Ultrasound-mediated targeted drug delivery: recent success and remaining challenges

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Presently, contrast-enhanced ultrasound (CEUS) imaging focuses on diagnostic clinical applications. In the future, however, therapeutic uses of CEUS will create a paradigm shift for patient care and the pharmaceutical industry. Ultrasound contrast agents (UCAs) are composed of shelled microbubbles that serve as superior diagnostic agents while traversing the smallest of blood vessels resulting in unparalleled, real-time spatial and temporal imaging of intact tissues and organs. These microscopic, gas-filled microspheres acting as intravascular indicators represent ideal carrier vehicles for local delivery of ultrasound-directed drug and gene therapies. The conceptually simple application of external acoustic energy in the transformation of these inert, microspheres into powerful therapeutic systems has seemingly unlimited potential.

Brief history of CEUS. All diagnostic imaging modalities use and require contrast effects to increase signal-to-noise ratios, permitting enhanced discrimination of the targets. Examples include the use of radiopaque contrast agents to create discrete image patterns using X-ray methods (ionizing radiation), thus creating enhanced detection of objects within the image plane. Similar to X-ray, positron emission tomography and radionuclide imaging procedures rely on radioactive emitters to highlight anatomy and provide information on cellular metabolism and physiology. Distinct from traditional imaging modalities, UCAs are designed to generate resonance reflection patterns to provide a strong signal-to-noise ratio based on enhanced acoustic scattering properties. Gas-filled microbubble agents are novel contrast agents based on the inherent compressibility of the gas core that gives rise to mechanical acoustic coupling and signal enhancement. This coupling is so strong that the primary acoustic properties of tissue are dramatically altered by the presence of even microscopic quantities of agent (one part in a million by volume).

The origins of CEUS imaging can be attributed to early communications of Dr. Claude Joyner. The subsequent observations of Gramiak and Shah (23) in 1968 established the modern field of contrast imaging. Now 44 years later, the CEUS field is poised to make a significant contribution in the areas of clinical diagnostic applications and ultrasound-directed, site-specific delivery systems.

Before the initial observations of Joyner and publications of Gramiak and Shah, medical ultrasound users did not use contrast agents for diagnostic purposes. UCAs are considered latecomers. During the last 20 years, numerous UCAs have undergone preclinical and clinical testing. Few have received Food and Drug Administration (FDA) approval for clinical use. The first FDA-approved commercial ultrasound agent was Albunex (12, 25, 36), approved in 1994. Presently in the USA there are two commercial UCAs in clinical use: Optison (41) (GE Medical Diagnostics, Princeton, New Jersey), approved in 1997, and Definity (Lantheus Medical Imaging, Billerica, New Jersey), similarly approved by the FDA in 2001. In Europe, SonoVue (Bracco Imaging) and Optison are approved for clinical applications. GE Medical Diagnostic’s UCA Sonazoid (54) is approved in Japan and Korea. Today there are no approved commercial UCAs in Central and South America.

Characteristics of UCAs. The UCAs used in the earliest reports of CEUS were free, unprotected gas bubbles with an exceedingly short half-life. With continued interest and technology development, coupled with increased clinical efficacy of CEUS, improved design and manufacturing provided wider ranges of safe, stable microbubble contrast agents.

From a materials perspective, three critical microbubble characteristics include clinical safety, stability, and, of course, size. Today’s UCAs exemplify the evolution of two primary routes of establishing stability: first, encapsulation of the gas
microbubble within a protective external shell and, second, the substitution of ambient air with fluorinated gases (51, 59). The prototypic protective shell is composed of one of the following: 1) protein, 2) phospholipid, 3) biocompatible polymer, or 4) surfactant molecules (Table 1) (51, 56). Cross-linking and/or chain entanglement in the shell further enhances the stability of microbubbles. When present, these features reduce the elasticity of the shell, leading to attenuated oscillation patterns upon sonication. This attenuation effect is most notable in microbubbles stabilized with synthetic polymer shells. Investigators have reported that the interactions between neighboring human serum albumin protein molecules present in Albunex and Optison are predominately noncovalent (25). Phospholipid-stabilized microbubbles are considered to be uncross-linked with insignificant chain entanglement (51). Phospholipid surfaces or shells are echogenic and sufficiently stable to permit a wide variety of products that exhibit complicated microstructures (19). Several recent reviews have been published regarding UCAs preparation (7, 56).

