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Gene Delivery Using Ultrasound Contrast Agents

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With the human genome product and continuing advances in molecular biology many therapeutic genes have been discovered. In the cardiovascular system, gene therapy has the potential to improve myocardial vascularization and ameliorate congestive heart failure. For successful development of clinical gene therapy, however, effective gene delivery vectors are needed. Ultrasound contrast agents can be used to develop new, more effective vectors for gene delivery. Ultrasound contrast agents lower the threshold for cavitation by ultrasound energy. Using physical properties of microbubbles and coating materials, genetic drugs have been incorporated into ultrasound contrast agents. Gene-bearing microbubbles can be injected IV and ultrasound energy applied to the target region. As the microbubbles enter the region of insonation, the microbubbles cavitate, locally releasing DNA. Cavitation also likely causes a local shockwave that improves cellular uptake of DNA. With transthoracic ultrasound, using commercially available diagnostic ultrasound system and an IV injection of gene-bearing microbubbles, high levels of transgene expression are observed in the insonated region of the myocardium. This new technology using microbubbles and ultrasound for gene delivery merits further study and development. (ECHOCARDIOGRAPHY, Volume 18, May 2001)

ultrasound contrast agents, microbubbles, gene therapy

Gene delivery is under development to treat a variety of human diseases. In the cardiovascular system, encouraging results are being obtained with the gene for vascular endothelial growth factor (VEGF).[1] Gene therapy with VEGF has been shown in animal studies as well as clinical trials to increase angiogenesis (new vessel formation) in ischemic tissue. Angiogenesis has therapeutic benefit to improve vascularization in the setting of decreased blood flow to atherosclerosis. Therapeutic benefits have been shown in the myocardium for coronary artery disease and in the extremities for peripheral vascular disease.[1,2] Vascular thrombosis has been moderated by using adenoviral transfer of genes for anticoagulant proteins such as hirudin.[3] Future antithrombotic targets for gene therapy include nitric oxide synthase,[4] cyclooxegenase, and prostacycline synthase for antiplatelet activity, TFPI for anticoagulant activity, and tPA or uPA for fibrinolytic activity.[5]

Vascular disease is an important target for developing gene transfer strategies. Delivery of the genes to the desired vascular tissue, however, can be difficult to achieve. Delivery of the VEGF gene has generally required direct injection of the genetic material into the tissue.[6] In the extremities, this can be achieved by direct injection of the plasmid DNA encoding the VEGF gene into the muscle.[2] In the heart this is more difficult and requires an invasive procedure to introduce the genetic material into the myocardial procedure. Surgical exposure of the heart has been used to open a window for direct injection of the VEGF gene into the myocardial tissue.[6,7] Another possible approach is via catheter delivery into the coronary arteries, but this has been less effective than direct injection.

For a therapeutic gene to be effective, it must reach the target cells, enter the nucleus of these cells, and then the gene must be expressed.[8] Gene expression and production of a protein product are generally necessary to pro-

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duce therapeutic effects.[9] In the case of VEGF, the gene must be transcribed, and from the messenger RNA, the VEGF protein translated. Therapeutic angiogenesis can be achieved with VEGF protein directly without the gene.[10] The advantage of using the gene instead, however, is that a more prolonged therapeutic effect can be achieved. While one molecule of VEGF protein may only act for a short while before it is metabolized, the gene may continue to produce VEGF for as long as the gene is transcribed.

New ultrasound contrast agents have been developed and some of these are now FDA approved.[11-13] This article explores some of the potential applications of ultrasound contrast agents for gene delivery. We shall attempt to explain how ultrasound contrast agents can be used for targeted localized gene delivery. These agents have applications for delivering genetic material such as VEGF for treating ischemic cardiovascular disease and other gene products for treating other diseases such as cancer and arthritis.

