JACC: CARDIOVASCULAR IMAGING
VOL. 2, NO. 7, 2009
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ISSN 1936-878X/09/$36.00
PUBLISHED BY ELSEVIER INC.
DOI:10.1016/j.jcmg.2009.05.006

EDITORIAL COMMENT

Ultrasound Mediated Destruction of DNA-Loaded Microbubbles for Enhancement of Cell-Based Therapies

New Promise Amidst a Confluence of Uncertainties?*

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Ultrasound contrast agents in current clinical use are small (1 to 4 μm) gas-filled "microbubbles" comprised of a perfluorocarbon gas that is encapsulated by a biocompatible shell (1). They freely transit through the microcirculation; high-powered ultrasound can be used to selectively destroy these microbubbles, with consequent release of their contents into the organ of interest. This acoustic responsiveness of microbubbles has been exploited,

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to great promise, for the targeted delivery of genes that are incorporated into the microbubble shell prior to systemic injection. The hypothesis is that destruction of deoxyribonucleic acid-loaded microbubbles by a focused ultrasound beam during their microvascular transit through the target area will cause localized release of the genes upon disruption of the microbubble shell, sparing nontarget areas, while concentrating the payload to the site of intended treatment. However, the exact mechanisms of gene incorporation are somewhat unclear (2).

Mechanistic uncertainties notwithstanding, using microbubbles as vectors for delivering plasmid deoxyribonucleic acid poses several advantages: The method appears to be safe, easy and can be repeated; plasmids in bubbles resist degradation in blood, can be targeted to the intended treatment site, and avoid safety issues of other gene delivery systems (3,4). Efficacy of ultrasound-targeted microbubble destruction (UTMD)-mediated gene delivery has been proven in animal studies using various types of microbubble preparations and reporter gene constructs in different organ systems (4–11). Early studies demonstrated capacity to safely deliver and direct reporter gene expression in the heart (5,6) and other tissues (7). Fewer studies have extended UTMD to the delivery of therapeutic genes into the heart (8). Interestingly, while the concept of UTMD-directed gene delivery was first proven in the heart, cardiac applications appear to have since been "left out" from subsequent studies examining clinically relevant therapeutic effects of this delivery method. Focus seems to have migrated to extracardiac applications (9–11).

So why does the heart appear to be excluded from the recent momentum for UTMD-mediated gene therapy? Some clues emerge when examining initial reporter gene studies with UTMD for the heart, mostly using luciferase plasmid driven by a cytomegalovirus promoter, in which transgene expression is quantified in relative light units (RLU). Cardiac expression of luciferase after UTMD-mediated gene therapy seems to be inefficient; peak expression varies from <1,000 to about 3,500 RLU/min/mg protein (6) compared to luciferase transgene expression on the order of 10^5 RLU/min/gm (12) in noncardiac tissue when delivered via other nonviral vectors (these methods have not as yet been directly compared with UTMD-mediated gene delivery). Whether this low efficiency for heart transduction is due to the promoter, technical fallacies in the application of UTMD, differences in the extent of plasmid incorporation into the microbubble shell resulting from varying microbubble synthesis protocols, and/or inherent

*Editorials published in *JACC: Cardiovascular Imaging* reflect the views of the authors and do not necessarily represent the views of *JACC: Cardiovascular Imaging* or the American College of Cardiology.

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differences in organ susceptibility to microbubble-mediated gene therapy is unclear, but highlights how little we know about the mechanisms by which ultrasound destruction of plasmid-bearing microbubbles really works.

In this issue of *iJACC*, Fujii et al. (13) rekindle enthusiasm for UTMD-directed gene therapy for the heart. The authors apply this approach to address a challenge encountered in cell-based therapies for the heart; namely limited cell engraftment after exogenous delivery of reparative cells, and seek to test the general hypothesis that improving the microenvironment supporting exogenously delivered cells will improve cell engraftment. In distinction to previous studies, mice were used, and a myocardial infarction model of chronic total coronary occlusion was employed as the disease model. Also unique was the selection, for one of the experimental groups, of plasmid encoding for stem cell factor (SCF) as the therapeutic gene. Separate groups of mice with chronic coronary occlusion were intravenously administered microbubbles bearing plasmid for green fluorescent protein (GFP), vascular endothelial growth $factor_{165}$ ($VEGF_{165}$), SCF, or blank plasmid, and hearts were exposed to UTMD using a clinical echocardiographic scanner. Fluorescent microscopy confirmed expression of the GFP, although the magnitude (% of cells) and site of the expression (infarct vs. noninfarct zone) and the transfected cell type(s) are not reported. Relative to controls, SCF levels were higher in the region remote from the infarct, whereas VEGF levels were higher in the infarct zone, but it is difficult to parcel out how much of each protein was increased due to the infarct model versus incremental expression by the transgene itself. Histologic analysis showed increased microvascular density in the treated groups, although it is not stated whether this occurred in the infarct or remote zone. Remarkably, 2-dimensional echocardiographic data indicated treatment-related decreases in infarct expansion and infarct thickness and significant improvements in left ventricular systolic function and perfusion.

