixtures were added to medium below the coverslips within the ring, and ultrasound was applied through medium from above the coverslips. To avoid standing waves, the six-well plate was partially submerged in a degassed water bath with an ultrasound-absorbing rubber pad underneath.

In Vivo Ultrasound-mediated Gene Delivery

For vivo ultrasound-mediated gene delivery to hind limb tumor xenografts, mice were anesthetized and placed prone on a 37°C heating pad with ultrasound-absorbing rubber placed underneath the hind limb. CBLuc-MB mixtures were prepared as described above by using 200 μ g of CBLuc and 1×10^9 cationic MBs ($n = 7$), 1×10^9 neutral MBs ($n = 7$), or no MBs ($n = 5$). To ensure a controlled, steady intravenous injection, a catheter (MicroMarker Tail Vein Access Cannulation kit; VisualSonics, Toronto, Ontario) was placed into a lateral tail vein of the anesthetized mouse and the CBLuc-MB mixtures were administered through a catheter by using an infusion pump (GeniePlus; Kent Scientific, Torrington, Conn) with a flow rate of 100 μ L/min. Local ultrasound-mediated gene delivery was actuated by insonation of the hind limb tumor xenografts by using the Sonitron 2000 system and a 6-mm-diameter probe positioned directly on top of the tumor xenograft with ultrasonographic (US) gel in between. Sonoporation parameters were as follows: 1 MHz, 2 W/cm², and 50% duty cycle, with

a total ultrasound time of 5 minutes. As a negative control, tumor-bearing mice received intravenous administration of CBLuc-cationic MBs but ultrasound was not applied ($n = 5$). Mice were randomly assigned to one of these four treatment groups.

To evaluate the spatial distribution of gene transfer in this tumor model, in vivo ultrasound-mediated gene delivery with cationic MBs was performed on additional tumor-bearing mice ($n = 4$) in identical fashion except for the substitution of CBLuc for β -gal.

In Vivo Bioluminescence Assessment of in Vivo Ultrasound-mediated Gene Delivery

Efficiency of in vivo ultrasound-mediated gene delivery and transgene expression were assessed by longitudinal in vivo bioluminescence imaging of CBLuc activity by using the IVIS-200 imaging system (Caliper Life Sciences, Hopkinton, Mass) at 12, 24, 36, and 48 hours after transfection. Mice were anesthetized as described above. After intraperitoneal injection of D-luciferin (150 mg/kg; Biosynth, Staad, Switzerland), the substrate for CBLuc, mice were immediately placed

prone in the IVIS system, and serial 2-minute imaging frames were acquired until a peak in bioluminescent signal was reached. By using Living Image 3.0 software (Caliper Life Sciences), image analysis of all acquired frames was performed by quantifying maximum radiance (in photons/sec/cm²/steradian [sr]) of matching regions of interest manually drawn over the tumor-bearing hind limbs (D.S.W., with more than 4 years of image analysis experience) and subtracting background luminescence (31). The highest value of each acquisition session was used as measurement of transgene expression.

Ex Vivo Evaluation of in Vivo Ultrasound-mediated Gene Delivery

After mice were imaged 48 hours after in vivo ultrasound-mediated gene delivery of CBLuc, all animals were sacrificed, and tumors were removed and snap frozen for ex vivo luciferase assay. The rapid growth and early ulceration of SVR tumor xenografts necessitated the sacrifice of the mice at this time point. Because MBs are cleared through the reticuloendothelial cell system (32,33), the livers and spleens of mice from the cationic MB with ultrasound group were also excised to assay for nonspecific luciferase expression. Tissue samples were homogenized and lysed through sonication in cold Tper buffer (Invitrogen). Lysates were centrifuged (10000 g for 3 minutes), and supernatant was collected. CBLuc reporter gene expression was measured by using the aforementioned commercial luciferase assay and was normalized to total protein, as performed for the cell culture transfection studies.

