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Sonoporation: Mechanical DNA Delivery by Ultrasonic Cavitation

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Abstract

Development of nonviral gene transfer methods would be a valuable addition to the gene-therapy armamentarium, particularly for localized targeting of specific tissue volumes. Ultrasound can produce a variety of nonthermal bioeffects via acoustic cavitation including DNA delivery. Cavitation bubbles may induce cell death or transient membrane permeabilization (sonoporation) on a single cell level, as well as microvascular hemorrhage and disruption of tissue structure. Application of sonoporation for gene delivery to cells requires control of cavitation activity. Many studies have been performed using in vitro exposure systems, for which cavitation is virtually ubiquitous. In vivo, cavitation initiation and control is more difficult, but can be enhanced by cavitation nucleation agents, such as an ultrasound contrast agent. Sonoporation and ultrasonically enhanced gene delivery has been reported for a wide range of conditions including low frequency sonication (kilohertz frequencies), lithotripter shockwaves, HIFU, and even diagnostic ultrasound (megahertz frequencies). In vitro, a variety of cell lines has been successfully transfected, with concomitant cell killing. In vivo, initial applications have been to cancer gene therapy, for which cell killing can be a useful simultaneous treatment, and to cardiovascular disease. The use of ultrasound for nonviral gene delivery has been demonstrated for a robust array of in vitro and mammalian systems, which provides a fundamental basis and strong promise for development of new gene therapy methods for clinical medicine.

Introduction

The role of genetic factors in the development of human disease is by no means a new concept. Genetic phenomena were already described by ancient Greeks and Hebrews. The nineteenth century brought the pioneering work of Gregor Mendel, who laid the grounds of modern genetics, and in 1902 Garrod described alkaptonuria as the first "inborn error

Abbreviations: CAT, chloramphenicol acetyltransferase; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbant assay; FITC, fluoroisothiocyanate; GFP, green fluorescent protein; HIFU, high-intensity-focused ultrasound; PESDA, perfluorocarbon-exposed sonicated dextrose albumin; PRF, pulse repetition frequency; SW, shock wave.

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of metabolism." However, it was only during the last two decades that our understanding of genetic mechanisms and associated technology have advanced enough to attempt therapeutic genetic manipulations. The newcomer in the therapeutic armamentarium, gene therapy, can be defined as the treatment of human disease by introduction of genetic material into human tissues. The transferred DNA can replace a defective gene, enhance, or inhibit a preexisting cell function, or introduce a completely new function into the cell. Conceptually, gene therapy could be used in a vast number of human diseases, beginning with the inborn errors of metabolism and ending with cancer therapy. It can be envisaged that, with the completion of the Human Genome Project, the list of diseases that are amenable to genetic therapy will only increase.

Identification of genetic mechanisms responsible for disease and the design of new genetic strategies alone cannot guarantee efficient gene therapy. The necessary step in all forms of genetic manipulation is transfection, the introduction of genetic material into cells. This is a major limiting step because DNA is a large, highly charged molecule, and, therefore, cannot diffuse through the cell membrane. Once inside the cell, the DNA is expressed for various periods of time. In this sense, we could distinguish stable transfection (achievable by incorporation of the transferred gene into the genome) and transient transfection (when the genetic fragment is expressed, but not included in the chromosomes). Strategies for achieving both types of transfection exist, but current techniques pose most of the limitations to the clinical application of gene therapy. Until now, none of the methods tested in the clinical setting have allowed an effective, safe, minimally invasive delivery of the genetic material.

Transfection protocols can be divided into two broad categories—viral and nonviral (1). The major vectors used so far in clinical testing are adenoviral (for cardiovascular disease) and retroviral (especially for cancer therapy); adeno-associated and lentiviral vectors are currently under investigation. Adenoviral vectors are engineered by removing key sequences for replication from the viral genome (thus rendering the virus incapable of replication) and replacing them with the therapeutic gene (2). Once injected into the organism, the adenoviral vector binds to membrane receptors and is subsequently internalized. The substituted DNA is transferred to the nucleus where it remains episomal (is not included in the host cell chromosomes). Thus, transfection with adenoviral vectors is only transient but, on the other hand, poses no concern for mutagenesis. The major advantages of this vector are related to its ability to transfect nonreplicating cells and the high percentage rates of transfection. Retroviral vectors are restricted to cells that proliferate actively, but the genetic material is incorporated into the host genome, allowing stable transfection. Although viral-mediated transfection allows high transfection rates when compared to DNA alone, currently available viral vectors still have substantial drawbacks. The major concerns are related to potential toxicity, development of severe immunological reactions (for adenoviruses), and potential induction of new mutations (for retroviruses).

Forms of nonviral transfection are represented by lipofection, electroporation, particle bombardment, and sonoporation. Lipofection is one of the most widely used nonviral methods of transferring genetic material to living cells. Essentially, cationic lipids encapsulate the negatively charged DNA and facilitate transfer of the gene through the cell membrane (3). This method allows high transfection rates with minimal cellular toxicity, but as with the viral vectors, it does not allow control of spatial or temporal specificity of delivery. Electroporation refers to the transfer of DNA through membrane pores formed in high-voltage electric fields (4). This method allows some spatial targeting, but requires electrode placement, which can be invasive. Particle bombardment represents yet another way of "injecting" foreign DNA into cells, this time by coupling the gene to projectiles that are made to penetrate the membrane at high speed (5). This method also allows

accurate placement of DNA delivery, but appears to be limited to surface (e.g., skin) applications.

Sonoporation, the subject of this chapter and a recent review by Newman et al. (6), is a relative newcomer in gene transfer. This technique is designed to enhance cell permeability through the use of ultrasound. Uptake of both DNA and other macromolecules has been demonstrated by several investigators (7–11). However, research in the field gained momentum only after the identification of cavitation as the probable mechanism behind the increased cell permeability, and the demonstration of further enhancement of transfection efficiency by using cavitation nuclei, such as ultrasound contrast agents (8, 10, 12, 13). These findings opened tremendous opportunities for targeted gene transfer. Conceptually, gene vectors mixed with ultrasound contrast agents could be injected intravenously, and targeted gene transfer could be achieved by selective insonation of a predefined area. Indeed, promising results have been recently reported in animal models (14–18). By using this approach, the risk of systemic exposure (a major drawback of current clinical protocols of gene transfer) could be substantially reduced.

There are, of course, other approaches to local gene therapy (eluting polymers, coated stents, local delivery catheters, direct injection, etc.). However, most of the local delivery systems tested so far provide a high local-to-systemic ratio at only one particular location (either the vascular segment that is treated or the injection site). This is of course an advantage when treating a very localized disease, such as restenosis after angioplasty. On the other hand, most of the pathological processes amenable to gene therapy, even when localized (myocardial ischemia, tumors, etc.), would require a diffuse treatment of the affected organ that can be achieved only by enhancing the transfer at the microcirculation level. This might be achieved by sonoporation, which combines the capability of enhancing gene transfer with the possibility of restricting this effect to the desired area, at the desired time (spatial and temporal specificity).

To better present the use of ultrasound for transfection, we will first describe basic ultrasonic principles, consider the range of potential bioeffects of ultrasound, and briefly outline uses of ultrasound in biomedicine. Research on sonoporation, which is the biophysical phenomenon behind the transient enhancement of cell permeability, will be examined. The current status of ultrasound-aided DNA transfer will then be reviewed for in vitro and in vivo conditions. Finally, potential clinical applications and future directions will be summarized.

Ultrasound and Ultrasonic Biological Effects

Ultrasound

Ultrasound propagates through matter from a vibrating object as an oscillating pressure wave. This is fundamentally the same as audible sound, but frequencies f are higher than 20 kHz, which is above the range of human hearing. The pressure amplitude of a sinusoidal wave is typically specified in Pascal (Pa, kPa, or MPa, where 100 kPa is approximately atmospheric pressure). The speed c of propagation is about 1500 m s^{-1} in aqueous media and the wavelength is defined by c/f, which is about 1.5 mm at 1 MHz. Wavelength is an important determinant of the geometry of a wave propagating away from a vibrating object: objects smaller than a wavelength in extent yield spherically diverging waves, while relatively large objects can yield directional beams and focusing. The propagating wave transmits energy, and exposure is often specified in terms of the intensity (e.g., W cm^{-2}), which is proportional to the square of the pressure amplitude.

