Effective ultrasound-targeted microbubble destruction (UTMD)-mediated gene transfer into the livers of small and large animals


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Ultrasound (US)-targeted microbubble (MB) destruction (UTMD) can significantly enhance gene delivery in mouse livers when pDNA/MBs were injected into the portal vein (PV) with simultaneous US exposure using a focused transducer. However, this transducer was ineffective in enhancing gene transfer into rats. A 13-mm diameter unfocused transducer was designed and the delivery route of pDNA/MBs was modified into a specific liver lobe, resulting in >100-fold increase in luciferase expression in rats. To facilitate the translation into human applications, many technical issues were explored in large animal models. We applied 1.1 MHz US to targeted canine and swine liver lobes with simultaneous injection of pDNA/MBs into a PV segmental branch and occlusion of the inferior vena cava. For more effective treatment of large tissue volumes, a 52-mm apodized, dual element unfocused transducer was specifically constructed to reduce the near field transaxial pressure variations, producing a uniform field of US exposure. Together with a 15 kW-capacity US amplifier, a 692-fold and 1800-fold increases of gene expression in canines and swines were achieved at 2.7-MPa, respectively. Transaminase levels and histology analysis indicated minimal tissue damage. These results demonstrated that UTMD is highly promising for safe and efficient gene delivery into the liver.
INTRODUCTION

The potential for therapeutic ultrasound (US) to augment minimally invasive nonviral gene transfer has long been recognized. Effective US-mediated gene delivery relies on acoustic cavitation nucleated by exogenous microbubbles (MBs), or a method often described generically as ultrasound targeted microbubble destruction (UTMD). When cavitation nuclei are present, US exposures of suitable frequency and acoustic pressure can transiently increase the permeability of endogenous barriers such as capillary endothelium or cell membranes to otherwise impermeable materials (e.g., drugs or macromolecules). Thus, UTMD can potentially overcome many barriers of in vivo gene delivery, leading to significant enhancement of gene transfer efficiency. In addition to the advantages of nonviral gene transfer including reduced immunogenicity and avoidance of oncogenic events by random integration into the host genome, UTMD-mediated gene transfer has many of the attributes of an ideal gene transfer system. First, the cargos delivered are naked plasmid DNA (pDNA) or RNA molecules which are very easy to prepare with low cost, low toxicity, and high consistency. Second, UTMD can target gene transfer site-specifically. Third, UTMD method is relatively safe and well tolerated over a wide range of US frequencies and intensities.

Our group has extensively studied gene delivery of reporter and therapeutic genes into the liver. We have demonstrated previously that US can significantly enhance gene transfection in mouse livers when MBs and pDNA carrying a reporter luciferase gene, pGL4, were injected into the portal vein (PV) while US was applied to the liver lobes by using a simple, geometrically-focused US transducer. However, this transducer was nearly ineffective when used in attempts to enhance gene transfer into rats. It became apparent that the effective treatment volume of the focused transducer was simply too small. A larger effective diameter transducer was designed for rat liver treatments and the delivery route was modified by injecting pGL4/MBs into a specific liver lobe through a PV branch (vs. prior intra-portal injection) with US exposure targeted to a specific liver lobe. With these modifications, we achieved a 100-fold increase in average luciferase expression in rats. Since liver volume increases rapidly with animal size, this was an important milestone towards translating this new technology to large animals.

In order to facilitate the eventual translation of these technologies into human application, many technical issues, such as surgical procedures, appropriate MB volumes, and US parameters and instrumentation require exploration in larger animal models. We chose canines and swines as an appropriate large animal models because: (1) the size of the liver lobe of a small dog or pig is similar to a portion of the human liver; (2) the liver circulatory system is similar between dogs, pigs, and human; and (3) dog disease models, such as canine hemophilia, are available. New US systems including newly designed unfocused transducers were developed for treating large tissue volumes in large animals and human patients. Further, we investigated various US parameters, MBs, and surgery protocols to improve gene expression following US-mediated gene delivery into the livers of large animals.