So that we could evaluate the spatial distribution of gene transfer and transgene expression, hind limb tumors and surrounding tissue were excised en bloc and frozen in Optimal Cutting Temperature (OCT; Tissue-Tek, Fisher Scientific, Pittsburgh, Pa) 48 hours after in vivo ultrasound-mediated delivery of β -gal. Two adjacent frozen tissue slices (5 μ m) were obtained for each sample, placed on microscope slides, immediately fixed in 4% formalin, and

then washed in PBS. For β -gal staining, freshly prepared β -gal substrate solution (0.1% X-gal, 4 mmol/L MgCl₂, 1.5 mg/mL potassium ferricyanide, and 2.5 mg/mL potassium ferrocyanide in PBS) was added to one of the two slices, which were then incubated for 24 hours at 37°C. Histologic staining (hematoxylin-eosin, Fisher Scientific, Pittsburgh, Pa) of the adjacent sections was accomplished by standard procedures. After being washed in PBS, slides were mounted with mounting medium and coverslips. Whole-tissue scans (magnification, $\times 20$) were obtained by using the NanoZoomer slide scanner (Hamamatsu, Bridgewater, NJ), and higher-resolution images were prepared offline by using NDP.view software (Hamamatsu). As a negative control, hind limb tumor and surrounding tissue from mice that were not treated with ultrasound gene delivery were also excised, and slices were stained and imaged as above.

Statistical Analysis

For the cell culture experiments, a one-factor analysis of variance (ANOVA) with planned comparisons was performed. For longitudinal in vivo assessment of luciferase activity after in vivo ultrasound-mediated gene delivery, a 4 $\times$ 4 repeated-measures ANOVA of log₁₀ bioluminescence signal with fixed factors of treatment condition (ultrasound-mediated gene delivery with cationic, neutral, or no MBs; exposure to cationic MBs but no ultrasound), time (12, 24, 36, and 48 hours after transfection), and their interactions was performed. The Greenhouse-Geisser sphericity correction for within-subject correlation was used (34). Planned comparisons of treatment groups were also performed. For ex vivo analysis of luciferase activity, a one-factor ANOVA of log₁₀ bioluminescence signal with fixed factor of treatment type was performed, along with planned comparisons. Correlation between the in vivo and ex vivo bioluminescence data at 48 hours was calculated by using the Spearman rank correlation (ρ).

All statistical analyses were performed with Stata, release 9.2 (Stata,

College Station, Tex). For the multiple pairwise group comparisons in the cell culture study, a Bonferroni-adjusted two-sided *P* value of less than .007 (.05/7) was considered to indicate a statistically significant difference (34). For the ANOVA pairwise group comparisons and correlation for the in vivo and ex vivo results of the in vivo gene delivery studies, a Bonferroni-adjusted two-sided *P* value of less than .005 (.05/11) was considered to indicate a statistically significant difference.

Results

MB Characterization and Assessment of DNA Binding

Mean ζ potentials for cationic and neutral MBs were +15.8 mV $\pm$ 2.0 (standard deviation) and -0.3 mV $\pm$ 0.3, respectively. There were no statistically significant differences in cationic (1.4 μ m $\pm$ 0.1, 3.8 μ m $\pm$ 0.5) and neutral (1.4 μ m $\pm$ 0.1, 3.9 μ m $\pm$ 0.5) MB mean diameters in number or volume, respectively. In vivo blood half-life of neutral MBs (7.17 minutes $\pm$ 0.20) was 2.4 times (*P* = .045) that of cationic MBs (3.03 minutes $\pm$ 1.78).

Results of confocal microscopy (Fig 3a) visually confirmed charge-coupling of fluorescence-labeled plasmid DNA with cationic MBs while no binding was observed with neutral MBs. Quantitative assessment of DNA binding (Fig 3b) showed a mean loading capacity of 14.9 μ g $\pm$ 1.7 per 5 $\times$ 10⁸ cationic MBs (corresponding to 0.03 pg per cationic MB), which was significantly higher (*P* < .001) than the essentially no binding (0.27 μ g $\pm$ 1.5) to an equivalent number of neutral MBs.

Nuclease Protection Assay

Gel electrophoresis of CBLuc that was electrostatically bound to the surfaces of cationic MBs while exposed to deoxyribonuclease (DNase) I showed partial protection of the plasmid from nuclease degradation (lane 4, Fig 4). In contrast, CBLuc plasmids that were mixed with neutral MBs and thus were not directly

bound to the MB shells during DNase I treatment were fully degraded (lane 6, Fig 4).