The average size of the microbubble contrast agent plays a key role in utility. Microbubbles must be small enough to pass through the lung capillaries and less than the size of a human red blood cell (7.8 μm in diameter). Microbubble size affects the bubble distribution within the body and is directly related to the resonance frequency when exposed to acoustic energy. Generally, microbubbles less than 0.5 μm do not provide a significant contrast effect at clinical concentrations using standard commercial ultrasound transducer frequencies. It is a remarkably fortuitous coincidence that the typical microbubble size range of 0.5 to 8 μm possesses resonance frequencies within the range of commercial diagnostic imaging systems (0.2–15 MHz).

Mechanical resonance effects amplify the microbubble scattering response (and thus scattering efficacy) by an order of magnitude. All appropriately sized UCAs serve as surrogate red blood cells and pass unhindered through the smallest capillaries acting as true, nondiffusible, intravascular indicators. These agents when intravenously infused enter circulate freely throughout the vasculature of the body and provide unparalleled images of heterogeneity of tissue perfusion (18). The ability of the microbubbles to enhance the signal-to-noise ratio is dependent on critical properties of the gas core and the acoustic impedance mismatch it affords within the microvasculature. The compressibility of the gas core is important, the benefits of which can be altered via manipulation of the ultrasound transceiver parameters. The physics of microbubble behavior following exposure to ultrasound energy has been previously reviewed (15, 19, 44). In summary, all UCAs share the following common characteristics: 1) a core containing a relatively nondiffusible, highly compressible and acoustically reflective gas; 2) a protective shell; and 3) a diameter range of 0.5–10 μm.

Currently, the only FDA-approved clinical application of UCAs is left ventricular opacification. Contrast enhancement enables sonographers and physicians to more clearly identify left heart chamber anatomy and function. In a landmark article, Kurt et al. (28) concluded that CEUS significantly improved diagnostic accuracy, resource use, and patient management. Based on the proven safety and clinical utility of UCAs, their use has been endorsed by professional society guidelines: the American Society of Echocardiography in 2000 and 2008 (37, 38), the European Association of Echocardiography in 2009 (45), and the Intersocietal Commission on Adult Echo Laboratory guidelines, published in 2010 (26).

The promise of diagnostic ultrasound and CEUS imaging has not yet achieved the goal of quantitative organ perfusion (cardiac, liver, kidney, brain, etc.). While the development of diagnostic and therapeutic uses of UCAs is in its early stages, it is linked to a promising future. Many of the advantages of diagnostic ultrasound over traditional imaging modalities can be adapted to expand the use of UCAs and therapy: 1) use of nonionizing, acoustic energy; 2) high spatial and temporal resolution; 3) real-time processing and interpretation; 4) large established user base; 5) ease of operation; 6) portability; and 7) favorable economics. To present an advantageous alternative to computerized, nuclear, and positron emission tomography imaging in clinical practice, ultrasound systems require substantial improvements in quantitative volumetric analyses. Newer clinical systems will likely include three- and four-dimensional imaging coupled with contrast-enhanced software applications for organ perfusion imaging. And unique to CEUS is the ability to directly image angiogenesis within tumors and within carotid artery plaques. With these advancements, there will likely be an increase in capabilities that can be translated to therapeutic applications.