MATERIALS AND METHODS

Development of US Therapy Transducers for Mouse, Rat, Dog and Pig Models. Our consistent finding is that UTMD-enhancement of gene delivery in the liver requires acoustic pressures (henceforth abbreviated P, for peak negative pressure) of 2 MPa or more at 1 MHz and with cavitation nucleated by exogenous MBs. For use with small animals, P of 2-3 MPa is easily obtained by geometric focusing. However, focusing produces only a small treatment 'spot'; in our 'mouse transducer', the focal 'footprint' area is <0.1 cm² (Fig. 1A). Instantaneous treatment volumes and thus treatment rates are small. In larger animals, a bigger acoustic 'footprint' is required to treat larger liver volumes while MBs and pDNA remain in the tissue. We have scaled our therapeutic US transducers from a simple, lens-focused systems (Fig. 1A), to custom-made (Sonic Concepts Inc., Bothell, WA) single- or dual-element transducers for use by placing the transducer directly on the liver. Figs. 1B-1 and 1B-2 illustrate field plots for an unfocused, single-element H-158 'rat transducer' (effective beam area ~1.3 cm²). The H-158 transducer can produce a face P of ~3 MPa. Fig. 1C illustrates the relative pressure profile at the face of a Sonic Concepts H-105 dual element planar 'dog transducer' (effective beam diameter ~16 cm²). The dual element, apodized configuration greatly reduces near field transaxial pressure variations, producing a relatively uniform acoustic field. These newly developed novel transducers can now be used to effectively treat larger tissue volumes.
Fig. 1. Transaxial pressure profiles of focused 'mouse transducer' (A), unfocused 'rat transducer (B-1, B-2), or 'dog transducer (C). A. 55 mm lens-focused 'mouse transducer' transaxial pressure plot at the focal plane; effective beam footprint is ~0.07 cm². Vertical scale is pressure in dB; horizontal scale is distance from centerline (full range = 8 mm). The -6 dB beam diameter is ~3 mm. B. Single-element H-158 'rat transducer' pressure field plots; effective near-field beam 'footprint' is ~1.3 cm². B-1: Axial and transaxial field profile, where warm colors indicate high pressures and cool colors represent lower pressures. The plot shows the expected peaks and nulls along the acoustic axis. Vertical (transaxial) scale is 1.5 cm; horizontal (axial) scale is 30 cm, with the transducer at left. B-2: Transaxial profile at 6.2 mm from the face. The -6 dB beam diameter is ~13 mm. Vertical scale is dB (pressure); horizontal scale is distance from centerline (full range = 16 mm). C. Apodized dual-element H-105 'dog transducer' 3D representation of pressure field at 1 mm from face; effective beam footprint is ~16 cm². Relative pressure is encoded in color. Spatial scalars are 70 mm full range in each dimension. The dual-element design produces a near field of remarkable uniformity.

Plasmids and Microbubbles. A luciferase reporter plasmid with an SV40 promoter, pGL4 (luc2/SV40, Promega, Madison, WI) was employed in combination with different MBs in the gene transfer experiments first in mice and rats, and then eventually in swines and canines. Commercially available US contrast agent Definity® (Lantheus, North Billerica, MA) was used as the control MB, while an experimental MB, RN18 was synthesized using longer alkyl chain lipids (18 carbons vs 16 carbons in Definity®) to improve its stability and efficiency in gene transfection experiments. These MBs have a perfluoropropane gas core (C₃F₈), and a phospholipid shell containing variants of phosphotidylcholine (PC), phosphatidic acid (PA), and methoxy(polyethylene glycol)-5000 phosphotidylethanolamine (mPEG-5000-PE). For making cationic MBs, RC-5K, 18-carbon chain cationic lipids (DSTAP) was incorporated into the lipid shell. MBs were activated for 45-sec using a Vialmix® agitator immediately prior to mixing with the pDNA solution for gene transfection and for characterization using Multisizer3 (M3) Coulter Counter (Beckman Coulter, Brea, CA).