Ultrasound-mediated Transfection in Cell Culture

Transfection of vascular endothelial cells in cell culture by using ultrasound and cationic MBs yielded mean bioluminescence signal ($219.0 \text{ RLUs}/\mu\text{g}$ protein ± 92.5) that was 20-fold higher than that with neutral MBs ($10.9 \text{ RLUs}/\mu\text{g}$ protein ± 2.7 , $P = .001$) and was also significantly higher than that with the control experiments with no MBs ($5.2 \text{ RLUs}/\mu\text{g}$ protein ± 1.2 , $P = .0005$) and no ultrasound ($3.5 \text{ RLUs}/\mu\text{g}$ protein ± 1.9 , $P = .0008$) (Fig 5, Table 1).

In Vivo Assessment of in Vivo Ultrasound-mediated Gene Delivery

CBLuc activity from hind limb tumor xenografts was observed as early as 12 hours after ultrasound-mediated gene delivery, with relative preservation of bioluminescence signal at all ensuing time points (Fig 6, Table 2). There was no significant main effect of time ($P = .606$), nor a significant interaction between time and treatment condition ($P = .125$).

Comparisons of treatment conditions across all time points revealed that the use of cationic MBs in in vivo ultrasound-mediated gene delivery resulted in significantly higher gene transfer efficiency ($[5.9 \pm 2.2]$ to $[9.3 \pm 5.2] \times 10^4$ photons/sec/cm²/sr, $P < .0001$) relative to the use of neutral MBs ($[2.4 \pm 0.8]$ to $[2.9 \pm 1.1] \times 10^4$ photons/sec/cm²/sr) or no MBs ($[0.3 \pm 0.2]$ to $[1.0 \pm 1.3] \times 10^4$ photons/sec/cm²/sr). Ultrasound-mediated gene delivery with cationic MBs resulted in 2.4–3.2-fold higher mean in vivo bioluminescence signals relative to gene delivery with neutral MBs at various imaging times (Fig 6, Table 2). The negative control group of mice receiving CBLuc and cationic MBs but no ultrasound showed background in vivo bioluminescence, confirming that ultrasound is necessary to trigger sonoporation. Low-level functional transgene expression was observed with in vivo

Figure 3

ultrasound-mediated gene delivery in the absence of MBs, but transfection efficiency was enhanced overall with the use of cationic or neutral MBs ($P < .0001$). In vivo bioluminescence signal was relatively confined to the site of insonation (Fig 7). There was no visible signal at known sites of MB clearance (32)—specifically, the liver and spleen.

Ex Vivo Confirmation of in Vivo Ultrasound-mediated Gene Delivery and Histologic Assessment of Spatial Distribution

In vivo bioluminescence imaging results were confirmed by ex vivo assay of excised tumors for CBLuc activity. Mean bioluminescent signal of tumors from the cationic MB ultrasound-mediated gene

Figure 4

Figure 4: Gel electrophoresis results from nuclease protection assay show that binding of plasmid DNA to cationic but not neutral MBs partially protects plasmid DNA from endonuclease degradation. Lane 1 demonstrates the electrophoretic banding pattern of CBLuc without exposure to DNase I. Incubation of CBLuc with DNase I for 15 minutes and then EDTA to stop the reaction resulted in loss of all bands, indicating complete degradation (lane 2). Simultaneous coinubation of CBLuc with DNase I and EDTA resulted in no degradation and preservation of all bands (lane 3). To test the effect of MB binding, CBLuc was incubated with cationic (lanes 4 and 5) or neutral (lane 6) MBs prior to exposure to DNase I. CBLuc fractions that were bound (lane 4) and not bound (lane 5) to cationic MBs were separated by filtration prior to electrophoresis. Electrophoresis of the excess nonbound CBLuc revealed complete loss of CBLuc bands (lane 5), indicating complete degradation, while electrophoresis of CBLuc that was bound to cationic MBs showed preservation of most bands (lane 4), indicating partial protection from endonuclease degradation. Lane 6 shows complete degradation of CBLuc incubated with neutral MBs; none of these plasmids were MB bound. Lane 7 exhibits electrophoresis of a 1-kb DNA standard.