Applications of Ultrasound Contrast Agents in Gene Delivery

Briefly, let us consider the barriers to effective gene delivery. Therapeutic genes are macromolecules with several thousand base pairs and molecular weights over 1 million Daltons.[14] These materials generally are relatively rapidly metabolized by serum esterases and are, therefore, not stable to IV administration, unless the genetic material is stabilized in some fashion.[15] Genes, like macromolecules, are generally too large to pass across the capillary fenestrations of blood vessels unless assisted by some mechanism. For systemic delivery, the large size of genes is then an obstacle to delivery to tissues beyond the endothelial cell barrier lining most blood vessels. After genes reach the correct tissues, they must pass across cell membranes and enter the cells' nucleus. This is no easy step as cells have designed efficient mechanisms for processing exogenous molecules. Once cells take up macromolecules, these are generally digested within lysosomes within the cells.[16]

Animal and human studies have shown that relatively efficient gene expression can be achieved by direct injection of a gene into muscle.[17-19] Muscle cells appear able to take up exogenous genes and produce the protein from the gene. However, direct injection of naked (uncoated) DNA has generally been less successful in other tissues. Furthermore, direct injection is invasive and technically difficult to achieve in some tissues such as the heart. A variety of coating materials has been tested to improve delivery of genetic materials. These include liposomes, cationic (positively charged polymers), and viruses.[20-27] Some viruses have evolved over millions of years for delivering genetic materials into living cells. Some studies have shown that viruses are efficient gene delivery vectors, that is, high levels of gene expression can be achieved using viruses to deliver genes. Immune response to virus can limit the effectiveness of gene therapy with a viral vector.[28] Viruses are antigenic and can cause allergic reactions. A death occurred in one recent clinical trial of gene therapy using a viral vector, and this may have been due to an immune reaction related to the viral vector.[29] Additionally, there is a concern about mutagenesis with some of the viral vectors.[30] Currently there is a need for safe synthetic vectors, which might be delivered intravenously to provide targeted gene delivery to a localized tissue.

Given the need for new, effective gene delivery vectors and the barriers to their development, how might ultrasound contrast agents fill this role? Ultrasound contrast agents can be designed as safe vehicles for encapsulating genetic materials.[31] Ultrasound energy can be used to cavitate (rupture) ultrasound contrast agents and deliver genes locally to a tissue.[32] Cavitation can be exploited to increase transvascular passage of macromolecules and cellular uptake or passage of therapeutic agents.[33,34] Ultrasound contrast agents can also be targeted to cell-specific receptors to hone in on a target, and ultrasound can then be applied to improve uptake of the genetic material.

In our assessment (ImaRx Therapeutics, Inc., Tucson, AZ, USA) of the potential of ultrasound contrast agents as gene delivery vehicles, we first performed studies of the effects of ultrasound alone on gene expression in cell culture.[35] Cells were exposed to ultrasound using a 1.0-megahertz continuous wave probe (RichMar, Inola, OK, USA) by immersing the head of the transducer directly into the cell culture medium overlying the cells. The effect of ultrasound exposure on the temperature of the cell culture medium was assessed. Cell survival studies were performed to study the effects of different levels of ultrasound power as well as the duration of insonation on cell survival. Transfection studies were performed us-

ing marker genes, which do not exert a therapeutic effect such as p-chloramphenicolacetyltransferase (CAT), beta-galactosidase (GAL), and green fluorescent protein (GFP) in several different cell lines such as HeLa, NIH t-3, and COS-1 cells. Figure 1 shows typical results from these studies. Ultrasound without microbubbles increased the gene expression in all of these cell lines. The enhancement of transfection occurred at levels of ultrasound of about 0.5 W/cm^2 and duration of exposure of only about 15 seconds and did not appreciably heat the cells nor adversely affect their survival. We extended these experiments in vivo into nude mice implanted with human melanomas. The tumors of these mice were injected with the gene for interleukin-2 (IL-2) using a lipid vector—in this case not a microbubble. The animals were sacrificed 72 hours later, the tumors excised, and the cells obtained from the tumor and then grown in cell culture. As shown in Figure 2, ultrasound increased gene expression in the tumors.