**Formulation of microbubbles and agents.** A critical aspect of the ultrasound-directed, site-specific microbubble gene or drug delivery system is the formulation of the microbubble and therapeutic agent(s). For example, Optison (16, 17, 49), Definity (53), SonoVue (34), and liposome (57) delivery systems are

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characterized by the premixing of plasmid DNA with the carrier vehicle. In one comparative study, phosphorodiamidate morpholino oligomers and plasmids were mixed with three agents: Optison, SonoVue, and Sonazoid (2). In other work, Sonazoid and small interfering RNA were added to a well containing cells and mixed gently just before exposure to ultrasound (39). There are also reports of therapeutics being incorporated directly into the bubble structure or durably bound to it. Literature citations include examples of plasmids incorporated into microbubbles during the synthesis of alumin-shell agents (20); similarly, agents have been incorporated into lipid microbubble delivery mixtures (9). In other in vitro experiments, plasmids were mixed with cells before the linking of compounds with microbubble vehicle (33). While the exact binding mode between the agent and the bubble surface remains unidentified in most cases, covalent interactions are one possible avenue to a durable linkage. Plasmid DNA (42) and small interfering RNA (30) have been covalently bound via biotin-avidin-biotin linkages in pegylated lipoplexes attached to phospholipid-shelled bubbles (62). Several recent reviews have discussed a variety of bonding scenarios that may exist between microbubbles and or small molecules (2, 34, 53, 57). Today, a clear understanding of the interaction between microbubbles and plasmid DNA remains elusive and is ripe for investigation.

The challenges associated with delivery of therapeutically relevant quantities of drugs via these bubble carriers in vivo has been described in some detail (27). An interesting exercise is therefore to consider the maximum drug payload a microbubble could theoretically carry to a target site in the body. If a 3-MDa plasmid DNA were stretched out to its maximum diameter of 48 nm, it would appear to a 3-μm diameter bubble as illustrated in Fig. 1, which is drawn to scale. If such a circular plasmid were to lie as a completely flat ring on the surface of the bubble, there would be enough bubble surface area to accommodate ~12,000 DNA molecules. Of course, plasmid DNA does not exist to any meaningful extent as a flat ring, which means that considering sterics alone, there is room on a 3-μm bubble for many more than 12,000 DNA molecules. The capacity to carry small molecule drugs would be much higher. A microbubble in this size range with a protein shell would contain a few to several million protein molecules, and a bubble with a lipid shell would comprise even more. Obviously, the actual capacity for a bubble to carry a plasmid or other agent is not driven by sterics alone but also significantly depends on the nature of the binding site and the availability of functionality that is required for bonding interactions. Nevertheless, it is an interesting analysis when considering the potential for drug payload.

Mechanism of action. Because of the composition of the UCA, intravenous injection methods are required to provide access to the vascular space and visceral organs. Upon exposure to low amplitude acoustic fields within the vasculature, the microbubbles are known to oscillate in a linear fashion, resonating within the diagnostic frequency range. As higher amplitudes are introduced, significant nonlinear responses are evoked. This produces acoustic signatures that provide marked signal-to-noise enhancements supersedes all other nonlinear responses produced in organ tissues. Extraction of the nonlinear acoustic response component is the basis of a number of microbubble-specific software imaging modes designed to highlight the signal over noise. At higher exposures, all within the diagnostic exposure limit, the microbubble oscillation becomes increasingly pronounced, resulting in rupture of the microbubble shell. With the ability to selectively direct this acoustic energy, it is believed that microbubble rupture may serve as a basis for localized therapeutic delivery systems.

Although the interaction between microbubbles, genes/drugs, acoustic energy, and the membrane of endothelial cells is complex, it has in part been elucidated using in vitro models. Transient, reversible permeability of cell membranes regularly occurs following the interaction of acoustic energy and microbubbles, creating enhanced cellular entrance of a gene/drug via membrane channel permeability or endocytosis. This ultrasound-induced permeability exists without significant detrimental cellular bio-effects (27). A series of relatively short ultrasound pulses are generally employed to avoid damage to the cell cycle and irreversible membrane disruption. Much experimental effort remains to be performed with regard to the use of appropriate ultrasound dosimetry while avoiding cellular damage. Ultimately, ultrasound energy and associated acoustic pulsing regimes will be designed for specific microbubble and tissue characteristics. Details of the physical processes involved remain incompletely understood, thus allowing for significant future optimization.