delivery group (12.2 RLUs/ μ g protein $\pm$ 2.8) was significantly higher ($P = .0038$) than that from the neutral MB ultrasound-mediated gene delivery group (7.0 RLUs/ μ g protein $\pm$ 2.3). Excised tumors treated with ultrasound but without MBs showed significantly lower levels of CBLuc activity (0.68 RLUs/ μ g protein $\pm$ 4.6) versus the cationic and neutral MB ultrasound-mediated gene delivery groups ($P < .001$). Negligible levels of luciferase activity were found in the no ultrasound negative control tumors (0.14 RLUs/ μ g protein $\pm$ 0.12). Similarly, very low levels of luciferase activity were detected in the livers (0.38 RLUs/ μ g protein $\pm$ 0.11) and spleens (0.21 RLUs/ μ g protein $\pm$ 0.11) excised from mice treated with

ultrasound-mediated gene delivery by using cationic MBs, confirming only minimal nonspecific gene expression at the sites of primary MB clearance. Ex vivo bioluminescence imaging data obtained 48 hours after in vivo ultrasound-mediated gene delivery ($\rho = 0.88$, $P < .0001$).

Spatial distribution of transgene expression after ultrasound-mediated delivery of β -gal with cationic MBs was assessed with β -gal and hematoxylin-eosin staining of excised hind limb tissue. Positive β -gal staining was present in tumor cells, adjacent muscle, and overlying skin in the region of insonation (Fig 8). Control tumors and

surrounding tissues that were not treated showed no transgene expression.

Discussion

Clinical translation of gene therapy has been impeded by the absence of an efficient, safe, and noninvasive means to selectively deliver therapeutic genes to sites of disease. While viral vectors are limited by safety concerns, nonviral platforms are disadvantaged by low transfection efficiencies (35). Ultrasound-mediated gene delivery with MBs holds promise to fulfill this void, particularly given the accepted use of contrast-enhanced US in clinical diagnostic imaging. Furthermore, its reliance on selective

Figure 5: Bar graph shows results of ultrasound (US)-mediated transfection of CBLuc with cationic, neutral, or no MBs in vascular endothelial cells in cell culture. CBLuc activity evaluated 24 hours after ultrasound-mediated gene delivery was substantially enhanced with use of cationic MBs relative to neutral MBs (20 fold) and no MBs (42 fold). Negligible luciferase activity was observed in the negative control group, which was exposed to CBLuc-cationic MB mixtures but without application of ultrasound. Error bars = standard deviations. * $P < .005$ relative to the neutral MB, no MB, and no ultrasound groups.

ultrasound exposure to actuate gene transfer affords advantages in spatial and temporal control (36). Last, this method provides an opportunity to monitor gene delivery, as MBs simultaneously function as US contrast agents.

We investigated whether the use of cationic MBs, which can bind, protect, and transport plasmid DNA, in ultrasound-mediated gene delivery enhances transfection efficiency. The cationic MBs used could each bind approximately 0.03 pg of DNA, which is similar to the 0.04 pg per cationic MB reported previously (14). This electrostatic binding approach confers advantages in versatility and ease of use compared with other methods (eg, covalent bonding of DNA to MB surfaces, incorporation of DNA into MB shells during synthesis [15,23]), as plasmids encoding for various therapeutic genes and other nucleotide-based therapeutics, such as oligonucleotides or small interfering RNA (29,37), can easily be substituted

and spontaneously charge-coupled to cationic MBs by simple coinubation. However, the finite surface area of MB shells limits the amount of DNA that can be electrostatically bound. Ongoing research explores novel methods to increase the DNA loading capacity of cationic MBs (28).