Gene Transfer Experiments. All procedures were performed according to the guidelines for animal care of both the National Institutes of Health and Seattle Children’s Research Institute (SCRI), with protocol approval of Institutional Animal Care and Use Committees (IACUC) of both SCRI and Fred Hutchinson Cancer Research Center (FHCRC). Mice and rats were obtained from Jackson laboratory, dogs (12-15kg) were housed at FHCRC and Domestic swines (8-12kg) were obtained from Washington State University (Pullman, WA) and housed at SCRI. Midline laparotomy was performed in animals and the pDNA/MB solution was injected either via portal vein (for mice), or portal vein segmental branch (for rats, pigs and dogs) with or without inferior vena cava (IVC) occlusion. US was applied simultaneously with the injection procedure on the liver surface (20 cycle pulses, 50 Hz pulse repetition frequency, 2.7 MPa peak negative pressure) for a set exposure time by scanning the transducer across the target lobe. For canine and swine experiments, diagnostic US was used to make sure the pGL4/MB solution was injected into the correct liver lobe, and to ascertain the MBs were destroyed after therapeutic US application. After 24 hrs, animals were sacrificed, and the treated and control lobes were sectioned and processed for luciferase expression. Blood and tissue samples were collected for liver enzyme tests and histological analysis.

RESULTS AND DISCUSSION

Gene Transfer into a Specific rat liver lobe via targeted treatments. Previously we have achieved significant enhancement of gene transfer efficiency (~250 fold) in mice by using a simple focused US transducer. However, it was nearly ineffective (~3 fold; Fig. 2B) in enhancing gene expression in rats. As the first step towards successful scaling up, the single-element H-158A transducer (effective beam area ~1.3 cm²) was designed specifically for treatment of the rat liver (Fig. 2A). Additionally, the plasmid solution was directly injected into a specific liver lobe through a portal vein branch (vs. prior intraportal injection). Similarly, the US exposure was
applied to the target lobe rather than the whole liver. With these innovative treatment strategies and pGL4 plasmid, luciferase expression was significantly enhanced over 0-30 Vol% MB concentrations with a plateau between 0.5 and 30 Vol% (Fig. 2B&C). The increase of gene delivery efficiency also depended on the acoustic peak negative pressure ($P$), achieving over 100-fold enhancement at 2.5 MPa compared with plasmid only controls (Fig. 2D).

Fig. 2. UTMD-mediated gene transfer targeting a specific rat liver. (A) Schematic illustration of US facilitated gene transfer targeting a specific liver lobe. (B) Comparison of enhancement of luciferase expression using focused or unfocused near-field transducers. (C) Dependence of transgene expression on Definity® concentrations. (D) The effect of US peak negative pressure on luciferase expression and correlation between luciferase expression (closed circles) and transient liver damage measured by aminotransferase levels (ALT (open squares) & AST (open triangles)) at 24 hours. (E) Comparison of the histology features of rat livers injected with pDNA and MB via portal vein and simultaneously treated with 2.7 MPa US exposure. The livers were harvested on day 0, 1, 5 12 30 after treatment for H&E staining. Original magnification X100, inserts X400.

These data demonstrate our successful scale-up from mice to rats. Since liver volume increases rapidly as animal size increases, this was an important milestone on the path toward treating still-larger animals.