A key element of the mechanism of microbubble enhanced delivery is termed sonoporation. Sonoporation occurs locally because of the oscillating microbubble within the vascular space. These oscillations are relatively pronounced and almost certainly indicate compromise of the bubble shell. The specific acoustic pressure/microbubble interactions are likely to be independent of the original microbubble shell material apart from differences in the thresholds for initiating large amplitude oscillations resulting from differences in shell elasticity. High-amplitude oscillations can also induce microscopic bio-effects such as microvascular leakage, allowing improved delivery of drugs by extravasation. In this process, because of the proximity of the endothelial wall, the mechanical oscillation of the microbubbles produce microjets that transiently create hydrophilic pores within the previously impermeable cell membrane, permitting transport of xenomolecules into the cytoplasm. In addition to membrane channel transport, endocytosis also appears to play an important role (22, 27). The mechanisms surrounding endocytosis appears to involve the induction of surface pores or depressions, resulting from acoustic energy/
microbubble interactions, which enhance transcellular transport. Based on experimental data, it is proposed that, at least under certain delivery conditions, endocytosis is the mechanism associated with macromolecules in the size range of 70–500 kDa (35). Today, there is a great deal of interest in identifying the exact mechanisms of transmembrane transport initiated via the acoustic therapeutic methodology.

Clinical applications of therapeutic microbubble delivery systems. The microbubbles serve as a general purpose intravascular vehicle for drug payloads. Consequently, the therapeutic field is replete with reports from a variety of investigators who described a wide range of unique applications. Significant research efforts have focused on the clinical disease states of cardiovascular medicine, diabetes, and oncology.

Thrombolysis. A persistent target for therapeutic ultrasound is that of thrombolysis. Birnbaum et al. (6) demonstrated the administration of albumin microbubbles associated with transcortaneous ultrasound potentiated arterial thrombolysis without the additional use of tissue plasminogen activator (tPA). The controls treated with either ultrasound or microbubbles did not induce reperfusion. Culp et al. (13) reported their results using thrombolytic agents associated with therapeutic ultrasound in which an experimentally induced, autogenous venous clot was injected into arteries of swine. The treatment consisted of intravenous injection of epifibatide, an antiplatelet drug of the glycoprotein IIb/IIIa inhibitor class (63). The experimental design included the presence or absence of microbubbles, both using transcortaneous ultrasound. The results indicated that the microbubbles, when associated with epifibatide, resulted in significantly increased thrombus dissolution. In a subsequent study, the authors reported the use of therapeutic ultrasound for thrombolysis using nontargeted versus platelet-targeted microbubbles (platelet targeting ligand in the shell of the microbubble) in combination with brief, high mechanical index power to induce thrombolysis within the left anterior descending artery. Both targeted and nontargeted microbubbles coupled with ultrasound resulted in improved thrombolysis, with platelet-targeted treatment being the most successful (13). In addition, using a fibrinolytic agent, Laiung et al. (29) compared the administration of Alteplase, a tPA, alone or mixed with a phospholipid-shelled UCA versus a similarly loaded liposome. Similar to the microbubble protocol, external acoustic energy was used to induce fragmentation of the Alteplase-loaded liposomes. Although the results indicated the success of all methods, the Alteplase-loaded liposome combination proved to be most efficacious. Overall, the potential clinical value of targeted therapy is impactful due to the ability to limit systolic adverse events; the use of Alteplase exemplifies this selective approach to localized therapy and avoided excessive systemic bleeding (13). Most recently, researchers at VU University, The Netherlands, have reported promising results from a pilot study of the Sonolysis trial. Here thrombolysis was achieved in patients with first acute ST elevation myocardial infarction by administration of low-dose tPA along microbubbles with three dimensional-guided high myocardial infarction therapeutic ultrasound (52). In achieving coronary reperfusion with limited negative impact, both safety and efficacy were demonstrated.