To try to understand the mechanism responsible for the enhancement of gene expression in cells with ultrasound, we analyzed gene expression in cells exposed to ultrasound using a cell culture model.[36] We found that ultrasound upregulated the expression of a number of cell repair genes. It could be that this upregulation of gene expression enhances the expression of exogenous genes as well. It also could be that mechanical factors (e.g., cell permeability, etc.) are also important.

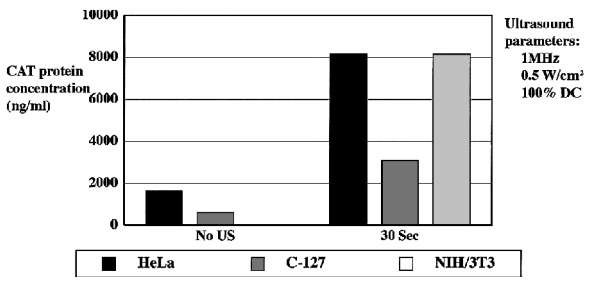

Figure 1. *The effects of SonoPoration on transfection rates of mammalian cells. In these experiments, the plasmid for chloramphenicolacetyltransferase (CAT) gene with a liposome vector was administered to three different mammalian cell lines (HeLa, C-127, and NIH/3T3). Ultrasound energy was applied to the cells and CAT gene expression was measured 48 hours later using CAT assay. As shown in the figure, ultrasound has a dramatic effect on increasing the efficiency of transfection. Of note, the NIH/3T3 cells, which were the most difficult to transfect without ultrasound, showed the largest effective increase in transfection efficiency.*

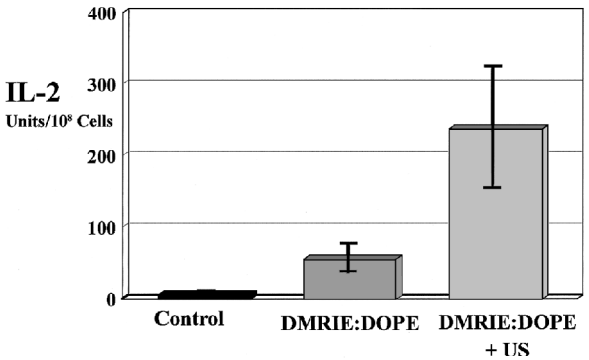

Figure 2. *The effect of ultrasound on gene therapy of mice tumors. In these experiments, the gene for the cytokine, interleukin 2 (IL-2) was injected into the tumor using a lipid carrier (DMRIE:DOPE). Ultrasound caused a severalfold increase in gene expression in the tumors, resulting in increased production of IL-2 in the tumor.*

While ultrasound contrast agents are used diagnostically to reflect sound, in gene therapy they may also be used to increase absorption of sonic energy. Ultrasound contrast agents are mainly based on microbubbles.[37] Microbubbles are elastic and compressible, have a much lower density than water, and create an acoustic impedance mismatch from biological tissues and fluids.[38] Because of these properties, microbubbles are efficient reflectors of ultrasound, and hence useful as ultrasound contrast agents. Microbubbles also lower the threshold of energy for cavitation.[39] In cavitation, ultrasound energy is concentrated into a microdomain.[40] Cavitation creates small shock waves, which will increase cell permeability. Cavitation destroys the microbubbles and can be used to release materials entrapped within the microbubbles or coated onto the surface of the microbubbles. Microbubbles and other materials (e.g., gaseous precursors that convert to gas at a temperature close to 37°C) that have sufficiently different acoustic impedance from tissues and biological fluids may be referred to as acoustically active. Such materials can be used to increase the absorption of acoustic energy within the tissue or blood to cause local therapeutic effects.