As an ultrasound wave propagates, several phenomena occur, which can potentially cause physical perturbations of biological cells and tissue. The primary mechanisms for bioeffects of ultrasound are heating and cavitation. Heating occurs as the ultrasonic energy is absorbed in a medium. At 1 MHz, absorption in tissue can be quite important in biomedical applications, and at 1 MHz, for example, intensity can by halved for each 10 cm of propagation (i.e., for a typical 0.3 dB cm^{-1} MHz^{-1} absorption coefficient). The lost intensity appears mostly as heating of the tissue, which is normally more important for higher frequencies. The amount of heating depends not only upon generating mechanisms, but also on the geometry of energy deposition, and on the heat transfer properties of tissues via conduction, convection, and radiation. The picture is further complicated by the generation of heat during cavitation and by the heating of the ultrasonic probe itself.

Cavitation is a nonthermal interaction between a propagating pressure wave and a gaseous inclusion in aqueous media. For low-pressure amplitudes, a gas bubble pulsates at amplitudes that depend strongly on its size, and are maximized at the resonance frequency of the bubble. At 1 MHz, a resonance size free bubble in water is about 7 μ in diameter, with cavitation phenomena typically being more important at lower frequencies. Large bubbles or air–water interfaces simply act as reflective surfaces for ultrasound waves. At higher pressure amplitudes, the transformation of cavitation nuclei, which are normally inactive and difficult to detect, into active cavities and bubbles gives the appearance of a threshold for vigorous cavitation. In addition, proliferation of bubble populations can multiply cavitation effectiveness. As a part of cavitation activity, microbubbles implode into minute sites of intense heat, light (sonoluminescence), free radical production, and shock wave generation, which can introduce secondary mechanisms for bioeffects.

Bioeffects of Ultrasound

The knowledge of bioeffects attributable to ultrasound extended remarkably during the past decades, as concerns regarding the safety of diagnostic and therapeutic ultrasound triggered research into the area (see Table 1). A detailed review of ultrasound bioeffects is beyond the scope of this chapter; the interested reader is referred to general information on bioeffects of ultrasound (50–53).

The bioeffects of ultrasound generally may be categorized as thermal and nonthermal. Heating by more than a few degrees above normal biological temperatures can perturb biological systems; for example, by enhancing metabolism and perfusion of tissue. At high intensities, rapid heating can cause simple cooking of tissue. Generation of heat by ultrasound has several applications in medicine and biology, from cleaning tanks to induction of hyperthermia for surgery and tumor ablation, but heating may also be associated with deleterious effects, especially in tissues undergoing organogenesis or with poor heat-transfer mechanisms (54). However, it is generally assumed that thermal mechanisms play only a minor role (if any) in the ultrasound-induced increase in cell permeability. A detailed review of thermal effects of ultrasound is provided by the 1992 report of the National Council on Radiation Protection and Measurements (51).

Any process that can produce a biological effect without a significant degree of heating (i.e., less than about 1°C above physiological temperature) is a nonthermal mechanism. In the case of ultrasound, nonthermal bioeffects are coined "mechanical." However, one should keep in mind that the term "mechanical effect" includes also processes that are not of mechanical nature, but arise secondary to mechanical interaction between ultrasound and tissues, such as chemical reactions initiated by free oxygen species generated during cavitation and sonoluminescence.

Table 1. Selected Mechanical Bioeffects of Ultrasound

	Effects	Comments	Refs.
Cellular level			
Free-radical generation		Intracellular event?	(19)
Permeability	Increased ion conductance		(20, 21)
	Increased permeability to macromolecules		See Sonoporation section
Excitability	Enhancement and suppression		(22)
Agglutination	Erythrocyte agglutination		(23)
Growth	Transient decrease	Delayed cell lysis?	(24)
DNA	Single-strand break		(25)
	Increased sister chromatid exchange	Unconfirmed	(26, 27)
	Mutation frequency		(28)
Cell death			See Sonoporation section
Systemic level			
Cardiac	Premature contractions, decreased aortic pressure		(29, 30)
Capillary	Petechiae	Diagnostic ultrasound with contrast agent	(31, 32)
Vascular	Vasoconstriction, vessel rupture		(33)
Lung	Microvascular hemorrhage		(34–36)
Intestine	Microvascular hemorrhage		(37, 38)
Fetal development	Abnormal head development	Thermal mechanism Only at high pressure	(39)
Neural	Hearing and tactile perception	Radiation pressure effect	(38, 40)
Therapeutic effects			
Thrombolytic	Enhanced thrombolysis		(41, 42)
Antitumoral	Enhanced effect of cytotoxic agents		(43, 44)
Drug delivery	Local drug delivery		(11, 45, 46)
Gene therapy	Enhancement of gene transfer		See Lysis and Sonoporation
Ultrasonic surgery			(47)
Healing	Stimulated bone growth		(48, 49)

The concentration of ultrasonic energy by cavitation bubbles yields a potential for biological effects in their vicinity whenever bubbles or cavitation nuclei are present in a biological medium exposed to ultrasound. Cavitation bioeffects can be confusing and elusive to study owing to the requirement of preexisting gaseous inclusions and to difficult-to-predict cavitation thresholds. In some in vitro situations, cavitation readily occurs at modest pressure amplitudes, because of the presence of cavitation nuclei or suitably sized bubbles. In contrast, in vivo conditions in mammals typically minimize cavitation nuclei (owing to active filtering and sterilization by physiological processes), with the notable

exceptions of the lungs and intestine. Cavitation causes mechanical perturbation in the vicinity of active bubbles, which can lead to membrane effects on individual cells, and to capillary rupture in vivo. The membrane effects range from sonoporation, which is the transient opening of holes in the membrane, to cell lysis or even fragmentation. Sonoporation is the effect of interest in this chapter, because cells exchange molecules with the surrounding medium while the transient holes are open and then survive for further study.

As could be expected, there is a strong influence of tissue characteristics on the bioeffects of ultrasound at any given level of insonation. With respect to mechanical bioeffects, the most important factor seems to be the presence or absence of natural gas bodies. In addition differences between species and differences related to the developmental age have been described (52).

It can be inferred that tissues naturally containing gas–liquid interfaces would be more susceptible to mechanical effects because cavitation depends on the presence of gas bodies. Indeed, microscopic hemorrhage in the lungs and intestine was consistently demonstrated after ultrasound exposure across species (34–37, 55). Several mechanisms have been proposed to explain lung hemorrhage: cavitation in the gas-rich environment in the capillaries adjacent to the alveoli, migration of pockets of air from the lung parenchyma into the pleural fluid and pleural damage upon insonation, shearing of the air–tissue interface, and a variation of the spallation effect (i.e., rapid ejection of liquid into air when a shock wave hits the air–liquid interface) (52).

The occurrence of mechanical effects in tissues that do not naturally contain gas bodies is more difficult to explain. It is well known that cavitation is very difficult to induce in vivo, basically because the filtration system of mammalian organisms allows the presence of cavitation nuclei in only minute amounts, and only in particular sites. This observation concurs with the finding that the cavitation threshold for water doubles upon filtration to 2 μm (56). However, it was hypothesized that in animals, bubbles can form spontaneously in low-surface tension fluids or by exclusion of water from hydrophobic surfaces.

Most of the studies performed with insonation within the low megahertz range (thermal effects are minimized at these frequencies) showed the presence of a threshold for the occurrence of mechanical bioeffects, that approximately coincides with the threshold for cavitation (27). Parameters characterizing ultrasound obviously play a role in the generation of biological effects. Beyond the intuitive role of ultrasound wave intensity, exposure time, and central frequency, more subtle parameters such as type of application (continuous vs. pulsed), pulse repetition frequency (PRF), and duty cycle also influence the ultrasound effects. Clarke and Hill (57) were among the first to study the complex interplay between intensity, mode of exposure, and duty cycle. The threshold relationship between intensity and bioeffects observed with continuous wave exposure held true also when pulsed waves were used. However, the PRF and the duty cycle also played a role in the extent of effects generated.