Chemotherapy. While a significant focus of ultrasound-directed therapy has been on applications for cardiovascular diseases, numerous studies have emerged for the treatment of cancer. The well-recognized adverse systemic effects associated with the use of chemotherapeutic agents presents an ideal opportunity for the introduction of ultrasound-directed therapies. Consistent with this concept, Heath et al. (24) delivered cisplatin and cetuximab coupled with microbubbles to xenografts in immunodeficient mice. They reported a significant reduction in tumor size compared with the controls; histological analysis confirmed increased numbers of apoptotic cells following treatment with ultrasound-directed therapies and microbubbles compared with chemotherapeutic agents used alone.

Although a unique advantage associated with ultrasound-directed therapy is the ability to direct therapy locally, the related cellular permeation effects may improve uptake and efficiency of drugs. This effect was demonstrated by Lin et al. (31) in a series of studies in tumor-bearing mice. Following the completion of sonication therapy, the authors administered doxorubicin (Dox). Results indicated that accumulation of the Dox was significantly increased, and related tumor growth rate was slowed, following the ultrasound-directed therapies. Similarly, Sorace et al. (55) treated tumor-bearing mice (2LMP, a human breast cancer) with Calcein, a fluorescent, nonpermeable molecule, and Taxol, using multiple ultrasound-directed therapeutic parameters. Variations in frequency, mechanical index, pulse repetition period, and ultrasound duration were assessed. Additionally, microbubble dosing schedules were varied to assess induced changes in membrane permeability. The authors stated that membrane permeability appeared to be dependent on ultrasound parameters; resultant cancer cell death rate was increased by over 50% compared with chemotherapy treatment alone.

Overall, the potential to increase the therapeutic index of agents that induce systemic toxicities is a unique advantage of using ultrasound-directed therapy and microbubbles. Based on these studies, the use of agents with high toxicity may be revisited using ultrasound-directed, site-specific delivery. And with regard to the use of small molecules, the pharmaceutical industry may reconsider and reintroduce therapies that were previously shelved because of systemic toxicity or therapeutic inefficiencies.

Renal therapy. Renal function is often a target of end-organ damage associated with diabetes and hypertension. Specifically, in cases of ischemia, there is an increase in inflammatory markers associated with reduced renal perfusion. Previous work has demonstrated that microbubbles bind to activated leukocytes (45). The mechanisms of albumin versus lipid microsphere retention within the diseased kidney appear to be different. While interactions between lipid shells and leukocytes are mediated by serum compliment, albumin shell interaction is the result of leukocyte β2-integrins (32). These differences may provide an advantage for renal therapeutics. If the microbubble and associated drug/gene payloads are preferentially retained within the kidney, perhaps vasodilators and related anti-inflammatory agents will provide a novel therapeutic approach while reducing systemic effects of therapy.

