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EDITORIAL COMMENT

Getting Good Vibes

The Therapeutic Power of Microbubbles and Ultrasound*

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Microbubbles are microspheres containing a shell-encapsulated gas, which are used as intravascular ultrasound contrast agents in diagnostic echocardiography (1). Although microbubbles may vary in their shell composition, all expand and contract (vibrate) when exposed to specific frequencies of ultrasound at appropriate acoustic pressures (2). Increasingly, bioeffects caused by ultrasound-induced microbubble vibration in vivo have been harnessed for therapeutic gain.

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Ultrasound-induced microbubble vibrations enhance gene delivery and thrombolysis ("sonothrombolysis"), and transiently open the normally impervious blood-brain barrier in pre-clinical models (3–5). In the realm of gene delivery, ultrasound-targeted microbubble vibrations that culminate in microbubble destruction hold the promise of a noninvasive, nonimmunogenic, nonviral means of delivering genes to a target via simple intravenous injection. This approach offers an ideal "theranostic tool," using navigation of the ultrasound beam to destroy the microbubbles only at the site where genes should be delivered (thus achieving targeting) while ultrasonically imaging the target to confirm microbubble delivery and destruction.

Shohet et al. (6) were the first to report that transthoracic ultrasound during intravenous delivery of microbubbles bearing the beta-galactosidase gene resulted in higher rat myocardial beta-galactosidase expression compared with controls. This group subsequently published studies describing effects of ultrasound-targeted destruction of microbubbles carrying therapeutic genes on pancreatic beta cell regeneration and function (7). Others have subsequently reported therapeutic gene delivery using ultrasound-targeted microbubble destruction (UTMD) to promote angiogenesis in limb ischemia (8). We recently reported growth inhibition of murine squamous cell carcinomas after intravenous delivery of lipid microbubbles bearing the thymidine kinase suicide gene, in the presence of ganciclovir and ultrasound (9). Such studies have demonstrated that this method critically requires the dual presence of ultrasound (i.e., gene-loaded microbubbles are not sufficient) and microbubbles (i.e., ultrasound and naked plasmids are not sufficient). Nonetheless, the other requirements for gene delivery via UTMD with respect to what parameters optimize efficacy are largely unknown, reflecting our incomplete understanding of the mechanisms underlying UTMD.

What, in fact, are the "active ingredients" that mediate the gene therapeutic effects of UTMD? Some of what we do know: microbubble acoustic behavior ranges from "stable cavitation," where the microbubble vibrates at harmonic multiples of the transmitted ultrasound frequency, to "inertial cavitation," whereby the microbubble vibrates asymmetrically and violently erupts (2,10). Such microbubble behaviors occurring in proximity to cells cause transient, self-sealing nanoscale pores to form in cell membranes (sonoporation), endocytosis, and enhancement of endothelial layer permeability (11–13). These mechanisms could promote drug or nucleic acid uptake by the cell. Unlike other nucleic acid

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carriers that rely on endocytosis for cell internalization, sonoporation facilitates nonendosomal uptake of macromolecules, which could spare macromolecules from a potential endosomal "dead-end" fate. Major mechanisms for UMTD may be a combination of hydrodynamic sequelae of microbubble oscillation/disruption on cell membranes (microjetting and/or microstreaming) (10); direct mechanical effects of a vibrating microbubble alternately stretching and invaginating a cell membrane (11); and induction of calcium influx and subsequent increases in endothelial permeability (14). It should be noted that the putative mechanisms mentioned here derive from observations made largely in vitro and under ultrasound conditions with low to modest acoustic pressures (<1 MPa). Thus, the relevance of these mechanisms to the in vivo models that have shown successful gene therapy, in which high acoustic pressures have been used, is unclear.