Biomedical Applications of Ultrasound

Ultrasound and its bioeffects have been studied and exploited in biomedical applications for over 70 years. In diagnostic ultrasound imaging, frequencies from about 2 to 20 MHz are used under conditions of minimal bioeffects. Continuous or pulsed wave beams can detect blood or other motion by the Doppler effect. Pulse-echo systems with carefully focused and scanned beams of ultrasound are utilized for medical imaging in obstetrics, cardiology, and radiology. Typically, pulsed ultrasound has relatively low-time average intensities, leading to relatively low heating. Although the pulses can have relatively

high pressure amplitudes (greater than 2 MPa), the relative lack of cavitation nuclei in vivo leads to relatively low risk of cavitation. A possible exception to this is the recent introduction of contrast agents into diagnostic ultrasound. These agents consist of suspensions of stabilized gas bodies of diameters suitable for passage through the circulation and also for return of strong echoes. Diagnostic ultrasound with contrast agents, therefore, introduces cavitation phenomena into an otherwise high threshold environment, with the potential for new therapeutic (or deleterious) bioeffects. For example, drug delivery to the interstitium can be accomplished by ultrasound-induced capillary rupture when microbubbles are present in the circulation (45).

Therapeutic applications often exploit ultrasound-induced heating. This effect is used for physical therapy of muscle and joint disease, using broad-beam ultrasound applicators, and for surgery, using high-intensity-focused ultrasound (HIFU) (54). Nonthermal effects of ultrasound are also of value. Low-frequency (e.g., 20 kHz) ultrasound probes are used for cavitation-induced lysis and disruption of cells. Focused high amplitude shock waves are used for lithotripsy of kidney and gall bladder stones. These shock waves typically involve single cycle pulses of about 100 kHz ultrasound with negative pressures in excess of 5 MPa. Because of these high pressures, lithotripter shock waves can also cause cavitation in tissue, an unwanted side effect that may be exploited for therapeutic purposes. If cavitation nucleation can be controlled or enhanced in vivo, HIFU and even diagnostic ultrasound also have potential for nonthermal therapy via the cavitation mechanism. These exciting new possibilities for medical therapy have stimulated much recent research on sonoporation and DNA delivery with ultrasound.

Sonoporation

Membrane damage is a well-known biological effect of ultrasonic gas body activation or cavitation (27, 50). The mechanical action of the cavitation bubbles typically causes cell lysis and disintegration. However, sublethal membrane damage also occurs, in which large molecules in the surrounding medium are able to pass in or out of the cell, followed by membrane sealing and cell survival. This allows foreign macromolecules to be trapped inside the cell. This ultrasound-mediated increase in cell membrane permeability has been termed "sonoporation," a term that recognizes some similarity to electroporation. It should be noted that sonoporation represents transient permeabilization, which can be indicated by trapping large fluorescent molecules inside the viable cells (the molecules are excluded by viable cells and leak out of nonviable cells), and is different from the commonly noted permeabilization indicated by trypan blue or propidium iodide stains, which stain lysed, nonviable cells.

Sonoporation was initially demonstrated using 20 kHz sonication (58), although this finding was not immediately noted as a new ultrasound bioeffect (it was intended as a method for intracellular pH measurement). Ameboid mold cells were sonicated in the presence of large fluorescent Dextran molecules. Cell recovery was about 40% of the original number, and 10% of these were loaded with the large molecules. Sonication treatment (20 kHz) has also been used to load mammalian cells with fluorescent Dextran (59). Ten to 20% of sonicated hepatoma, mouse myeloma, HeLa, and fibroblast cell lines were found to be fluorescent after sonication with 10 mg/mL of 40 kD fluorescent Dextran. In another study using 20 kHz ultrasound, Johannes and Obe (60) showed that sonication of cultured CHO cells in the presence of endonuclease enzymes led to the production of chromosome aberrations in about 20% of subsequently cultured cells. Without sonication, few cells internalized the enzymes or had aberrations, indicating that the enzymes entered the cells via sonoporation.

Following the development of lithotripsy, research on lithotripsy-mode ultrasound quickly broadened to encompass a variety of biological effects (61). An important factor found for shock wave treatments was that some cells were transiently permeabilized, allowing them to take up large molecules normally excluded by the cell membrane. In an in vitro study, Gambihler et al. (62) evaluated the accumulation of fluorescein-labeled Dextran (3900–2,000,000 molecular weight) in L1210 cells exposed in the presence of the Dextran. The large molecules, normally excluded by the cell membrane (except for pinocytosis into vacuoles), were found in surviving cells with a diffuse distribution within the cytoplasm. The sonoporation effect of lithotripter shock waves may be valuable for selectively treating tumors with large toxic molecules (63). The ribosome inactivating proteins gelonin and saporin were apparently transferred into L1210 mouse leukemia cells and HELA cells by shock wave exposure, resulting in greatly enhanced cell killing. These plant toxins are also usable in vivo, and significant remission of SSK2 fibrosarcoma tumors was obtained in 40% of mice treated by systemic injection of the toxins and localized shock wave treatment.

Sonoporation of red blood cells was examined in relation to cavitation-induced hemolysis by Miller et al. (64) in whole blood, which simulates in vivo conditions but allows the convenient logistics of in vitro experiments. Fluorescent-labeled Dextran (FITC-dextran at 580,000 MW) was added to suspensions of canine erythrocytes, and the mixture was exposed to lithotripter shock waves of 14.8 MPa mean pressure amplitude. Exposure at 5 or 50% hematocrit in PBS or 50% in plasma yielded hemolysis and FITC-Dextran uptake in surviving cells. An air bubble was needed in the chamber to obtain substantial effects, implicating the cavitation mechanism. Hemolysis increased with increasing numbers of shock waves. The numbers of cells with fluorescent Dextran uptake remained roughly constant for 250–1000 shock waves, but this represented an increasing percentage of the surviving cells. These interesting exposure–response trends could be modeled by simple theory for random interaction of the cells with bubbles. The number of cells S, of the original number S_0, surviving n shock waves declines exponentially at a rate of a per shock wave, is given by

$$S = S_0\, e^{-an}. \tag{1}$$

The number of fluorescent cells F arising at a rate b from the surviving cells, and lysed at rate a, is then given by

$$F = S_0\, e^{-an}(1 - e^{-bn}). \tag{2}$$

This theoretical model has the interesting property that 100% of the survivors will eventually become loaded with fluorescent molecules as n increases toward infinity. This theory was fitted to actual measurements (64) as shown in Fig. 1. About one cell became fluorescent for every three cells lysed. The percentage of survivors that were fluorescent, plotted in the lower panel of Fig. 1, was calculated as F/S times 100%.

Sonoporation by shock waves has also been demonstrated by Zhong et al. (65). Mouse lymphoid cells were treated in the presence of fluorescent Dextran, and the percentage of transiently permeabilized cells reached 11.2% after 100 shock waves. The effects were associated with acoustic emissions indicative of cavitation. High-speed photography was used to show creation and collapse of bubbles with maximum sizes in the range of 100–200 μm, which emitted shock waves upon creation and collapsed up to 1 ms later.

The exact process by which cavitation causes transient permeabilization of cell membranes may be related to cavitation-generated stress waves. Laser pulses produce bubbles

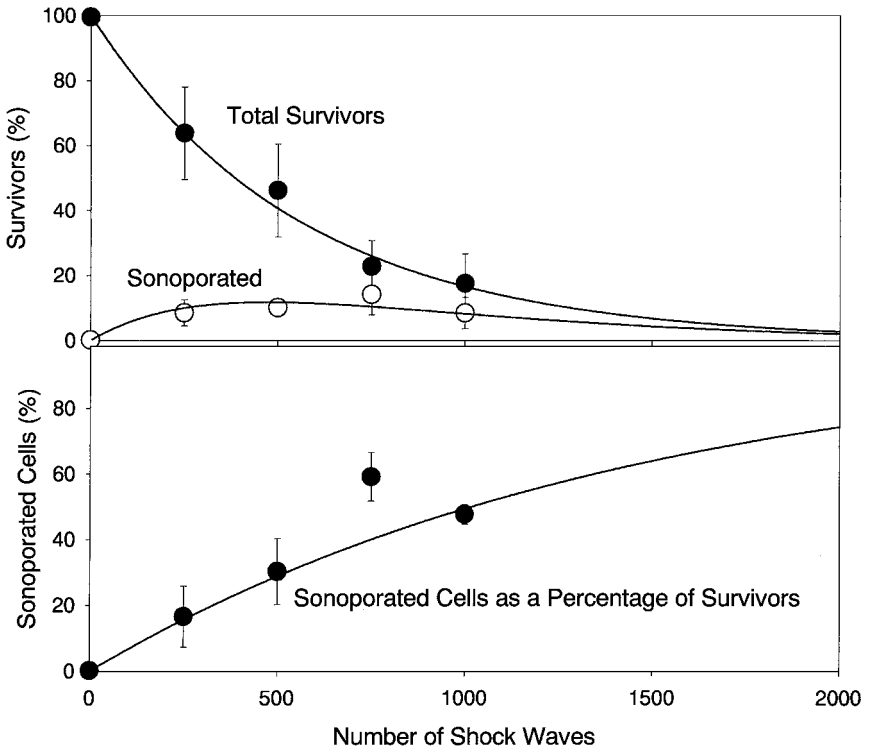

Fig. 1. The percentage of erythrocytes lysed and sonoporated (upper panel) for lithotripter shock wave treatment of whole blood with added fluorescent Dextran. The lower panel shows the percentage of sonoporated survivors. The plotted curves represent a theoretical model of the process, which was fitted to the data (see text). Reproduced with permission from Miller et al. (64).