Blood-brain barrier. Successful treatment of brain glioma is limited because of the difficulty of providing a therapy that is capable of crossing the blood-brain barrier (BBB). Tight cellular junctions prohibit therapeutic yields of most agents; therefore, the effective dosing regimens may be associated with increased systemic cytotoxic events. Ultrasound-directed therapies have the potential to achieve increased brain tissue...
delivery yields. Ting et al. successfully delivered the chemotherapeutic 1,3-bis(2-chloroethyl)-1-nitrosourea using microbubbles and focused ultrasound (FUS) to successfully cross the BBB. By encapsulating 1,3-bis(2-chloroethyl)-1-nitrosourea within the microbubble, the authors extended the circulatory half-life of the drug by a factor of 5-fold while decreasing the liver accumulation similarly. By embedding chemotherapeutic agents within the microbubbles, the “protected” payload was allowed to circulate for a prolonged period thus permitting repeated ultrasound therapeutic cycles facilitating the disruption of the BBB. Because albumin uptake by neurons has been shown to be neurotoxic, ostensibly it may seem counterintuitive that using ultrasound-directed therapies with microbubbles to permeabilize the BBB would be indicated for albumin-shelled microbubbles. However, Alonso et al. (1) has reported the fate of albumin bound to Evans blue dye after ultrasound-mediated permeabilization of the BBB was accumulated predominantly in activated microglia, astrocytes, and endothelial cells, thus preventing albumin-induced neurotoxicity. Further investigations by Baseri et al. (4) determined a variety of sonication parameters required for safe BBB permeability. The authors used an ultrasound frequency of 1.525 MHz with a pulse length of 20 ms and a pulse repetition frequency of 10 Hz. They tested peak rarefractional pressures between 0.15–0.98 MPa and determined the safe acoustic pressure to be between 0.3 and 0.46 MPa. These calculations were made using ultrasound-directed therapies with FUS in in vivo murine brain tissue; subsequent histological investigations were performed. These studies have been advanced to include nonhuman primates (61).

Treat et al. (60) have conducted extensive studies investigating magnetic resonance-guided, microbubble-mediated, FUS drug delivery to increase the permeability and payload yield across the BBB. In 2007, the investigators conducted three studies with similar technology using Dox with an albumin-shelled commercial microbubble. The first set of experiments did not include Dox and was designed to determine the amount of FUS energy required to disrupt the microbubbles and create transmembrane porosity in the BBB as confirmed by contrast-enhanced MRI. The second and third sets of experiments included Dox with the stated goal of optimizing the acoustic protocols and dose ranging for the commercial albumin-shelled microbubbles. The different concentrations of microbubbles revealed enhanced Dox uptake and transmission at the cost of increased tissue damage (60). In a follow-up study performed by the same authors, the integrity of the tight junctions in a posttreatment scenario was investigated focusing on rat brain microvessels. The authors administered the albumin-shelled microbubbles coupled with FUS directed to two locations within the brain. Successful disruption of the BBB led to leakage of injected horseradish peroxidase and lanthana- num chloride and the absence of the biomarkers present only with intact tight junctions of the BBB. At 4-h posttreatment, the leakage had ceased and membrane barrier function was restored as determined by biomarker analysis (48).

Despite years of preclinical research, the exact mechanism of BBB permeation using ultrasound-mediated microbubbles therapy remains to be elucidated. It is known that microbubble cavitation results in mechanical changes within the microvasculature and thereby alters the integrity of the BBB components (4). This may involve both a paracellular and transcel-

lular barrier effect following FUS-UCA interaction (60). There is evidence that a redistribution and loss of immunosignal occurs at the tight junction protein sites (such as occludin, claudin-5, and zonula occludens-1) and persists up to 4 h posttreatment (48). Moreover, the vesicular transport of tracer molecules across the endothelial cells increases, contributing to increased penetration of molecules located within the basement membrane, surrounding pericytes, arteriole smooth muscle cells, and perivascular neuropil (47). Although many questions remain to be answered, the advancement of this technology will bring the potential to treat a number of formerly terminal conditions of the brain, perhaps not just limited to cancer.

**Gene therapy.** In addition to delivering small molecules via ultrasound-directed therapies, significant progress has been made in delivering plasmid DNA products. Because of insertional genome issues associated with viral-mediated plasmid therapies (3), delivery of naked plasmids using ultrasound-directed therapy appears to be promising without the accompanying limitations associated in earlier viral methods. Dana-lou et al. (14) investigated the use of external ultrasound therapy without microbubbles to increase uptake of plasmid DNA coded for LacZ in wild-type and dystrophic mice. No response was observed in the ultrasound-only treatment group; however, a 3-fold transfection of the gene and a 22-fold increase in level of expression was noted in animals treated using ultrasound and microbubble therapies.