Preparation and Characterization of Custom-Made MBs. MBs play a significant role in achieving efficient and high level transfection in UTMD-mediated gene delivery. In order to further improve gene transfer efficiency and reduce cost of the reagent, we have initiated the study of generating custom-made therapeutic MBs. Commercially available US contrast agent Definity® was used as the control MB. The lipid shell composition is modified to produce the next generation MBs with improved binding affinity to plasmid DNA (pDNA) and increased MB stability that will further enhance cavitation effects with exposure to US. Figure 3A shows the lipid composition of 4 different new MBs, including 2 neutral MBs (RN16 and RN18) and 2 cationic MBs (RC40S and RC5K). All of these MBs have a perfluoropropane gas core ($C_3F_8$), and a phospholipid shell containing variants of phosphotidylcholine (PC), other lipid components, and polyethylene glycol (PEG) stabilizers. The use of different alkyl backbones (C16 vs. C18), and the use of different PEG stabilizers (PEG40S vs. PEG5K) were investigated. Longer alkyl chains are believed to augment stability as they allow for better lipid packing in the MB shell. Employing different PEG stabilizers can change the hydrophilicity and reduce surface tension, which may prevent MBs from coalescing. As shown in Fig. 3B, Definity® MBs were slightly more concentrated than RN16 and RN18 MBs, however, the C18 MBs (RN18) have larger sizes as well as higher stability than C-16 MBs (RN16 and Definity®). In order to increase the loading of negatively charged pDNA onto the MBs, 18-carbon chain cationic lipids (DSTAP) was incorporated into the lipid shell. Cationic MBs RC40S contained a short PEG stabilizer (PEG40S), whereas RC5K contained a longer PEG stabilizer (mPEG-5000). The cationic MBs are less concentrated and are slightly bigger than Definity MBs. Fluorescently labeled pDNA-MB binding was analyzed by flow cytometry. A significantly higher percentage of fluorescent MB population and mean fluorescence intensity (MFI) were obtained with cationic MBs compared with neutral MBs (Fig. 3C), indicating much enhanced pDNA loading on cationic MBs. Using both luciferase and GFP reporter plasmids, we found that the presence of MBs dramatically enhanced gene delivery in vitro, with RN18 MBs observed to most effectively enhance expression as much as 177-fold (Fig. 3D). Cationic MBs had a higher binding affinity to plasmid DNA; however, different US parameters may be required to maximize the effect in enhancing gene transfection.
Preliminary Success in Scale-Up of Gene Transfer in a Specific Dog Liver Lobe. We performed sonographic wash-in/wash-out studies to scale pDNA and MB delivery to the dog livers. The best retention of the pDNA/MBs complexes was achieved with hepatic vein occlusion before bolus portal injection of pDNA/MBs complexes. With transient hepatic vein occlusion, the MBs distributed evenly into a specific liver lobe and persisted for several minutes. The majority of the MBs were destroyed by exposing the lobe to therapeutic US. These pilot experiments set the stage for the subsequent study of delivery of reporter gene constructs.

We then undertook a preliminary dog gene transfer experiment, using the H158 rat transducer as the best available at the time. We injected pGL4 plasmid and MBs into a segmental portal vein branch, and occluded a branch of the hepatic vein controlling outflow from the targeted lobe to promote pDNA and MB retention during US exposure. Most of the MBs were destroyed by treatment as assessed in sonographic images. Treated and control liver lobes were harvested 24 hours later and cut into smaller sections which were evaluated for luciferase gene expression. Significant enhancement in luciferase activity in the treated lobe was obtained compared to untreated lobe (247 vs. 13 RLU/mg protein; Fig. 4, Dog 2, 3 and 5).

When the H105 ‘dog transducer’ became available, we next performed a dog experiment using a P− of ~2 MPa. This
treatment (Dog 7) produced luciferase activity similar to those obtained using the H158 transducer. Next, pGL4 plasmid DNA was mixed with either 1.5 ml (Dog 8, Lobe 1) or 3 ml (Dog 8, Lobe 2) Definity and injected into two separate target liver lobes which were insonated at 2.7 MPa. Luciferase expression was enhanced 48-fold and 161-fold with 1.5 mL and 3.0 mL Definity MBs, respectively, compared to the control liver lobe. Gene expression levels in some sections were enhanced over 600-fold (Dog 8, Lobe 2 and Dog 9). Up to 4,700 fold enhancement was achieved in our most recent dog experiment (Dog 12).9

**Scale-up of Gene Transfer in a Specific Pig Liver Lobe.** The difficulty of scaling up the UTMD-mediated protocol to large animals and human applications lies on the large tissue volumes that need to be treated as well as the relatively small volume of plasmid solution that can be used. The scale-up study requires proportionally increased amount of pDNA/MBs delivered to tissues and an equivalent increase in US energy.