Figure 3 shows the effects of cavitation on microvascular permeability.[41] As microbubbles are cavitated by ultrasound, the local shock waves increase capillary permeability. This process has been shown experimentally to increase transcapillary passage of macromolecules or nanospheres codelivered with the microbubbles.[41,42] Microvascular permeabiliza-

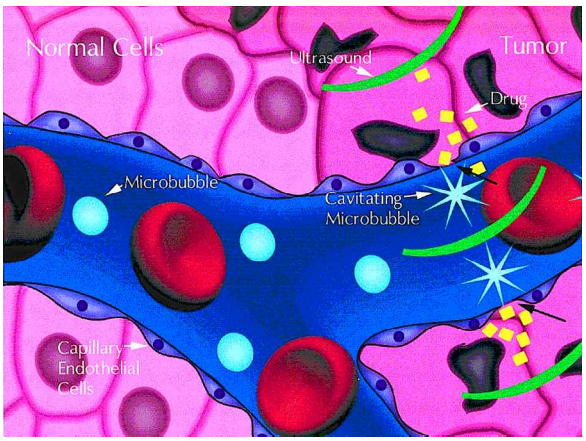

Figure 3. *The effects of cavitation on microvascular permeability. Nanospheres and drugs can be delivered into the interstitium as the microbubbles cavitate. This process can be used to improve local drug delivery. Black arrows indicate gaps formed between the capillary endothelial cells by the energy of cavitation. (Adapted from reference 41)*

tion caused by cavitation of microbubbles may be an important mechanism for gene delivery with ultrasound contrast agents. Experimental studies by Dr. Sanjiv Kaul's group at the University of Virginia show that capillary permeability effects occur in experimental animals with microbubbles using a diagnostic ultrasound transducer and a mechanical index of about 1.6 megapascals.[41,42]

Microbubbles with a cationic surface have been produced by ImaRx Therapeutics, Inc., to bind genetic materials (Fig. 4). These microbubbles avidly bind DNA.[43] Applications of ultrasound energy in the range of 1 megapascals rupture the microbubbles and release the

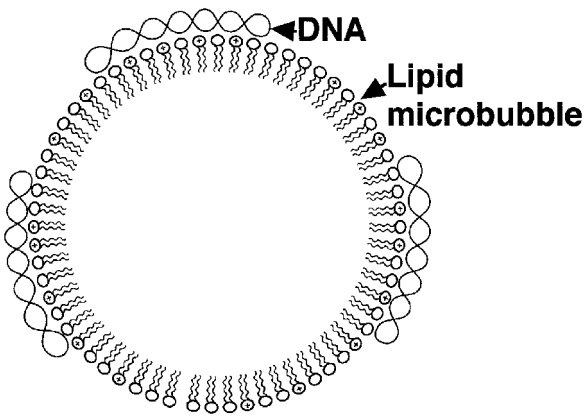

Figure 4. *DNA/microbubble interaction. Using electrostatic interaction, genetic material may be bound noncovalently to the surface of microbubbles.*

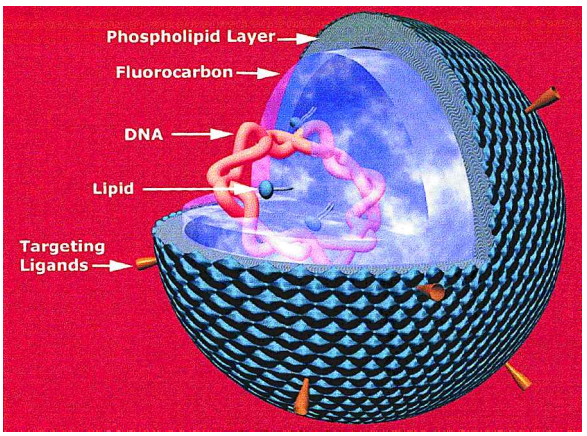

Figure 5. *Liquid perfluorocarbon gene carrier. The outer surface is stabilized by amphipathic lipid. Targeting ligands have been incorporated onto the head groups of the lipids. The genetic material is stabilized by cationic lipids. Electron microscopy studies have shown that the DNA is condensed as an electron-dense granule within the center of the nanoparticle. The diameter of these particles is about 100-200 nanometers.*