What exactly happens between ultrasound, microbubbles, and the microvessel wall that facilitates gene transduction? Do the key events reside in the capillaries, the arterioles, or the venules? Imaging studies of microbubble–microvessel wall interactions have yielded some insight. Using intravital microscopy, Price et al. (15) demonstrated that ultrasound-induced ruptures of microbubbles transiting the capillaries of exteriorized rat spinotrapezious muscle caused capillary ruptures that dispersed 205- to 503-nm colloidal particles into a tissue area of about $25 \times 10^3 \mu$m. It was estimated that the extent of microvascular disruption could be as little as 1.5% of all capillaries to achieve particle coverage of 50% of total muscle area, suggesting that relatively few capillary ruptures could distribute macromolecules across a significant area of tissue. Intravital microscopy of mouse cremaster muscle during delivery of fluorescent plasmid DNA–loaded microbubbles in the presence of high-pressure ultrasound demonstrated perivascular deposition of DNA, with only 10% to 15% of depositions associated with visible microvascular hemorrhage, suggesting that gross hemorrhage was not an absolute prerequisite for extravascular penetration of plasmid DNA (16). More recently, high-speed imaging of rat cremaster muscle showed microvascular deformations caused by intravascular microbubbles vibrating in response to ultrasound, with microvessel distention and invagination, and liquid jets directed away from the near vessel wall (17). One can only speculate, but also graphically imagine, what such perturbations on the endothelial surface of microvessels might do to individual endothelial cell membrane permeability, vascular endothelial lining permeability, other endothelial functions, or even cytoplasmic or nuclear trafficking of genes.

In this issue of *iJACC*, Xie et al. (18) add yet another observation regarding UTMD that adds both additional information as well as mystery to the question of how vibrating microbubbles facilitate gene transfer. The study investigated whether microbubble targeting to an endothelial epitope would increase UTMD-mediated gene transduction. Lipid microbubbles bearing an antibody against the leukocyte adhesion molecules P-selectin or intercellular adhesion molecule-1 (ICAM-1) were charge coupled to plasmid DNA encoding for the luciferase reporter. A battery of in vitro flow chamber studies, intravital microscopy of activated murine cremaster muscle, and in vivo imaging studies of ischemic murine hindlimb confirmed that the plasmid-loaded targeted microbubbles adhered to inflamed tissue. P-selectin–targeted microbubbles carrying the luciferase gene were intravenously delivered to mice after hindlimb ischemia-reperfusion, during simultaneous ultrasound delivery, with 3 different acoustic pressures tested in separate groups. Using bioluminescence imaging and real-time polymerase chain reaction (PCR) for luciferase mRNA as readouts, it was found that compared with nontargeted microbubbles, P-selectin–targeted microbubbles resulted in higher luciferase expression *only* at the lower acoustic pressures (0.6 MPa). At the higher acoustic pressures (1.0 and 1.8 MPa), targeting did not confer additional gene transduction.

Although the authors did not report a statistical comparison of transduction between the experimental groups as a function of acoustic pressure, the data in Figure 4 suggest that high acoustic pressure trumps all: although P-selectin targeting enhanced transduction at the lower acoustic pressure of 0.6 MPa, the extent of transduction was still *lower* than that achieved with targeted- or nontargeted microbubbles at the higher acoustic pressures. Further, whereas the authors suggest that the lower acoustic pressures combined with microbubble targeting could be preferable to the high acoustic pressures due to lesser microvascular hemorrhage (albeit at the cost of less transduction), it was interesting that there were no differences among the acoustic pressure groups with respect to long-term histological

fibrosis or vascular permeabilization. Thus, if the goal is to achieve maximal gene transduction while avoiding significant long-term toxicity, one cannot definitively conclude from this study whether lower acoustic pressure + microbubble targeting or high acoustic pressure (without targeting) is the preferred approach.

But ignoring for a moment the superior transduction results at high pressures, the more interesting question posed by this study is why did P-selectin targeting improve transduction at the lower acoustic pressure? P-selectin expression post-ischemia occurs predominantly in the venules, which present less endothelial surface area compared with the capillaries. Moreover, molecular imaging studies have shown that relatively few targeted bubbles actually adhere to the target relative to the number of targeted microbubbles injected (19). How is it, then, that the presumably few microbubbles adhering to the venular endothelium could have mediated a 5-fold increase in gene transduction? What is the in vivo microvascular acoustic behavior of a microbubble at the 0.6-MPa (1.6-MHz) ultrasound that was delivered, which likely lies somewhere between stable and inertial cavitation regimes? Are the adhered microbubbles bursting? The in vitro cavitation data presented by the authors indicate that 0.5-MPa 1-MHz ultrasound destroys freely floating lipid microbubbles by the eighth pulse train. However, an adhered microbubble that is spatially confined in a tiny venule might behave very differently; it could stably oscillate when insonified at 0.6 MPa and potentially create the conditions that have been associated with increased individual cell membrane permeability, enhanced endocytosis, and "loosening" of junctions between endothelial cells (4,11–13). Also, whether plasmid availability at the target site requires microbubble breakage and/or "liberation" from the lipid shell is unknown. The fact that transduction is greater at higher acoustic pressures might suggest that microbubble breakage is required to ensure plasmid availability.