by optical breakdown of the medium, and the resulting generation of ultrasonic stress waves has been shown to transiently permeabilize cell membranes (66). In studies of this effect in red blood cells (erythrocytes), an important role was demonstrated for aquaporins (water channels) in the membranes (67). Human erythrocytes, which have aquaporins, showed uptake of fluorescent Dextran after stress wave treatment, while chicken red blood cells, which do not have aquaporins, did not. If the channels were blocked in human erythrocytes, the stress wave induced uptake of fluorescent Dextran was eliminated. Another important factor is the gradient (or rise time) of the stress waves (68). Stress wave gradient was varied by applying ArF excimer or ruby laser pulses to polyamide or polystyrene targets, and transient permeabilization was observed by fluorescent Dextran uptake in cells resting on the target material. An 8-ns rise time was much more effective for cell loading than a 40-ns rise time for about the same peak stress (about 60 MPa). A single laser-generated stress wave was sufficient to achieve maximal cell loading of the large molecules.

A common exposure system used for in vitro research on cavitation bioeffects is the rotating tube system. Cells in suspension are affected by bubbles, which cycle back and forth across the tube as it rotates. Sonoporation of cultured cells has been demonstrated with this system using fluorescent Dextran as the test macromolecule (8). Chinese hamster ovary cells were exposed to 2.25 MHz ultrasound in sterile 4.5-ml polyethylene chambers

and tested for cell lysis, sonoporation, and DNA transfection. Ten percent of Albunex® (Mallinckrodt Inc., St. Louis, MO), a gas-body-based ultrasound contrast agent, was added to assure cavitation nucleation, and the chambers were rotated at 60 rpm to promote cavitation activity during the 1-min exposures. Sonoporation was observed for spatial peak pressure amplitudes as low as 0.1 MPa, and significant lysis occurred above 0.2 MPa. Up to half of the surviving cells were fluorescent after exposure at 0.8 MPa. The effects essentially disappeared when the Albunex® was omitted from the suspension and the tube was not rotated. The ultrasonic frequency has an important role in sonoporation (69). After continuous wave exposure at frequencies of 1.0, 1.68, 2.25, 3.3, 5.3, and 7.15 MHz, sonoporation was noted for spatial peak pressure amplitudes as low as 0.1 MPa up to 3.3 MHz, increasing to 0.39 MPa at 7.15 MHz. Significant lysis occurred for 0.14 MPa exposures at 1.0 MHz, but not for the other frequencies with the relatively low-pressure amplitudes of exposure used. The fluorescent cell count increased roughly in proportion to increasing Albunex® concentration. The sonoporation effect appears to be a form of membrane damage, and although the cells exclude trypan blue dye immediately after exposure, they have poor proliferation ability in culture. The plating efficiency of cells exposed to 0.28 MPa at 2.25 MHz and sorted by a flow cytometer were only 19% (3.6% SD) for fluorescent cells, compared to 67% (1% SD) for non-fluorescent-exposed cells and 62% (6% SD) for sham-exposed cells.

Ward et al. (70) observed ultrasound induced cell lysis and sonoporation for 2 MHz, 0.2 MPa exposure of cell suspensions in a 200 rpm rotating tube system. Addition of ultrasound contrast agent was essential for robust effects. Optison™ (Mallinckrodt Inc., St. Louis, MO), an improved agent with greater persistence owing to the use of perfluoropropane gas, provided much greater enhancement of effects than did Albunex®, which contains air. In further work with Optison™ (71), the bubble to cell spacing appeared to have an important role in the magnitude of effects. The spacing was inferred from a static model (i.e., neglecting ultrasonic forces on the bubbles or between cells and bubbles) for different cell and bubble concentrations. The percentage of affected cells declined with concentration, which was approximately as the inverse third power of the static spacing.

Sonoporation has also been demonstrated for monolayer cells (i.e., a nonrotating system) in contact with Optison™ gas bodies for treatment by 3.5 MHz diagnostic ultrasound (72). Epidermoid carcinoma cells were grown on thin Mylar sheets, which formed the upper window of a thin (1 mm) disc-shaped chamber. Fluorescent Dextran and Optison™ were added to the medium, and the gas bodies were allowed to rise to the cells before exposure. Up to about 10% of the cells in a 1-mm field of view were fluorescent, indicating sonoporation, after 1-min exposure in the spectral Doppler mode or two-dimensional scan mode. Pressure amplitudes of 0.23 MPa in Doppler mode (5 μs pulses) and 0.39 MPa (0.46 μs pulses) in scan mode were about equally effective. This finding indicates that even diagnostic ultrasound might be useful in therapeutic applications of sonoporation.

DNA Delivery by Sonoporation

The ability of ultrasound to load cells with large molecules, which survive for subsequent culture, opens the possibility of DNA transfection and expression of foreign gene products. The earliest work on this method was performed in vitro and in plant cells. Recent research has expanded to include the possibilities for gene therapy in mammalian cells by sonoporation in vivo.

DNA Delivery in Vitro

Sonication treatment (20 kHz) has been used to transfect cells with plasmid DNA (59). Murine fibroblasts lacking the gene for the production of the enzyme thymidine kinase were used as the target cells for "therapy" with a plasmid (pPVTK4) coding for the enzyme. After sonication treatment in the presence of the plasmid, survival was about 70%. Transfection was demonstrated by culture with medium containing hypoxanthine, aminopterin, and thymidine (HAT) to select against colonies lacking the thymidine kinase gene. An average of 23 transformed colonies was counted per million viable cells, and repeated passage of selected cells indicated that some stable transfection had occurred.

Sonication of plant cells also results in loading of DNA plasmids into the cells. Joersbo and Brunstedt (73, 74) found that plasmid DNA could be transferred into sugar beet and tobacco protoplasts by 20 kHz sonication as evidenced by chloramphenicol acetyltransferase marker gene expression. Furthermore, viruses could also be loaded into cells by this method (75). Sugar beet protoplasts were sonicated with beet necrotic yellow vein virus particles. Infection was analyzed by ELISA assay of the virus protein coat and was maximal about 3 days after inoculation. Zhang et al. (76) reported transient expression of β-glucuronidase in tobacco leaf segments after sonication with the marker gene plasmid. Stable transfection was obtained with salmon sperm DNA as a carrier for sonication with the plasmid, but not in controls with plasmid or sonication alone. Wyber et al. (77) reported a 20-fold enhancement of transfection for 20 kHz sonication of yeast cells together with LEU2 reporter gene plasmids plus salmon sperm DNA (as a carrier DNA) relative to controls. Cavitation activity was measured by the iodine release assay, and correlated with loss of cell colony forming units (viability).

Since sonication treatment at 20 kHz is not commonly used in medicine (except in certain surgical procedures such as liposuction), the potential for DNA transfer with medically relevant MHz frequency ultrasound is of considerable interest. Kim et al. (7) developed an exposure system with 1 MHz ultrasound delivered into culture flasks and plates. Primary fibroblasts from rat hind limb muscles were cultured and exposed with a β-galactosidase control vector or with a plasmid (pMC1neo poly(A)) containing an antibiotic resistance gene. Variations in transfection efficiency were found with temperature, pressure amplitude above 0.2 MPa, exposure duration, and on–off timing. Highest transfection rates were found for about 0.4 MPa and 20 s exposures, with about 50% survival. Stable transfection rates averaged 0.34% of surviving cells. Treatment at 3.5 MHz resulted in no DNA transfer, which implicates cavitation-induced sonoporation as the mechanism. The involvement of cavitation was further demonstrated by augmenting nucleation with Albunex® ultrasound contrast agent (10). A human condrocyte cell line was transfected with a marker plasmid coding for the green fluorescence protein (GFP) using 1 MHz ultrasound transmitted into culture dishes. Scoring of fluorescent cells was accomplished after cell harvest by flow cytometry. Transfection increased above an apparent threshold of 0.12 MPa (spatially averaged over the culture dish) and reached about 50% of viable cells at 0.41 MPa for 20 s (Fig. 2).