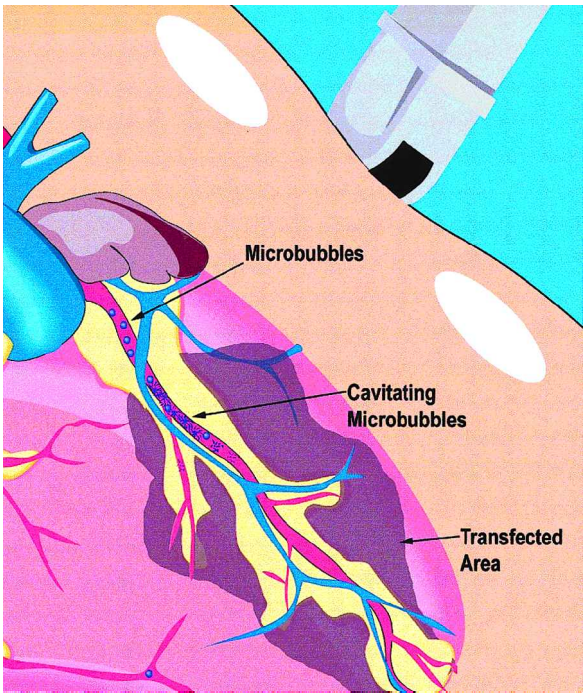

Figure 6. *Schematic diagram of gene therapy to the heart. A diagnostic ultrasound transducer is placed on the patient's chest. An ultrasound contrast agent bearing genetic material has been administered intravenously. As the microbubbles enter the region of insonation they distribute within the myocardial tissue via the vascular bed. The microbubbles cavitate within the capillaries of the myocardial tissue releasing the genetic material.*

DNA. Using gel electrophoresis, we have shown that the DNA is intact and stable after cavitation with ultrasound and release of the DNA from the microbubbles. Dr. Thomas Porter of the University of Nebraska has also shown that albumin-coated perfluorobutane microbubbles will bind oligonucleotides.[44-47] Dr. Fuminori Moriyasu's group at the University of Kyoto has prepared cationic gelatin-coated gas-filled polymeric microspheres and shown that these will bind genetic materials.[48] In addition to the microbubbles shown in Figure 4, ImaRx Therapeutics, Inc., has produced gaseous precursor materials for gene delivery using phospholipids and perfluorocarbons such as perfluorohexane and perfluoropentane. As shown in Figure 5, the structure of these agents is different from the microbubbles. Electron microscopy studies have been performed and showed that the DNA is entrapped in the center of the fluorocarbon material and condensed into small electron dense structures within fluorocarbon cores. These agents, like the microbubbles, have different acoustic impedance from water and can be used to absorb ultrasound energy as cavitation nuclei. As shown in Figure 5, targeting ligands have also been incorporated onto the surface of these gaseous precursor agents. Fibroblast growth factor (FGF) has been covalently bound to lipids coating the surface of these agents. FGF is a biologically important ligand, expressed on endothelial cells in angiogenesis associated with cancer as well as atherosclerosis. In vitro transfection studies in cell culture using cells expressing the FGF receptor have shown significant enhancement of gene expression from fluorocarbon vectors bearing the FGF ligand.

In vivo studies have been performed in rats with intravenous injection of microbubbles as well as gaseous precursor agents binding genes. Most studies have been performed using marker genes, that do not exert a therapeutic effect such as p-chloramphenicol acetyl transferase (CAT), beta-galactosidase (b-GAL), and green fluorescent protein. When gene-carrying microbubbles are injected IV and ultrasound is applied to the animal's thigh, preferential gene expression is attained in the muscle of insonation (ImaRx Therapeutics, Inc.).[31] Very low levels of gene expression are observed in animals administered these agents unless they are insonated by ultrasound. Moriyasu's group has administered cationic gelatin-coated microsphere agents binding the CAT gene IV to rats and applied ultrasound to the animals' livers. High gene expression was achieved in the liver of the animals exposed to ultrasound but minimal, if any, expression in animals administered the agents but not exposed to ultrasound.[48] Thomas Porter has administered albumin-coated microbubbles binding oligonucleotides encoding the antisense sequence to FGF. In these experiments, the antisense construct to FGF is designed to decrease fibroblast proliferation. Arterial injury was created in the pig model, and the antisense carrying microbubbles were administered intravascularly and ultrasound was applied to the region of arterial damage. Porter was able to demonstrate deposition of antisense material in the vessel wall in the region of insonation by ultrasound.[45] Decreased fibroblast proliferation was observed in the region of insonation presumably due to inhibition of FGF by the antisense oligonucleotides.[44]