Whatever the "active ingredient(s)" within a given treatment strategy, microbubble targeting is important and incremental, but the effects of targeting do not appear to supersede the permissive effects on gene transduction that are conferred by acoustic conditions associated with inertial cavitation. The observations reported by Xie et al. (18) add to our awareness of the principles (e.g., targeting) that may help guide development of gene therapy protocols using microbubbles and ultrasound. Importantly, this study also underscores the need to gain mechanistic insight in order to rationally optimize gene theranostic approaches utilizing ultrasound-mediated microbubble destruction.

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REFERENCES

1. Kaul S. Myocardial contrast echocardiography: a 25-year retrospective. Circulation 2008;118:291–308.
2. de Jong N, Bouakaz A, Frinking P. Basic acoustic properties of microbubbles. Echocardiography 2002;19:229–40.
3. Bekeredjian R, Grayburn PA, Shohet RV. Use of ultrasound contrast agents for gene or drug delivery in cardiovascular medicine. J Am Coll Cardiol 2005;45:329–35.
4. Sheikov N, McDannold N, Sharma S, Hynynen K. Effect of focused ultrasound applied with an ultrasound contrast agent on the tight junctional integrity of the brain microvascular endothelium. Ultrasound Med Biol 2008;34:1093–104.
5. Xie F, Lof J, Matsunaga T, Zutshi R, Porter TR. Diagnostic ultrasound combined with glycoprotein IIb/IIIa targeted microbubbles improves microvascular recovery after acute coronary thrombotic occlusions. Circulation 2009;119:1378–85
6. Shohet RV, Chen S, Zhou YT, et al. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. Circulation 2000:101;2554–6.
7. Chen S, Ding J, Yu C, Yang B, Wood DR, Grayburn PA. Reversal of streptozotocin-induced diabetes in rats by gene therapy with betacellulin and pancreatic duodenal homeobox-1. Gene Ther 2001;14:1102–10.
8. 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;101:295–303.
9. Carson AR, McTiernan CF, Lavery L, et al. Gene therapy of carcinoma using ultrasound-targeted microbubble destruction. Ultrasound Med Biol 2011;37:393–402.
10. Postema M, van Wamel A, ten Cate FJ, de Jong N. High speed photography during ultrasound illustrates potential therapeutic applications of microbubbles. Med Phys 2005:32:3707–11.
11. van Wamel A, Kooiman K, Harteveld M, et al. Vibrating microbubbles poking individual cells: drug transfer into cells via sonoporation. J Control Release 2006;112,149–55.
12. Meijering BD, Juffermans LJ, van Wamel A, et al. Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation. Circ Res 2009;104:679–87.
13. Kooiman K, Emmer M, Foppen-Harteveld M, van Wamel A, de Jong N. Increasing the endothelial layer permeability through ultrasound-activated microbubbles. IEEE Trans Biomed Eng 2010;57:29–32.

14. Zhou Y, Kumon RE, Cui J, Deng CX. The size of sonoporation pores on the cell membrane. Ultrasound Med Biol 2009;35:1756–60.
15. Price RJ, Skyba DM, Kaul S, Skalak TC. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. Circulation 1998;98:1264–7.
16. 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;29:1759–67.
17. Chen H, Kreider W, Brayman AA, Bailey MR, Matula TJ. Blood vessel deformations on microsecond time scales by ultrasonic cavitation. Phys Rev Lett 2011:106:034301.
18. Xie A, Belcik T, Qi Y, et al. Ultrasound-mediated vascular gene transfection by cavitation of endothelial-targeted cationic microbubbles. J Am Coll Cardiol Img 2012;5:1253–62.
19. Villanueva FS, Jankowski RJ, Klibanov S, et al. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells: a novel approach to assessing endothelial function using myocardial contrast echocardiography. Circulation 1998;98:1–5.

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