Head-to-head comparisons between ultrasound-mediated gene delivery performed by using cationic MBs and that performed by using neutral MBs showed significantly enhanced transfection efficiency with use of cationic MBs in both cell culture (20 fold) and mouse hind limb tumor models (2.4–3.2 fold). The in vivo results are furthermore supported by the shorter blood half-life of the cationic MBs. Our results are in contradistinction to findings of a recent pilot study (38) that showed no significant difference in transgene expression after ultrasound-mediated gene delivery to skeletal muscle with cationic versus neutral MBs. In this study (38),

Table 1

Summary of CBLuc Activity 24 Hours after in Vitro Ultrasound-mediated Transfection of CBLuc to Endothelial Cells

Group	CBLuc Activity (RLUs/μg Protein)
Cationic MBs with ultrasound	219.0 ± 92.5*
Neutral MBs with ultrasound	10.9 ± 2.7
No MBs with ultrasound	5.2 ± 1.2
Cationic MBs without ultrasound	3.5 ± 1.9

Note.—Data are mean CBLuc activities ± standard deviations.

* $P < .005$ for cationic MB group compared with neutral MB, no MB, and no ultrasound groups.

MB-plasmid mixtures were administered by direct intramuscular injection, which minimizes the transfection

Table 2

Summary of Serial in Vivo Bioluminescence Values after in Vivo Ultrasound-mediated Delivery of CBLuc to Hind Limb Tumor Xenografts

Group	12 Hours	24 Hours	36 Hours	48 Hours
Cationic MBs with ultrasound ($n = 7$)	8.1 ± 4.0	7.4 ± 3.3	9.3 ± 5.2	5.9 ± 2.2
Neutral MBs with ultrasound ($n = 7$)	2.6 ± 1.2	2.6 ± 1.7	2.9 ± 1.1	2.4 ± 0.8
No MBs with ultrasound ($n = 5$)	1.0 ± 1.3	0.6 ± 0.6	0.4 ± 0.3	0.3 ± 0.2
Cationic MBs without ultrasound ($n = 5$)	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2

Note.—Data are mean maximum radiances $\pm$ standard deviations in units of $\times 10^4$ photons/sec/cm²/sr. Pooling across time points, all pairwise comparisons between groups showed significant differences ($P < .0001$).

Figure 6

Figure 6: Graph shows serial in vivo bioluminescence as a measure of transfection efficiency observed 12, 24, 36, and 48 hours after in vivo ultrasound (US)-mediated transfection of CBLuc to hind limb tumor xenografts by using cationic ($n = 7$), neutral ($n = 7$), or no MBs ($n = 5$). Efficiency of ultrasound-mediated gene delivery was substantially higher with use of DNA-binding cationic MBs than with neutral MBs (2.4–3.2 fold) or no MBs. Negative-control mice ($n = 5$) that received intravenous CBLuc-cationic MB mixtures but without application of ultrasound showed background bioluminescence. Mean maximum radiances with standard deviations (shown here with error bars) are detailed in Table 1. $E+4 = 10^4$. * $P < .0001$ for all pairwise comparisons between treatment groups, without significant main effect of time.

advantages afforded by using plasmid-binding cationic MBs.

The enhancement in transfection efficiency with cationic MBs was more pronounced in the cell culture studies. Possible explanations for this include the paucity of endonucleases in culture media, possibly more DNA initially administered in vitro on a per-cell basis,

and the inverted design of the cell culture experimental setup. As the cationic MBs floated, they transported and concentrated bound plasmid DNA to the surfaces of the downward-facing cells. Nonbound plasmids mixed with neutral MBs, in contrast, distributed throughout the volume of the media, lowering the DNA concentration at the

cell surfaces. Moreover, compared with the static cell culture setting, the hemodynamic forces in vivo may cause some bound DNA to be released once introduced into the circulation.

Achieving a suitably high local concentration of plasmid DNA at the desired site of gene delivery is particularly challenging in vivo. Systemically

administered free plasmid DNA is rapidly degraded by serum endonucleases and is removed by the reticuloendothelial system (22,39,40). Efforts to overcome these effects by increasing initial administered DNA dose are limited by toxicity concerns. Thus, a mechanism to preserve the integrity of genetic cargo while in circulation is critical to the success of any gene delivery platform. Our nuclease protection assay results provide supporting evidence that the enhanced transfection efficiency with use of cationic MBs in *in vivo* ultrasound-mediated gene delivery may be partially due to inhibition of endonuclease degradation. The exact underlying mechanism is unknown but may be related to steric hindrance of endonucleases by MBs, preventing access to bound DNA.