Seeding of the medium with 10% gas-body-based ultrasound contrast agent was required to promote transfection of CHO cells at low amplitudes in the rotating tube exposure system, clearly demonstrating that the transfection was mediated by ultrasonic cavitation (8). Luciferase reporter plasmid at 20 μg/mL was added to the suspension during exposure, and cells were assayed for proliferation ability and for luciferase gene expression 2 days after exposure. Cell proliferation was greatly reduced above the cavitation threshold. Luciferase production was significant for 0.20 MPa exposure, and reached 0.33 ng per 10^6 cells at 0.8 MPa exposure. The luciferase production was greater for

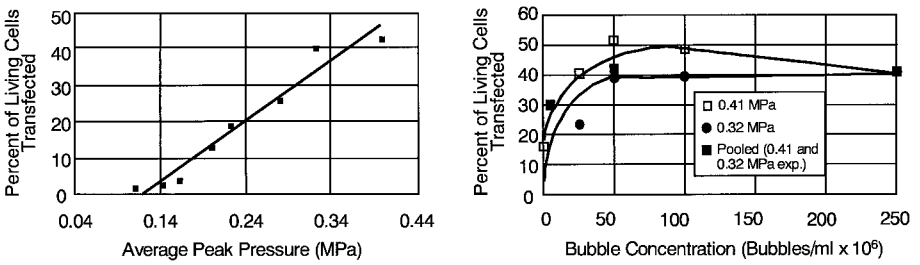

Fig. 2. Transfection rate of living cells after ultrasound exposure as a function of average peak pressure of the 20-s burst of 1 MHz ultrasound (left panel). Transfection rate of living cells after exposure plotted against Albunex concentration at the time of exposure (right panel). Reproduced with permission from Greenleaf et al. (10).

cells exposed in medium supplemented with serum than for cells exposed in serum-free medium.

Tata et al. (9) examined GFP marker plasmid transfer in two human cancer cell lines LnCap and PC-3. Ultrasound treatment was similar to that used in physical therapy with 932 kHz frequency and a 20% duty cycle. The ultrasound beam was directed upward into culture dishes with an average intensity of 0.33 W cm^{-2}. Loss of cell viability was greatest for continuous or low pulse repetition frequency (PRF) exposure, but remained near control levels for PRFs above 500 Hz. Similarly, as indicated by fluorescent-labeled plasmids in viable cells, the uptake of the DNA was greatest from continuous to 200 Hz PRF. Gene expression followed a similar pattern, with maximal expression for 50–150 Hz PRF, and no increase in GFP expression for a clinically relevant PRF of 1 kHz. This response pattern likely reflects the variation in occurrence and amount of cavitation activity in this system.

Lithotripter shock waves are also effective for in vitro transfection of cell suspensions. Expression of β-galactosidase gene in a reporter plasmid was observed in 0.1–0.5% of viable plated cells after 48 h (78). The expression increased with increasing concentration of DNA during exposure and with increasing numbers of shock waves. The effect was suppressed by application of 10 MPa overpressure, which is a positive test for cavitation. The method was applicable to many different cultured cell lines including HeLa cells, mouse L-M fibroblasts, monkey kidney cells, and L1210 mouse leukemia cells. The GFP reporter plasmid was utilized by Miller et al. (79) to show the numbers of cells transfected by shock waves in vitro. A plasmid solution was added to the B16 melanoma cells in suspension at 20 μL mL^{-1} and 0.2 mL of air was retained in the 1.2-mL exposure chambers. After 800 SW in vitro, cell counts after 2 days of culture were only 5.3% of shams, with 1.4% of the cells expressing GFP. An important feature of the in vitro results was that the trends for transfection appeared to follow the same favorable trends noted previously for fluorescent Dextran loading of erythrocytes (64).

The transfection efficacy of lithotripter shock wave treatment was compared to 1.18 MHz focused ultrasound exposure by Huber et al. (80). While lithotripter shock waves induced up to an eightfold increase in marker gene expression in the remaining 5% viable cells (relative to controls), focused ultrasound induced up to an 80-fold increase at 45% viability. Viability decreased and DNA transfer increased for increasing pressure amplitude up to 5 MPa at 40% duty cycle, and increasing sonication time up to 10 min. For varying PRF, 100 Hz appeared to be most effective. These results suggest that focused

ultrasound could compete with lithotripter shock wave treatment in ultrasonically aided gene transfection, although in vitro results may not be faithfully reflected for in vivo conditions. Huber and Pfisterer (81) examined this question using 1.18 MHz focused ultrasound on prostate tumor cells in vitro and in vivo (see below for in vivo results). In vitro, 1 MPa exposures with 4 ms burst duration and 100 Hz PRF produced 55- to 220-fold increases in β-galactosidase expression in different cell lines including human melanoma and Dunning rat prostate tumor cell lines.

Ultrasound might be useful for gene therapy in cardiology applications. Lawrie et al. (82) cultured porcine vascular smooth muscle cells and endothelial cells and performed luciferase marker plasmid transfer with naked or lipid-charged DNA with or without 1 MHz ultrasound exposure of the monolayers. The transfection period was 3 h in duration, while ultrasound was applied at 0.4 W cm^{-2} only for 60 s, 30 min into the transfection period. The ultrasound application enhanced subsequent luciferase expression by 7.5 times for naked DNA, 2.4 times for lipid-charged DNA in the muscle cells and 3.3 times for lipofection of endothelial cells. Substantial heating, briefly reaching 51°C, was produced by the ultrasound but simulation of this heating had no effect on the subsequent luciferase expression. However, the ultrasound, but not the heating alone, induced a reduction in cell proliferation, which implicates the cavitational mechanism in the results. This implication was confirmed in experiments with 10% added Albunex® or Optison™ ultrasound contrast agents (13). Up to 300-fold enhancement over naked DNA transfection was obtained for 956 kHz ultrasound. High-pressure amplitude exposure (about 2 MPa) was further associated with hydrogen peroxide production, which is a test for inertial collapse of the bubbles with free-radical production. However, free-radical generation is not required for transfection, since the hydrogen peroxide production was eliminated for lower exposure at about 1 MPa, for which the transfection efficiency was retained. The addition of Optison™ also enhanced lipofection (Tfx-50 lipoplexes) and polyplexes (LT-1) of the smooth muscle cells.

Manome et al. (83) have examined transfer of naked DNA into tumor cells in vitro and in vivo using 1 MHz continuous ultrasound (see below for in vivo results). In vitro, 30-s ultrasound treatment enhanced β-galactosidase and antibiotic-resistance reporter gene expression in mouse adenocarcinoma cells both in transient and stable transfection assays. Stable transfection for 10^5 cells cultured for 10 days with geneticin selection averaged 34 cells for 30-s ultrasound exposure.

A clinical spectral Doppler ultrasound system, as well as a laboratory system, was used by Koch et al. (84) to enhance transfection by cationic liposomes. The GFP reporter plasmid was used with rodent and canine glioma cells. For the clinical system, exposure was performed at 2 MHz, 0.5 W cm^{-2} average intensity in 12 well culture plates, and fluorescent cell counts made 24 h later. Up to 32.7% transfection was obtained for 90-s exposure, compared to 7.4% in controls. For the laboratory system, cells were grown on polypropylene membranes and exposed in a chamber with an ultrasound absorber to simulate free-field exposure conditions. If exposures were performed at 0.6 MPa pressure amplitude which did not produce cavitation (indicated by a lack of subharmonic emission), then no effects were found on viability or transfection. However, if Levovist® (Schering AG, Berlin) ultrasound contrast agent was added to the exposure chamber at 2–200 μg/mL, significantly increased transfection was observed. In addition, cell viability was reduced, for example, to 39.3% for the higher contrast agent concentration. Although the contrast agent was not used with the spectral Doppler system, it seems likely that cavitation activity was responsible for the observed effects.