We have performed cardiac experiments for gene delivery at the University of Michigan. Cationic phospholipid-coated perfluorobutane microbubbles were prepared to bind the CAT gene. The DNA was added to the preformed microbubbles, agitated gently and the DNA was bound by the microbubbles. The mean size of the microbubbles was about 2-microns. The material was administered via peripheral vein and ultrasound was applied to the animal's heart using a 1.0 megahertz transducer with a Sonos Model 5500 ultrasound system and an insonation energy level of 1.7 MegaPascals as shown in Figure 6. The dog was sacrificed 48 hours after the gene delivery experiment, the heart was excised, and CAT gene expression assayed. High CAT levels were observed within the myocardium within the region of insonation, but not within the myocardium not exposed to ultrasound.[49]

Conclusions

Ultrasound has a direct effect on gene expression that may be used to enhance gene expression without the use of exogenous microbubbles. A synergistic effect is attained with the use of microbubbles and ultrasound and cavitation is a likely mechanism. Acoustically active materials, microbubbles, and gaseous precursor agents have been developed that bind or entrap genetic materials. Targeting ligands have also been incorporated onto the surface of these agents for cell-specific delivery. Acoustically active gene delivery vehicles appear to hold promise for gene delivery. These

materials can be injected IV and targeted gene delivery is attained within the tissue exposed to ultrasound. Myocardial targeted gene delivery has been shown from IV injection of gene-carrying microbubbles in concert with cardiac ultrasound. This new technology holds the promise to deliver genes more selectively than other methods and less invasively than direct injection. Studies are currently in progress with therapeutic genes in experimental animals. Successful clinical development of ultrasound-mediated gene delivery with acoustically active carriers will entail additional experimental studies as well as clinical trials. The ability to focus ultrasound and cause local cavitation with these new gene carriers may provide a powerful new tool for gene delivery.