Similar research protocols using gene therapy have been performed in the domain of cardiovascular disease (21, 50, 64). Shohet et al. (50) implemented ultrasound-mediated gene delivery to achieve a 10-fold increase in protein expression of the myocardium compared with controls. The experimental design of Zhigang et al. (64) included an acute ligation of the left anterior descending coronary artery that leads to an acute myocardial infarction. The animals were treated with VEGF-coded genes at 3 days postinfarction. The three experimental groups included ultrasound-directed and microbubble therapy with the VEGF gene bound to microbubbles and a series of controls. The combination of ultrasound, microbubbles, and VEGF bound to the microbubbles resulted in enhanced VEGF expression and, accordingly, increased microvascular density within the myocardial infarct region compared with controls. An extension of this study was performed by Fujii et al. (21), in which the authors induced angiogenesis in mice and treated via ultrasound-targeted gene therapy in the post-myocardial infarction period. At day 7 post-coronary artery ligation, the mice received ultrasound-directed therapy with VEGF plasmids or a stem cell factor. Twenty-one days post-myocardial infarction, contrast echocardiography was used to measure myocardial perfusion and left ventricular function. Additional data included direct measurement of protein expression within the myocardium. The results indicated that the ultrasound-directed microbubble gene combination therapy efficiently delivered both stem cell factor and VEGF to the infarcted heart muscle, resulting in an increased expression of the specified genes associated with increased vascular density derived from VEGF; myocardial perfusion and ventricular function were notably improved, consistent with therapeutic efficacy (21).

As in previous research performed with ultrasound-directed therapies and small molecules, additional research has focused on optimization of gene delivery. Chen et al. (8) reported acoustic parameters associated with gene delivery, including...
adenoviral and plasmid DNA targeted to the myocardium. The luciferase reporter gene was incorporated into liposome-based microbubbles and served as the reference standard in experiments where the acoustic parameters were then varied. The independent variables included electrocardiogram triggering, variable ultrasound frequency, and power output (as measured by a mechanical index). Luciferase expression was measured 4 days posttreatment. The authors noted that low frequencies of acoustic energy (1.3 MHz) coupled with maximal mechanical indices and electrocardiogram gating allowed reperfusion of the capillary vasculature and gene expression. Furthermore, the authors identified similar levels of expression in the rat myocardium with adenoviral and plasmid DNA genes. Notably, the adenoviral DNA resulted in expression of the gene within the liver although plasmid DNA revealed no detectable liver expression. Song et al. (53) also conducted optimization studies investigating microbubble concentration using a novel focused high-intensity therapeutic ultrasound system for delivery of the reporter gene luciferase to the mouse liver.

Grayburn et al. (8, 9, 10, 11, 20) have provided leadership in the area of ultrasound-directed microbubble therapy by delivering proinsulin genes to the pancreas of experimentally induced diabetic rats. The diabetic model was produced by pretreating rats with streptozotocin (STZ), a naturally occurring chemical known to be particularly toxic to the β-cells of pancreatic islets. The plasmas were administered with microbubbles using external ultrasound activation to permit delivery of the genes into the pancreas and specifically to induce regeneration of β-cells. In 2010, the authors reported results in which STZ-induced diabetic rats underwent therapy with cDNA encoding for Ngn3, Pax4, Nkx2.2, Nkx6.1, MafA, or NeuroD1, all with the rat insulin promoter RIP3.1. Plasmas were incorporated within lipid microbubbles and delivered 48 h after STZ administration. Whereas Ngn3, Pax4, Nkx2.2, Nkx6.1, and MafA led to regeneration of islets with abnormal architecture, treatment with NeuroD1 led to regeneration with near normal morphology. Restoration of normalized serum glucose along with insulin and C-peptide levels were used as metrics for successful transduction. These were closely monitored and were observed to return to a normal physiological state at day 30 following treatment. The comparable set of animals serving as controls remained diabetic and did not normalize (8). A follow-up study was conducted and published in 2012 in which cyclin D2, cyclin-dependant kinase 4, and glucagon-like peptide 1 were similarly delivered to the diabetic rat pancreas (46, 11). The stated goal was to induce mitosis among the surviving β-cells by delivering both cyclin D2 and cyclin-dependant kinase 4. With the use of this combination of gene therapy, it was possible to create a complex that is associated with cell proliferation at the G1 phase (59), including the effects of glucagon-like peptide 1, which stimulates cyclin D2 expression (10, 11, 46). The results indicated that the delivery of these three genes induced near normal β-cell mass recovery and islet regeneration that persisted for 6 mo. The immunofluorescent staining results indicated that the return to near normal glycemia was associated with resident adult pancreatic progenitor cells (11). This signifies a relatively long-lasting effect produced by microbubble gene therapy heretofore impossible without the adverse safety profile of viral vectors.