At assessment of the spatial distribution of *in vivo* ultrasound-mediated gene delivery, transgene expression was found in not only tumor cells but also adjacent muscle and skin. However, macroscopically, the *in vivo* bioluminescence images and *ex vivo* results from the CBLuc sonoporation studies showed no reporter gene expression at sites distant from the region of insonation, including the liver and spleen. Consistent with the literature (13,41), these data suggest that *in vivo* ultrasound-mediated gene delivery is non-specific at the cellular level but is targetable regionally to where ultrasound is applied. Delivery specificity may be improved by ultrasound technology advances that allow for more focused application of the ultrasound beam. Additionally, experiences with molecularly targeted MBs in molecular imaging may be leveraged to engineer cationic MBs with targeting ligands to further localize the cavitation nuclei and their genetic cargo to disease sites (42).

Limitations of this study include the following: First, although mice were randomized to treatment groups, there may have been minute differences in tumor perfusion and volume among groups that may account for some of the transfection efficiency differences. Second, *in vivo* bioluminescence imaging of mice was performed only up to 48 hours after transfection;

Figure 7: Representative images of *in vivo* bioluminescence 36 hours after ultrasound-mediated gene delivery to hind limb tumor xenografts by using intravenous administration of CBLuc coupled or mixed with, *A*, cationic MBs, *B*, neutral MBs, or, *C*, no MBs. *D*, Mice in this group received CBLuc-cationic MB mixtures, but ultrasound was not applied. CBLuc activity was relatively confined to the region of insonation. The greatest *in vivo* bioluminescence was observed in, *A*, mice treated with ultrasound-mediated gene delivery using cationic MBs.

thus, the total duration of CBLuc expression remains unknown. Third, the potential adverse effects of ultrasound-mediated gene delivery and custom-made MBs were not evaluated. Fourth, we did not determine where plasmids were delivered intracellularly or relative to the endothelium. Last, it is unknown whether the transfection efficiency advantages found with cationic MBs would persist in alternative experimental conditions; our results may be specific to the parameters utilized here.

Practical applications: Our results suggest that plasmid-carrying cationic MBs enhance ultrasound-mediated transfection efficiency in cell culture and in a mouse tumor model, supporting a potential role for cationic MBs in advancing ultrasound-mediated gene delivery as a method for efficient and targetable nonviral gene therapy. Cationic MBs serve multiple functions as cavitation nuclei for sonoporation, as well as intravascular carrier vehicles for systemic gene delivery and protection of bound genetic payload against endonuclease degradation.

Acknowledgments: The authors gratefully acknowledge the assistance of Ying Ren, MD; Ian Y. Chen, MD, PhD; and Thierry Bettinger, PhD.

Disclosures of Potential Conflicts of Interest:

D.S.W. No potential conflicts of interest to disclose. **C.P.** No potential conflicts of interest to disclose. **M.A.P.** Financial activities related to the present article: none to disclose. Financial activities not related to the present article: has received payment for a lecture, as well as travel expenses, from VisualSonics. Other relationships: none to disclose. **R.P.** No potential conflicts of interest to disclose. **J.R.** No potential conflicts of interest to disclose. **S.S.G.** No potential conflicts of interest to disclose. **M.S.** No potential conflicts of interest to disclose. **J.K.W.** No potential conflicts of interest to disclose.

References

1. Miller DL, Pislaru SV, Greenleaf JE. Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somat Cell Mol Genet* 2002;27(1-6):115-134.
2. Tachibana K, Uchida T, Ogawa K, Yamashita N, Tamura K. Induction of cell-membrane porosity by ultrasound. *Lancet* 1999;353(9162):1409.
3. Brayman AA, Coppage ML, Vaidya S, Miller MW. Transient poration and cell surface receptor removal from human lymphocytes *in vitro* by 1 MHz ultrasound. *Ultrasound Med Biol* 1999;25(6):999-1008.
4. Duvshani-Eshet M, Baruch L, Kesselman E, Shimoni E, Machluf M. Therapeutic ultrasound-mediated DNA to cell and nucleus: bioeffects revealed by confocal and atomic force microscopy. *Gene Ther* 2006;13(2):163-172.