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References

1. Tio RA, Lebherz C, Scheuerman T, et al: Local and systemic evidence for VEGF production after direct intramyocardial injection with naked DNA encoding for VEGF. (abstract) *Eur Heart J* 1998;19:588.
2. Baumgartner I, Pieczak A, Manor O, et al: Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* 1998;97:1114-1123.
3. Rade JJ, Schulick AH, Virmani R, et al: Local adenoviral-mediated expression of recombinant hirudin reduces neointima formation after arterial injury. *Nat Med* 1996;2:293-298.
4. Van der Leyen H, Gibbons H, Morishita R, et al: Gene therapy inhibiting neointimal vascular lesion formation: In vivo transfer of endothelial cell nitric oxide synthase gene. *Proc Natl Acad Sci USA* 1995;92:1137-1141.
5. Caplice NM, Simari RD, Holmes DR: Molecular biology and gene transfer in atherosclerosis in the stenting era. *Int J Cardiovasc Intervent* 1999;2:141-152.
6. Symes JF, Losordo DW, Vale PR, et al: Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. *Ann Thorac Surg* 1999;68: 830-837.
7. Losordo DW, Vale PR, Isner JM: Gene therapy for myocardial angiogenesis. *Am Heart J* 1999;138:132-141.
8. Forbes SJ, Hodgson HJ: Review article: Gene therapy in gastroenterology and hepatology. *Aliment Pharmacol Ther* 1997;11:823-836.
9. Haber E: Molecular Cardiovascular Medicine. Scientific American, Inc., New York, 1995, p. 80.
10. Witzenbichler B, Asahara T, Murohara T, et al: Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. *Am J Pathol* 1998;153:381-394.
11. Broillet A, Puginier J, Ventrone R, et al: Assessment of myocardial perfusion by intermittent harmonic power Doppler using SonoVue, a new ultrasound. *Invest Radiol* 1998;33:209-215.
12. Fritz TA, Unger EC, Sutherland G, et al: Phase I clinical trials of MRX-115. A new ultrasound contrast agent. *Invest Radiol* 1997;32:735-740.
13. Ernst H, Hahn EG, Balzer T, et al: Color Doppler ultrasound of liver lesions: Signal enhancement after intravenous injection of the ultrasound contrast agent Levovist. *J Clin Ultrasound* 1996;24:31-35.
14. Lasic DD: Liposomes in Gene Delivery. CRC Press, Boca Raton, 1997, p. 24.
15. Lechardeur D, Sohn KJ, Haardt M, et al: Metabolic instability of plasmid DNA in the cytosol: A potential barrier to gene transfer. Gene Ther 1999;6:482-497.
16. Laurent N, Wattiaux-De Coninck S, Mihaylova E, et al: Uptake by rat liver and intracellular fate of plasmid DNA complexed with poly-L-lysine or poly-D-lysine. *FEBS Lett* 1999;443:61-65.
17. McMahon JM, Wells KE, Bamfo JE, et al: Inflammatory responses following direct injection of plasmid DNA into skeletal muscle. *Gene Ther* 1998;5:1283-1290.
18. Anwer K, Shi M, French MF: Systemic effect of human growth hormone after intramuscular injection of a single dose of a muscle-specific gene medicine. *Hum Gene Ther* 1998;9:659-670.
19. Prentice H, Bishopric NH, Hicks MN, et al: Regulated expression of a foreign gene targeted to the ischaemic myocardium. *Cardiovasc Res* 1997;35:567-574.
20. Yang JP, Huang L: Direct gene transfer to mouse melanoma by intratumor injection of free DNA. *Gene Ther* 1996;3:542-548.
21. Fischer D, Bieber T, Li Y, et al: A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm Res* 1999;16:1273-1279.
22. Srinivasakumar N, Schuening FG: A lentivirus packaging system based on alternative RNA transport mechanisms to express helper and gene transfer vector RNAs and its use to study the requirement of accessory proteins for particle formation and gene delivery. *J Virol* 1999;73:9589-9598.
23. Yu W, Shimoyama A, Uneda T, et al: Gene transfer mediated by YKS-220 cationic particles: convenient and efficient gene delivery. *J Biochem* 1999;125:1034-1038.
24. Kasono K, Blackwell JL, Douglas JT: Selective gene delivery to head and neck cancer cells via an integrin targeted adenoviral vector. *Clin Cancer Res* 1999;5: 2571-2579.
25. Mediratta SK, Quezada A, Matar M, et al: Intratumoral delivery of IL-12 gene by polyvinyl polymeric vector system to murine renal and colon carcinoma results in potent antitumor immunity. *Gene Ther* 1999;6:833-839.
26. Grimm D, Kleinschmidt JA: Progress in adeno-associated virus type 2 vector production: promises and prospects for clinical use. *Hum Gene Ther* 1999;10: 2445-2450.
27. Morris MC, Chaloin L, Mery J, et al: A novel potent strategy for gene delivery using a single peptide vector as a carrier. *Nucleic Acids Res* 1999;27:3510-3517.
28. Chun S, Daheshia M, Lee S, et al: Immune modulation by IL-10 gene transfer via viral vector and plasmid DNA: Implication for gene therapy. *Cell Immunol* 1999;194:194-204.