Summary. There is ample evidence that ultrasound-mediated gene delivery in the presence of microbubbles is effective; however, there is much to be work to be done in demonstrating mechanisms of action. If sonoporation were the only means of gene delivery, only the cells of the vessel endothelium in close proximity to the microbubbles would be transfected. Unlike the case of sonothrombolysis, therapeutic effects requiring transfection following vascular administration would seemingly require the participation of delivery mechanisms other than, or in addition to, mechanical perturbation.

As evidenced in this limited review, there is increasing interest in understanding the potential for and mechanisms of this novel delivery method: ultrasound-directed, site-specific drug/gene delivery using microbubble contrast agents. It is likely in the near term that this technology will enter the clinical arena based on the ever-increasing scientific contributions from researchers. The fascinating preclinical studies discussed here represent only a glimpse into the future of clinical medicine.

In recent years, advocacy groups such as the International Contrast Ultrasound Society have made major inroads toward proving the vast diagnostic potential and safety of contrast ultrasound. However, the routine clinical uses of UCAs as delivery vehicles is still in its infancy in terms of clinical potential. In the near future, the regulatory status of the novel use of ultrasound-directed therapies will require focused attention. And certainly, within the scope of the ultrasound-directed microbubble therapeutic paradigm is the promise of gene therapy. This major step forward may represent the missing link that could revolutionize the treatment of inherited conditions.

Despite safety and toxicity concerns of adenoviral vectors, the concept of gene therapy made major progress as several first human studies were reported. In 2006, two patients received retro viral vector treatment to restore gp91phox function leading to the formation of the chemical compounds needed for white blood cells to kill bacteria (40). Shortly after this study, cancer was treated for the first time using the gene therapy of adoptive cell therapy. In this study, autologous lymphocytes were reengineered using a retrovirus to target and attack advanced metastatic melanoma in 25 patients (43). And in February 2012, three patients received gene therapy for the treatment of Leber’s congenital amaurosis, a rare inherited eye disease caused by an abnormality in the RPE65 gene (5). This procedure was conducted after following patients who had received similar treatment in the opposite eye for three years with no severe adverse effects from therapy. This groundbreaking research may provide a path for gene therapy applications using ultrasound-directed microbubble therapies.

To master each of the therapeutic applications presented here and introduce it into the clinic requires interdisciplinary collaboration. Much effort is being dedicated to making technological advancements in all aspects of the drug delivery platform: ultrasound hardware, software, and “wet wear.” Expertise in the fields of cellular physiology, genetics, physical chemistry, and acoustic physics must come together with shared goals. Under these currently forming relationships, it is conceivable that new strategic partnerships will be developed among industry, pharmaceutical companies, and academia to provide the necessary tools to accelerate the novel field of microbubble-based therapy.
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DISCLOSURES
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AUTHOR CONTRIBUTIONS

REFERENCES