Figure 8

Figure 8: Spatial distribution of transgene expression 48 hours after in vivo ultrasound-mediated gene delivery of β -gal with cationic MBs assessed with β -gal and hematoxylin-eosin (H & E) staining of excised hind limb tissue sections. Brightfield images show positive β -gal staining (arrows) in tumor cells, as well as in adjacent muscle and overlying skin in the region of insonation. Lettered boxes in top row of images correspond to areas of (a) tumor, (b) muscle, and (c) skin.

5. Kimmel E. Cavitation bioeffects. *Crit Rev Biomed Eng* 2006;34(2):105–161.
 6. Schlicher RK, Radhakrishna H, Tolentino TP, Apkarian RP, Zarnitsyn V, Prausnitz MR. Mechanism of intracellular delivery by acoustic cavitation. *Ultrasound Med Biol* 2006;32(6):915–924.

7. Collis J, Manasseh R, Liovic P, et al. Cavitation microstreaming and stress fields created by microbubbles. *Ultrasonics* 2010;50(2):273–279.
 8. Nyborg WL. Ultrasonic microstreaming and related phenomena. *Br J Cancer Suppl* 1982;5:156–160.

9. Greenleaf WJ, Bolander ME, Sarkar G, Goldring MB, Greenleaf JF. Artificial cavitation nuclei significantly enhance acoustically induced cell transfection. *Ultrasound Med Biol* 1998;24(4):587–595.
 10. Liang HD, Lu QL, Xue SA, et al. Optimization of ultrasound-mediated gene transfer (sonoporation) in skeletal muscle cells. *Ultrasound Med Biol* 2004;30(11):1523–1529.
 11. Kim HJ, Greenleaf JF, Kinnick RR, Bronk JT, Bolander ME. Ultrasound-mediated transfection of mammalian cells. *Hum Gene Ther* 1996;7(11):1339–1346.
 12. Meijering BD, Henning RH, Van Gilst WH, Gavrilovic I, Van Wamel A, Deelman LE. Optimization of ultrasound and microbubbles targeted gene delivery to cultured primary endothelial cells. *J Drug Target* 2007;15(10):664–671.
 13. Carson AR, McTiernan CF, Lavery L, et al. Gene therapy of carcinoma using ultrasound-targeted microbubble destruction. *Ultrasound Med Biol* 2011;37(3):393–402.
 14. Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. *Ultrasound Med Biol* 2003;29(12):1759–1767.
 15. Chen S, Ding JH, Bekeredjian R, et al. Efficient gene delivery to pancreatic islets with ultrasonic microbubble destruction technology. *Proc Natl Acad Sci U S A* 2006;103(22):8469–8474.
 16. Li YS, Davidson E, Reid CN, McHale AP. Optimising ultrasound-mediated gene transfer (sonoporation) *in vitro* and prolonged expression of a transgene *in vivo*: potential applications for gene therapy of cancer. *Cancer Lett* 2009;273(1):62–69.
 17. Bekeredjian R, Chen S, Frenkel PA, Grayburn PA, Shohet RV. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. *Circulation* 2003;108(8):1022–1026.
 18. Shohet RV, Chen S, Zhou YT, et al. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. *Circulation* 2000;101(22):2554–2556.
 19. Ferrara K, Pollard R, Borden M. Ultrasound microbubble contrast agents: fundamentals and application to gene and drug delivery. *Annu Rev Biomed Eng* 2007;9:415–447.
 20. Liang HD, Tang J, Halliwell M. Sonoporation, drug delivery, and gene therapy. *Proc Inst Mech Eng H* 2010;224(2):343–361.