29. Seachrist L: Rac continues searching for cause of gene therapy death. *Bioworld Today* 1999;10:235.
30. Haber E: Molecular Cardiovascular Medicine. Scientific American, Inc., New York, 1995, p. 83.
31. Unger EC, McCreery TP, Sweitzer R, et al: Ultrasound enhancement of gene expression and gene delivery using acoustically active carriers. (abstract) Macromolecular Drug Delivery Conference, Breckenridge, CO, 1999 p. 20.
32. Unger EC, McCreery TP, Sweitzer R, et al: Ultrasound enhanced gene expression and gene delivery. (abstract) International Conference on Gene Therapy and Molecular Biology & Medicine, Redwood City, CA, 1999.
33. Unger EC, McCreery TP, Schweitzer RH, et al: Acoustically active lipospheres containing paclitaxel: A new therapeutic ultrasound contrast agent. *Invest Radiol* 1998;33:886-892.
34. Unger EC, McCreery T, Sweitzer R, et al: MRX 501: A novel ultrasound contrast agent with therapeutic properties. *Acad Radiol* 1998;5:S247–S249.
35. Unger EC, McCreery TP, Sweitzer R: Ultrasound enhancement of gene expression from liposomal transfection. (abstract) Radiology 1997;205(S):S724.
36. Unger EC, McCreery TP, Sweitzer RH: Ultrasound enhances gene expression of liposomal transfection. *Invest Radiol* 1997;32:723-727.
37. Cosgrove DO, Blomley MJK: Echo-enhancing ('contrast') agents for ultrasound. In Dawson P, Cosgrove DO, Grainger RG (eds): Textbook of Contrast Media. Isis Medical Media, Oxford, UK, 1999, pp. 451-452.
38. Forsberg F: Physics of ultrasound contrast agents. In Goldberg BB (ed): Ultrasound Contrast Agents. Martin Dunitz Ltd, London, UK, 1997, pp. 9-13.
39. Apfel RE, Holland CK: Gauging the likelihood of cavitation from short-pulse, low-duty cycle diagnostic ultrasound. *Ultrasound Med Biol* 1991;17:179-185.
40. Miller MW, Miller DL, Brayman AA: A review of in vitro bioeffects of inertial ultrasonic cavitation from a mechanistic perspective. *Ultrasound Med Biol* 1996; 22:1131-1154.
41. Skyba DM, Price RJ, Linka AZ, et al: Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation* 1998;98:290-293.
42. Price RJ, Skyba DM, Kaul S, et al: Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation* 1998;98: 1264-1267.
43. Unger EC: Therapeutic applications of microbubbles. (abstract) Interventional Cardiology, Jerusalem 1999.
44. Porter TR, Xie F, Iversen P, et al: Intravenous antisense administered bound to perfluorocarbon exposed sonicated dextrose albumin microbubbles inhibits stenosis formation following coronary balloon injury. (abstract) *Circulation* 1998;98:I503.
45. Wong J, Mukharjee D, Porter TR, et al: Ultrasound power affects uptake of PESDA linked oligonucleotide deposition into myocardial tissue. (abstract) *Circulation* 1998;98:I503.
46. Porter TR, Li S, Iversen PL: The effect of microbubble gas composition and external ultrasound frequency on the non-invasive enhancement of antisense oligonucleotide delivery to the vascular wall in pigs. (abstract) *Circulation* 1997;96:I401.
47. Porter TR, Iversen PL, Li S, et al: Interaction of diagnostic ultrasound with synthetic oligonucleotide-labeled perfluorocarbon-exposed sonicated dextrose albumin microbubbles. *J Ultrasound Med* 1996;15: 577-584.
48. Matsumura T, Moriyasu F, Tabata Y, et al: Ultrasound-mediated transfection with cationic gelatin. (abstract) *J Ultrasound Med* 1998;16:S28.
49. Li P, McCreery T, Mathew B, et al: In vivo transfection and gene expression in canine myocardium after intravenous injection of microbubble linked plasmid DNA and exposure to ultrasound. (abstract) American Society of Echocardiography meeting, Chicago, IL 2000.