This is an ambitious study with ambitious results. It is an ambitious undertaking because the study applies a mechanistically poorly understood technology of UTMD to the heart, which has been historically difficult to transfect with this technique. And even more ambitious because it applies this fledgling gene delivery strategy to areas that are themselves bedeviled by fundamental mechanistic uncertainties and treatment conundrums, namely reparative cell therapy and attenuation of adverse left ventricular remodeling. That this confluence of uncertainties should result in such a dramatic biologic effect with significant clinical therapeutic implications is remarkable and should be cause for both optimism as well as healthy skepticism.

Some of the data in this study are difficult to causally connect in a physiologically coherent way and call for cautious interpretation and analysis of why the clinical effects were so dramatic. The results are all the more remarkable given that the dose of plasmid, when considering the microbubble-plasmid attachment protocol used, was likely less than that used in prior reporter gene studies (6). An easy explanation might be that the mouse myocardial model is more susceptible to UTMD-directed gene therapy than previously used rat models, and that there is species-specific heterogeneity in responsiveness to gene transduction by plasmid-bearing microbubbles. Another possible explanation is that even slight transgene expression was adequate to initiate a cascade of events which triggered downstream events that ultimately caused a functionally measurable physiologic response.

While plausible, such explanations are unlikely to account for the entire effect, and other features of the study merit mention in this regard. It should be noted, for example, that a model of chronic total occlusion was used. Since mice do not have abundant pre-formed collaterals, the microbubbles would have limited access to the infarct territory. This implies, therefore, that the "active ingredients" mediating the therapeutic effects must have, at least initially, emanated from the remote, noninfarct territory, which then raises mechanistic questions as to how events in a distant region mediated the therapeutic events in the infarct zone. One possible explanation suggested by the authors is that the treatment recruited "progenitor" cells, particularly since SCF, which recruits c-kit+ cells, was expressed more in the remote zone than the infarct zone. That SCF expression was higher in the remote zone makes sense given the greater access of microbubbles to the nonoccluded coronary artery territory. However, it is not so clear whether "progenitor cells" were mobilized, as the flow cytometry data presented were somewhat equivocal as to the presence of discrete subpopulations of c-kit+ cells. Also, c-kit+ cells in the heart could be bone marrow-derived endothelial progenitor cells, circulating endothelial progenitor cells and or resident cardiac progenitor cells (14), and it would be difficult to speculate what reparative processes could

have been set in motion as a function of a specific progenitor cell type. It is also difficult to reconcile how UTMD increased $VEGF_{165}$ levels only in the infarct zone, since microbubbles would have impeded access to the infarct territory in the setting of total coronary occlusion.

Overall, this study generates both optimism as well as caution for using plasmid-carrying microbubbles to produce clinically relevant biological effects on the heart using UTMD. The investigation is certainly hypothesis-generating, and creates a need to perform additional studies to shed greater light on some of the issues mentioned earlier. The questions raised by this study underscore how much remains to be learned about the mechanisms and effects of microbubble-mediated gene therapy, and therefore, how relatively little we know about ways to optimize the results. The myriad unknowns accompanying the mechanisms of myocardial repair and regeneration, ventricular remodeling, and angiogenesis, among the phenomena touched on by this study, and the interdependence of these phenomena, make a gene-therapy approach to augment any of these processes—by any vector—challenging indeed. Nonetheless, the potentially powerful advantages of UTMD-mediated gene therapy warrant pragmatic studies to standardize and optimize protocols, as well as further systematic exploration of its efficacy, mechanisms of action, and clinical indications. As to the specific goal of Fujii et al. (13) to enhance the local intramyocardial milieu for therapeutic cell engraftment, the next step would be to determine whether their UTMD protocol actually improves engraftment of exogenously delivered cells, although if the dramatic therapeutic effects reported in this study are reproduced, the need to actually deliver additional reparative cells may even be obviated.

Acknowledgments
The author would like to thank Catalin Toma, MD and Andrew Carson, PhD, for discussions related to this editorial.

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Key Words: contrast ultrasound ■ gene therapy ■ myocardial infarction.