DNA Delivery in Vivo

The observation of transient permeabilization by shock waves leads to a suggestion by Delius et al. (85) that extracorporeal shock wave lithotripsy might be useful for gene therapy. On the basis of the in vitro results at higher frequencies, and even diagnostic ultrasound with contrast agents, many forms of ultrasound appear to be capable of DNA transfer to varying degrees depending on the cavitation activity obtained. However, it is well known that cavitation activity is minimized in vivo relative to in vitro conditions. Therefore, actual in vivo testing is required to accurately assess the potential of ultrasound-enhanced gene therapy.

Kim et al. (7) tested the possible extension of their in vitro experiments to in vivo conditions. The β-galactosidase reporter plasmid was injected into both knee joints of rats, and one joint was treated with 1 MHz plus 30 kHz ultrasound at 0.4 MPa and 40 kPa, respectively, for 1 min. Three of four treated knees showed reporter gene expression after 4 days, while none was detected in the unexposed joints, as shown in Fig. 3.

In vivo testing of the potential for enhanced cancer gene therapy by lithotripter shock wave exposure was performed using the luciferase marker plasmid in the B16 mouse melanoma tumor by Bao et al. (86). Luciferase reporter vector was injected with a concentration of 2 mg mL^{-1} at 10% of the tumor volume. Air at 10% of tumor volume was also injected after the DNA in some tumors to enhance acoustic cavitation activity. Shock waves were applied at 5.2 MPa peak negative pressure amplitude to the tumors of mice placed in a waterbath to assure free-field conditions. Exposure to 800 shock waves, followed by immediate isolation and culture of tumor cells for 1 day, yielded 1.1 (0.43 SE) pg/10^6 cells for plasmid injection only and 7.5 (2.5 SE) pg/10^6 cells for plasmid plus air injection. Significantly increased luciferase production, relative to shams, occurred for 200, 400, 800, and 1200 shock wave treatments with plasmid and air injection. Exposure with the isolation of tumor cells delayed for a day to allow gene expression within the growing tumors gave increased luciferase production for 100 and 400 shock wave exposure without and with air injection. For immediate harvest, expression was enhanced roughly 15-fold relative to direct injection alone, and air injection gave a further sevenfold increase. For delayed isolation, a 350-fold enhancement was found for 400 SW plus air compared to simple direct injection. The GFP reporter plasmid was utilized to elucidate the numbers

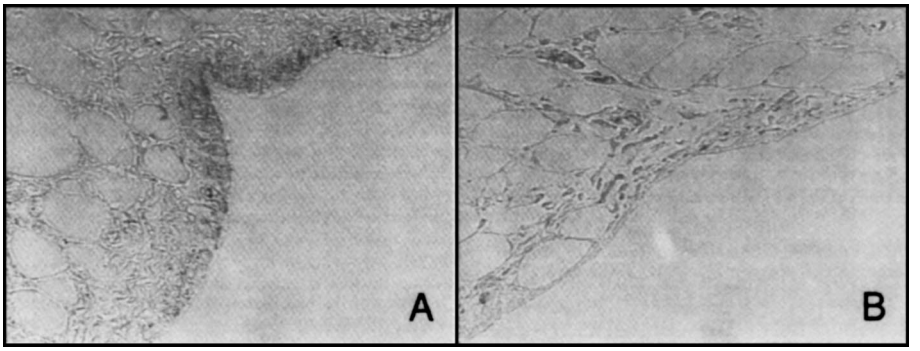

Fig. 3. Photomicrograph of rat knee joints after injection of a β-galactosidase plasmid and exposure to ultrasound in the presence of the contrast agent Albunex. Three out of four exposed knees showed β-galactosidase expression (A), while no expression was seen in control, unexposed (B) joints. Reproduced with permission from Kim et al. (7).

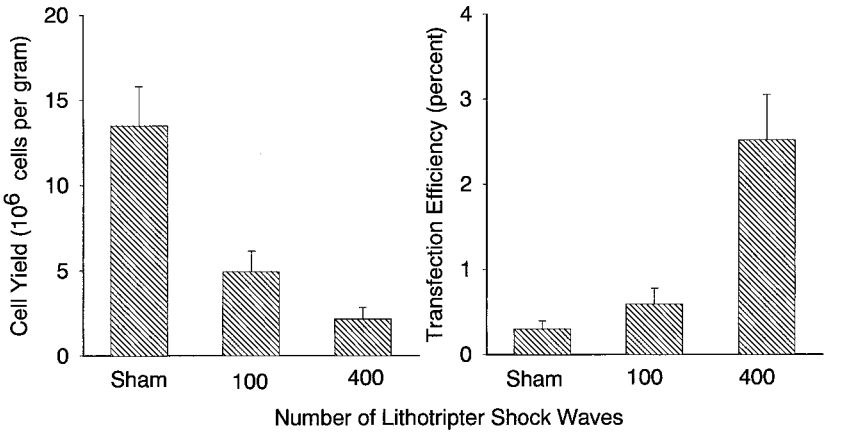

Fig. 4. Results for GFP transfection by lithotripter shock waves plus injected air in B16 mouse melanoma tumors. Cell yield after 2 days decreased with increasing exposure, but the percentage of the recovered viable cells which were fluorescent increased. Reproduced with permission from Miller et al. (79).

of B16 melanoma tumor cells undergoing transfection (79). The plasmid solution was injected intratumorally at 0.2 mg mL^{-1} DNA per milliliter of tumor and air was injected at 10% of tumor volume in vivo. For 400 SW exposure, viable cell recovery from excised tumors was reduced to 4.2% of shams and cell transfection was enhanced by a factor of about 8, reaching 2.5% of viable cell counts ($P < 0.005$ in t test). These results indicate tumor ablation plus enhancement of transfection for ultrasound SW treatment, as shown in Fig. 4.

Huber and Pfisterer (81) evaluated 1 MHz focused ultrasound enhancement of transfection of the β-galactosidase reporter plasmid into Dunning prostate tumors implanted in rats. Rats were exposed in a water tank to 1 MPa burst mode ultrasound (4-ms duration, 100 Hz PRF) after injection of 10 μg of the plasmid DNA. Staining of sections of exposed tumor revealed areas of β-galactosidase positive (blue stained) cells. DNA injection plus ultrasound produced a 10-fold increase in positive cells compared to intratumoral DNA injection alone, and a 15-fold enhancement in β-galactosidase protein assayed by ELISA. No transfection of tumor cells was obtained for intravenous injection of 100 μg of plasmid DNA with or without ultrasound exposure.

Manome et al. (83) also followed up on in vitro results with in vivo testing in colon carcinoma tumors implanted in mice. The tumors were injected with 200 μg β-galactosidase reporter plasmid and exposed to 1 MHz ultrasound at 10–20 W cm^{-2} 10 min later. Relative β-galactosidase activity 48 h after treatment increased approximately threefold for ultrasound, and was higher at the higher intensity. Expression peaked for 30-s exposure and declined for longer exposures up to 2 min. The mechanism of the enhanced transfection was not examined, but the intensities employed were sufficient to induce some cavitation activity at the injection site.

Ultrasound treatment appears to enhance transfection of tumor cells by cationic lipids in vivo (87). Without ultrasound, most transfection obtained with CAT reporter or interleukin-12 coding plasmids complexed to cationic liposomes occurred in the lungs. For ultrasound treatment of the tumor before or after intravenous injection of the plasmid complex resulting in 3- 270-fold enhancement of gene transfer to the tumor, depending on conditions. The tumor DNA delivery increased with increasing DNA dose, and

increasing exposure duration. The effect was maximized for ultrasound treatment up to 1.5 W/cm^2 about 1 min after injection of the DNA complexes. By the immunostaining of histological sections taken 15 min after treatment, the DNA delivery was localized to the endothelium within the tumor. The ultrasound treatment was termed sonoporation; however, the mechanism for this in vivo effect is not completely clear, because no cavitation enhancement was employed.

Ultrasound of relatively low power (2 W cm^{-2} at 1 MHz for 5 min) has been applied to subcutaneous mouse tumors for use in therapy (88). The treatment enhanced the effectiveness of chemotherapeutic bleomycin treatment and also the delivery of DNA plasmids to the tumor tissue. This ultrasound treatment was less effective for transfer and expression of a luciferase marker plasmid than was an electroporation procedure. It should be noted that the ultrasound was applied without added cavitation nucleation agents, and that the electroporation system required insertion of 10-mm wide steel strips into the tissue on two sides of the tumors, which complicates the interpretation of this comparison.