To treat large tissue volumes with US, as described in the previous section, we built the new unfocused H105 transducer with large effective beam area (16cm²). In addition, we use different MBs and surgical strategies to retain most of pDNA/MB locally during US application in order to maximize the effect of UTMD in gene transfection. Specifically, with occlusion of inferior vena cava (IVC), the MBs distributed evenly into a specific liver lobe and persisted for several minutes (Fig. 5A). With the pGL4/MBs localized, application of therapeutic US on the liver surface allowed for MBs to cavitate, creating temporary openings for the luciferase reporter plasmid, pGL4 to enter the cells. Fig. 5B shows the luciferase expression using Definity® and RN18 MBs in swines 24-hrs after treatment with 2.7Mpa US. Each dot represents a small section of the treated lobe, while the solid lines indicate the average luciferase expression. The average luciferase expression in the pig injected with Definity® (1220 RLU/mg protein) is significantly higher (1800-fold, p<0.001) than the sham-treated pig. New MB RN18 was used in two gene transfection studies. In the first pig, the average luciferase expression using RN18 (1350 RLU/mg) is also significantly higher (2000-fold, p<0.001), than sham. The second pig showed more significantly improved luciferase expression (4200 RLU/mg), which is 6300-fold (p<0.001) better than sham. In addition, the parts of the liver lobe that had more US exposure (solid dots, bottom half of liver) had much higher luciferase expression than the parts that had less exposure (hollow dots, upper half of liver) (7400 vs. 85 RLU/mg average luciferase expression). The parts of the liver lobe that had better than sham. The second pig showed more significantly improved luciferase expression (4200 RLU/mg), which is 6300-fold (p<0.001) better than sham. In addition, the parts of the liver lobe that had more US exposure (solid dots, bottom half of liver) had much higher luciferase expression than the parts that had less exposure (hollow dots, upper half of liver) (7400 vs. 85 RLU/mg average luciferase expression).

**Figure 5.** UTMD-mediated gene transfer into pigs using a large diameter transducer (H105) under 2.7MPa P°. (A) Surgical procedure of the pig experiments. (B) Luciferase gene expression using Definity® and RN18 MBs. Each dot represents a small section of the treated lobe 24-hrs after US exposure, while the solid lines are the average luciferase expression. Sham data are collected from pig injected with pGL4/Definity® MB but no US exposure. The numbers x below the data points indicate the fold enhancement of luciferase gene expression levels relative to sham control. (C) Representative images from H&E stained slides of pig livers 24-hr after control (upper panel), Definity® (middle panel), and RN18 (lower panel) injection and US exposure.

**Table 1.** Liver enzymes, ALT and AST, levels were collected 24-hrs after MB injection and US treatment. All samples are found to be within normal limits.

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<th>ALT Normal Range (30-70 U/L)</th>
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<tr>
<td>Sham</td>
<td>68</td>
<td>44</td>
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<td>Definity®</td>
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protein in exposed vs. unexposed areas), indicating that sufficient US exposure is necessary for successful gene transfection.

Transient liver damage was evaluated by analyzing liver enzyme levels in the blood 24-hrs after treatment. Table 1 showed that the ALT and AST levels from pigs injected with Definity® and RN18 MBs and exposed to US
were within normal levels, indicating that no tissue damage was produced with MB and US treatments. In addition, histological analysis showed that no necrotic or apoptotic cells were detected in the US-treated livers (Fig. 5C, middle and bottom panels) or the control liver (Fig. 5C, top panel); however, an increase in leukocyte infiltration was observed near the portal triad in UTMD treated liver lobes (Fig. 5C, middle and bottom panel). Based on these histological data, we believe that the US pressures used in our gene transfer experiments produced no or minimal damage in liver tissue, albeit were quite effective in achieving high level luciferase expression in treated liver tissues.

**CONCLUSIONS**

These studies demonstrate that UTMD can significantly enhance gene transfer efficiency in small and large animal models. Large tissue volumes can be acoustically treated effectively with optimized MB concentration, appropriate occlusion strategies and suitable US transducer technology. The experimental RN18 and RC5K MBs we developed are similar in concentration and size to Definity®, and could potentially be more effective in gene transfection. The US parameters used in this study showed significant enhancement in gene expression (up to several thousand fold) with minimal tissue damage. These results pave the way to achieve our goal of delivering therapeutic genes and represent an important developmental step towards US mediated gene delivery in large animals and clinical trials.

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