21. Luo D, Saltzman WM. Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat Biotechnol* 2000;18(8):893–895.
22. Lentacker I, De Geest BG, Vandenbroucke RE, et al. Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA. *Langmuir* 2006;22(17):7273–7278.
23. Frenkel PA, Chen S, Thai T, Shohet RV, Grayburn PA. DNA-loaded albumin microbubbles enhance ultrasound-mediated transfection *in vitro*. *Ultrasound Med Biol* 2002;28(6):817–822.
24. Tinkov S, Bekerredjian R, Winter G, Coester C. Microbubbles as ultrasound triggered drug carriers. *J Pharm Sci* 2009;98(6):1935–1961.
25. Hernot S, Klibanov AL. Microbubbles in ultrasound-triggered drug and gene delivery. *Adv Drug Deliv Rev* 2008;60(10):1153–1166.
26. Leong-Poi H, Kuliszewski MA, Lekas M, et al. Therapeutic arteriogenesis by ultrasound-mediated VEGF165 plasmid gene delivery to chronically ischemic skeletal muscle. *Circ Res* 2007;101(3):295–303.
27. Vannan M, McCreery T, Li P, et al. Ultrasound-mediated transfection of canine myocardium by intravenous administration of cationic microbubble-linked plasmid DNA. *J Am Soc Echocardiogr* 2002;15(3):214–218.
28. Borden MA, Caskey CF, Little E, Gillies RJ, Ferrara KW. DNA and polylysine adsorption and multilayer construction onto cationic lipid-coated microbubbles. *Langmuir* 2007;23(18):9401–9408.
29. Haag P, Frauscher F, Gradl J, et al. Microbubble-enhanced ultrasound to deliver an antisense oligodeoxynucleotide targeting the human androgen receptor into prostate tumours. *J Steroid Biochem Mol Biol* 2006;102(1-5):103–113.
30. Tlaxca JL, Anderson CR, Klibanov AL, et al. Analysis of *in vitro* transfection by sonoporation using cationic and neutral microbubbles. *Ultrasound Med Biol* 2010;36(11):1907–1918.
31. Ray P, Gambhir SS. Noninvasive imaging of molecular events with bioluminescent reporter genes in living subjects. *Methods Mol Biol* 2007;411:131–144.
32. Willmann JK, Cheng Z, Davis C, et al. Targeted microbubbles for imaging tumor angiogenesis: assessment of whole-body biodistribution with dynamic micro-PET in mice. *Radiology* 2008;249(1):212–219.
33. Perkins AC, Frier M, Hindle AJ, et al. Human biodistribution of an ultrasound contrast agent (Quantison) by radiolabeling and gamma scintigraphy. *Br J Radiol* 1997;70(834):603–611.
34. Girden ER. ANOVA: repeated measures. Thousand Oaks, Calif: Sage, 1991.
35. Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. *Nat Rev Genet* 2005;6(4):299–310.
36. Newman CM, Bettinger T. Gene therapy progress and prospects: ultrasound for gene transfer. *Gene Ther* 2007;14(6):465–475.
37. Otani K, Yamahara K, Ohnishi S, Obata H, Kitamura S, Nagaya N. Nonviral delivery of siRNA into mesenchymal stem cells by a combination of ultrasound and microbubbles. *J Control Release* 2009;133(2):146–153.
38. Nomikou N, Tiwari P, Trehan T, Gulati K, McHale AP. Studies on neutral, cationic and biotinylated cationic microbubbles in enhancing ultrasound-mediated gene delivery *in vitro* and *in vivo*. *Acta Biomater* 2012;8(3):1273–1280.
39. Houk BE, Hochhaus G, Hughes JA. Kinetic modeling of plasmid DNA degradation in rat plasma. *AAPS PharmSci* 1999;1(3):E9.
40. Liu F, Shollenberger LM, Conwell CC, Yuan X, Huang L. Mechanism of naked DNA clearance after intravenous injection. *J Gene Med* 2007;9(7):613–619.
41. Koike H, Tomita N, Azuma H, et al. An efficient gene transfer method mediated by ultrasound and microbubbles into the kidney. *J Gene Med* 2005;7(1):108–116.
42. Willmann JK, Paulmurugan R, Chen K, et al. US imaging of tumor angiogenesis with microbubbles targeted to vascular endothelial growth factor receptor type 2 in mice. *Radiology* 2008;246(2):508–518.