In vivo cardiovascular applications of sonoporation were also recently reported. Several studies addressed the possibility of preventing vascular proliferation (and thus of restenosis after angioplasty). In a porcine model of coronary artery injury, intravenous administration of an antisense nucleotide to *c*-myc in conjunction with PESDA (perfluorocarbon-exposed sonicated dextrose albumin) was associated with an apparent reduction in the degree of proliferation after 4 weeks (89). The results are difficult to explain, since the addition of ultrasound at 1 MHz for 30 or 180 s did not induce an antiproliferative effect. These findings could eventually be explained by additional injury due to cavitation in those vessels that were insonated. However, the study opens the interesting possibility of enhanced transfer of oligunucleotides via echographic contrast agents alone.

Two other recent reports suggest a role for sonoporation in the prevention of restenosis. In a rat model of carotid artery injury, Taniyama et al. (17) showed a decrease in neointima formation after delivery of the antiproliferative gene p53 in combination with 25% Optison and insonation at 1 MHz for 2 min. Two weeks after delivery, treated vessels showed significantly lower intima to media ratio in comparison with both a negative control and p53 plasmid alone. Yamasaki et al. (15) have shown also in a rat carotid artery injury model reduced proliferation after administration of a decoy for the transcription factor E2F in conjunction with Optison (25%) and insonation at 1 MHz for 2 min. It should be emphasized, however, that these are preliminary results, and further studies are needed to confirm the findings.

A totally different approach to insonation was taken by Amabile et al. (18). In a rabbit model of femoral artery injury, plasmid-mediated transfection of blue fluorescent protein was enhanced by intravascular ultrasound at 2 MHz. Interestingly enough, the combination of plasmid and ultrasound was associated with higher expression of the reporter gene when compared even to adenoviral-based transfection.

Gene delivery to the myocardium of rats was obtained with harmonic mode diagnostic ultrasound, a microbubble contrast agent and a viral β-galactosidase vector (14). The contrast agent was prepared in the laboratory and processed with the vector to attach the virus particles to the microbubbles. For exposure, 2 mL of this preparation was infused over a 30-min period via the jugular vein. Three frames from a 1.3 MHz transducer destroyed the microbubbles evident in the second-harmonic image, and three frame bursts were triggered to allow refill of the tissue between scans. After 4 days, expression of the reporter gene was assayed in histological sections and by measurement of enzyme activity. Staining and enzyme activity was detected in the myocardium after

echocardiographic destruction of the microbubbles mixed with the viral vector at about 10 times the levels found in controls (bubbles plus ultrasound, no vector; bubbles plus vector, no ultrasound; vector alone, no bubbles, no ultrasound). β-Galactosidase activity was found in the livers of all animals receiving the viral vector. Cavitation activity was clearly responsible for the effect because the procedure involved destruction of contrast agent microbubbles; however, it is uncertain whether the viral vector was delivered by mechanical sonoporation or by some other process. Echocardiographic microbubble destruction followed by vector infusion produced about twice the gene expression of controls, suggesting that disruption of the endothelial barrier during microbubble destruction might be a factor in the enhanced viral transduction.

Conclusions and Prospects

Ultrasound causes perturbation of cell membranes through the interaction of cavitation bubbles with the cells. This can lead to lysis or to sonoporation, in which permeabilized cell membranes heal and allow the cell to survive. Sonoporation appears to be injurious to the cells, but affords an opportunity to deliver large molecules from the extracellular medium to the cytoplasm. Sonoporation is conveniently studied using large fluorescent Dextran molecules, with DNA delivery a special case. Although cavitation activity generally decreases for increasing ultrasound frequency, sonoporation has been demonstrated for 20 kHz ultrasonic probes, for lithotripter shock waves and even for megahertz frequency ultrasound as used for medical therapy. With augmentation of cavitation activity by adding gas-body-based contrast agents to the exposure medium, sonoporation and DNA delivery has been produced by diagnostic ultrasound in cell suspensions and in monolayer culture systems. In vivo situations typically show minimal cavitation activity due to a dearth of cavitation nucleation sites; however, by augmenting nucleation with the inclusion of injected air or microbubble contrast agents, sonoporation and DNA delivery has been shown in several tumor models and in normal tissues.

A clear need in gene therapy is for novel vector delivery methods. Several gene delivery methods are now available, including direct plasmid injection into tumors, DNA-lipid conjugates, and viral vectors, but none appears to be fully developed nor does any one method have universal applicability. Viruses yield high transfer efficiency, but are immunogenic, or even mutagenic, and infect many nontumor cells (90, 91). The efficiency found for DNA lipid conjugates in vitro does not necessarily improve efficiency in vivo over simple injection of naked DNA (92). Direct plasmid injection is appealing for its simplicity and avoidance of viruses, but has yielded a low level of transfection in tumors (86, 93). Other novel methods include "gene gun" delivery of DNA on particulate projectiles (94), and in vivo electroporation with implanted electrodes (95).

This review has detailed the initial progress by several researchers toward development of ultrasound-enhanced gene therapy. Studies have shown ultrasound enhancement of DNA delivery over direct injection, lipofection, and even over viral transfection. Theoretically, sonoporation could help deliver genes with high spatial and temporal specificity. The importance of selective targeting in gene therapy cannot be overemphasized, considering the risks associated with systemic delivery of genetic vectors at high doses. Indeed, an effect that is desirable in one region (e.g., induction of apoptosis in tumor cells) is potentially fatal if generalized to other organs.

The development of "smart" bubbles that can recognize and attach to specific targets might enhance the spatial resolution even further, providing a real "ZIP code based" delivery of genetic material. In addition, ultrasound-induced bioeffects beyond sonoporation could enhance the effects of gene therapy within a single treatment modality. Delivery of DNA

outside blood vessels by capillary rupture might be valuable to target cells of a specific tissue. In cancer gene therapy, the concomitant tumor ablation with lithotripter exposure might provide augmentation of a gene mediated antitumoral therapy. Further research should elucidate the potential of the ultrasound enhanced DNA delivery and identify the most promising therapeutic applications.

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Literature Cited

1. Nabel EG. Circulation 1995; 91:541-548.
2. Schneider MD, French B. Circulation 1993; 88:1937-1942.
3. Gershon H, Ghirlando R, Gutman SB et al. Biochemistry 1993; 32:7143-7151.
4. Chang DC, Reese TS. Biophys J 1990; 58:1-12.
5. Daniell H. Methods Enzymol 1993; 217:536-557.
6. Newman CM, Lawrie A, Brisken AF et al. Echocardiography 2001; 18:339-347.
7. Kim HJ, Greenleaf JF, Kinnick RR et al. Hum Gene Ther 1996; 7:1339-1346.
8. Bao S, Thrall BD, Miller DL. Ultrasound Med Biol 1997; 23:953-959.
9. Tata DB, Dunn F, Tindall DJ. Biochem Biophys Res Comm 1997; 234:64-67.
10. Greenleaf WJ, Bolander ME, Sarkar G et al. Ultrasound Med Biol 1998; 24:587-595.
11. Mukherjee D, Wong J, Griffin B et al. J Am Coll Cardiol 2000; 35:1678-1686.
12. Porter TR, Iversen PL, Li S et al. J Ultrasound Med 1996; 15:577-584.
13. Lawrie A, Briskin AF, Francis SE et al. Gene Ther 2000; 7:2023-2027.
14. Shohet RV, Chen S, Zhou Y et al. Circulation 2000; 101:2554-2556.
15. Yamasaki K, Tachibana T, Taniyama Y. Circulation 2000; 102:II-247.
16. Komamura K, Tatsumi R, Miyatake K. Circulation 2000; 102:II-561.
17. Taniyama Y, Tachibana K, Yamamoto K. Circulation 2000; 102:II-164.
18. Amabile PG, Waugh JM, Lewis TN et al. Circulation 2000; 102:II-147.
19. Fu YK, Kaufman GE, Miller MW et al. Radiat Res 1979; 80:575-580.
20. Mortimer AJ, Dyson M. Ultrasound Med Biol 1988; 14:499-506.
21. Dinno MA, Crum LA, Wu J. Ultrasound Med Biol 1989; 15:461-470.
22. Mihran RT, Barnes FS, Wachtel H. Ultrasound Med Biol 1990; 16:297-309.
23. Pohl EE, Rosenfeld EH, Pohl P et al. Ultrasound Med Biol 1995; 21:711-719.
24. Kaufman GE, Miller MW. Ultrasound Med Biol 1978 4:139-144.
25. Miller DL, Thomas RM, Buschbom RL. Ultrasound Med Biol 1995; 21:841-848.
26. Liebeskind D, Bases R, Mendez F et al. Science 1979; 205:1273-1275.
27. Miller MW, Miller DL, Brayman AA. Ultrasound Med Biol 1996; 22:1131-1154.
28. Kaufman GE. Ultrasound Med Biol 1985; 11:497-501.
29. Dalecki D, Keller BB, Raeman CH et al. Ultrasound Med Biol 1993; 19:385-390.
30. MacRobbie AG, Raeman CH, Child SZ et al. Ultrasound Med Biol 1997; 23:761-765.
31. Skyba DM, Price RJ, Linka AZ et al. Circulation 1998; 98:290-293.
32. Miller DL, Quddus J. Proc Natl Acad Sci USA 2000; 97:10179-10184.
33. Hynynen K, Chung AH, Colucci V et al. Ultrasound Med Biol 1996; 22:193-201.
34. Child SZ, Hartman CL, Schery LA et al. Ultrasound Med Biol 1990; 16:817-825.
35. Tarantal AF, Canfield DR. Ultrasound Med Biol 1994; 20:65-72.
36. Zachary JF, O'Brien WD, Jr. Vet Pathol 1995; 32:43-54.
37. Miller DL, Thomas RM. Ultrasound Med Biol 1995; 21:249-257.
38. Dalecki D, Child SZ, Raeman CH et al. J Acoust Soc Am 1995; 97:3165-3170.
39. Ramnarine KV, Nassiri DK, McCarthy A et al. Ultrasound Med Biol 1998; 24:575-585.
40. Tsirulnikov EM, Vartanyan IA, Gersuni GV et al. Ultrasound Med Biol 1998; 14:277-285.
41. Nilsson AM, Odselius R, Roijer A et al. Ultrasound Med Biol 1995; 21:833-840.

42. Tachibana K, Tachibana S. Circulation 1995; 92:1148.
43. Harrison GH, Balcer-Kubiczek EK, Eddy HA. Int J Rad Biol 1991; 59:1453-1466.
44. Saad AH, Hahn GM. Ultrasound Med Biol 1992; 18:715-723.
45. Price RJ, Skyba DM, Kaul S et al. Circulation 1998; 98:1264-1267.
46. Unger EC, McCreery TP, Sweitzer RH et al. Invest Radiol 1998; 33:886-892.
47. ter Haar G. Ultrasound Med Biol 1995; 21:1089-1100.
48. Bender LF, Janes JM, Herrick JF. Arch Phys Med Rehabil 1954; 35:555.
49. Duarte LR. Arch Orthop Trauma Surg 1983; 101:153-159.
50. Nyborg WL, Carson PL, Carstensen EL et al. Exposure Criteria for Medical Diagnostic Ultrasound: II Criteria Based on all Known Mechanisms. Report No. 140, National Council on Radiation Protection and Measurements, Bethesda, MD, 2001.
51. Nyborg WL, Carson PL, Miller MW et al. Exposure Criteria for Medical Diagnostic Ultrasound: I. Criteria Based on Thermal Mechanisms, National Council on Radiation Protection Report No 113, Bethesda, MD, 1992.
52. American Institute of Ultrasound in Medicine. J Ultrasound Med 2000; 19:69-168.
53. ter Haar G, Duck FA. (eds.). The Safe Use of Ultrasound in Medical Diagnosis, British Medical Ultrasound Society/The British Institute of Radiology, London, 2000.
54. Whittingham TA. In Ultrasound in Medicine, FA Duck, AC Baker, HC Staritt (eds.), Institute of Physics Publishing, Philadelphia, 1998, pp. 129–148.
55. Dalecki D, Child SZ, Raeman CH et al. Ultrasound Med Biol 1997; 23:777-781.
56. Roy RA, Atchley AA, Crum LA et al. J Acoust Soc Am 1985; 78:1799-1805.
57. Clarke PR, Hill CR. J Acoust Soc Am 1970; 47:649-653.
58. Fechheimer M, Denny D, Murphy RF et al. Eur J Cell Biol 1986; 40:242-247.
59. Fechheimer M, Boylan JF, Parker S et al. Proc Natl Acad Sci USA 1987; 84:8463-8467.
60. Johannes C, Obe G. Mutat Res 1997; 374:245-251.
61. Delius M. Shock Waves 1994; 4:55-72.
62. Gambihler S, Delius M, Ellwart JW. J Memb Biol 1994; 141:267-275.
63. Delius M, Adams G. Cancer Res 1999; 59:5227-5232.
64. Miller DL, Williams AR, Morris JE et al. Ultrasonics 1998; 36:947-952.
65. Zhong P, Haifan L, Xi X et al. J Acoust Soc Am 1999; 105:1997-2009.
66. Doukas AG, Flotte TJ. Ultrasound Med Biol 1996; 22:151-164.
67. Lee S, McAuliffe DJ, Zhang H et al. Ultrasound Med Biol 1997; 23:1089-1094.
68. Mulholland SE, Lee S, McAuliffe DJ et al. Pharm Res 1999; 16:514-518.
69. Miller DL, Bao S, Morris JE. Ultrasound Med Biol 1999; 25:143-149.
70. Ward M, Wu J, Chiu J. J Acoust Soc Am 1999; 105:2951-2957.
71. Ward M, Wu J, Chiu J. Ultrasound Med Biol 2000; 26:1169-1175.
72. Miller DL, Quddus J. Ultrasound Med Biol 2000; 26:661-667.
73. Joersbo M, Brunstedt J. Plant Cell Rep 1990; 9:207-210.
74. Joersbo M, Brunstedt J. Physiol Plantar 1992; 85:230-234.
75. Joersbo M, Brunstedt J. J Virol Methods 1990; 29:63-69.
76. Zhang L, Cheng L, Xu N, Zhao N et al. Biotechnology 1991; 9:996-997.
77. Wyber JA, Andrews J, D'Emanuele A. Pharma Res 1997; 14:750-756.
78. Lauer U, Burgelt E, Squire Z et al. Gene Ther 1997; 4:710-715.
79. Miller DL, Bao S, Gies RA et al. Ultrasound Med Biol 1999; 25:1425-1430.
80. Huber PE, Jenne J, Debus J et al. Ultrasound Med Biol 1999; 25:1451-1457.
81. Huber PE, Pfisterer P. Gene Ther 2000; 7:1516-1525.
82. Lawrie A, Briskin AF, Francis SE et al. Circulation 1999; 99:2617-2620.
83. Manome Y, Nakamura M, Ohno T et al. Hum Gene Ther 2000; 11:1521-1528.
84. Koch S, Pohl P, Cobet U et al. Ultrasound Med Biol 2000; 26:897-903.
85. Delius M, Hofschneider P, Lauer U et al. Lancet 1995; 345:1377.
86. Bao S, Thrall BD, Gies RA et al. Cancer Res 1998; 58:219-221.
87. Anwer K, Kao G, Proctor B, Anscombe I et al. Gene Ther 2000; 7:1833-1839.
88. Tomizawa M, Ebara M, Saisho H et al. Cancer Lett 2001; 173:31-38.
89. Porter TR, Kricsfeld D, Xie F. J Am Soc Echocardiogr 2000; 13:434.

90. Cooper MJ. Semin Oncol 1996; 23:172-187.
91. Berinstein NL. Biological therapy of cancer, In The Basic Science of Oncology, 3rd edn., IF Tannock, RP Hill (eds.), McGraw Hill, New York, Chap. 18, 1998.
92. Nomura T, Nakajima S, Kawabata K et al. Cancer Res 1997; 57:2681-2686.
93. Yang JP, Huang L. Gene Ther 1996; 3:542-548.
94. Rakhmilevich AL, Turner J, Ford MJ et al. Proc Natl Acad Sci USA 1996; 93:6291-6296.
95. Nishi T, Yoshizato K, Yamashrio S et al. Cancer Res 1996; 56:1050-1055.