Ultrasound in Med. & Biol., Vol. 24, No. 4, pp. 587–595, 1998

0301-5629/98 $19.00 + .00

PII S0301-5629(98)00003-9

● *Original Contribution*

ARTIFICIAL CAVITATION NUCLEI SIGNIFICANTLY ENHANCE ACOUSTICALLY INDUCED CELL TRANSFECTION

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(*Received* 9 *January* 1997; *in final form* 19 *January* 1998)

Abstract—The efficiency of ultrasound-mediated gene transfection was enhanced three- to fourfold, compared to previous results, through the use of green fluorescent protein reporter gene, cultured immortalized human chondrocytes and artificial cavitation nuclei in the form of Albunex®. Cells were exposed to 1.0-MHz ultrasound transmitted through the bottom of six-well culture plates containing immortalized chondrocytes, media, DNA at a concentration of 40 μg/mL and Albunex® at 50×10^6 bubbles/mL. Transfection efficiency increased linearly with ultrasound exposure pressure with a transfection threshold observed at a spatial average peak positive pressure (SAPP) of 0.12 MPa and reaching about 50% of the living cells when exposed to 0.41 MPa SAPP for 20 s. Adding fresh Albunex® at 50×10^6 bubbles/mL prior to sequential 1-s, 0.32- or 0.41-MPa exposures increased transfection with each exposure, reaching 43% transfection after four exposures. Efficient *in vitro* and *in vivo* transfection now appear possible with these enhancements. © 1998 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Transfection, Gene therapy, Ultrasound, Albunex®, Bubble nucleation, Gene therapy.**

INTRODUCTION

Gene therapy (artificial introduction of exogenous genes) promises effective treatment of a wide variety of diseases, both inherited and acquired. Diseases such as severe combined immunodeficiency disease (SCID), cystic fibrosis and some forms of cancer have been treated with gene therapy to varying degrees of success (Hanania et al. 1995); but apart from these few examples, gene therapy is far from the panacea it was imagined to be. Gene therapy's failure to live up to its potential can be attributed, in part, to the lack of a high efficiency, *in vivo* method of gene transfer that can be used throughout the body, not merely in anatomically isolated regions (Lyerly and DiMaio 1993).

Gene therapy is based on deceiving the body's cells by introducing therapeutic nucleotides. Foreign deoxyribonucleic acid (DNA) with appropriate promoters and enhancers is placed into a target cell, and the cellular machinery translates the foreign DNA and manufactures the coded protein as if it were its own. The inserted foreign DNA can therefore be chosen so that the translated protein has some therapeutic value. Gene therapy requires transfection techniques to insert this all-important foreign DNA into the cell.

Transfection generally refers to the uptake and expression of foreign DNA by a cell. Transfection can be transient or stable (Lyerly and DiMaio 1993). Transient transfection occurs when the foreign DNA is expressed by the cell but is not incorporated into the genomic DNA of the cell. Because of this lack of incorporation, the DNA generally is not passed to the daughter cells upon cell division. Stable transfection results when the foreign DNA is incorporated into the genomic DNA of the cell, and the genetic material is passed on to the daughter cells. Although stable transfection is preferable in some instances, potentially successful *in vivo* gene transfer techniques only need to have high transient transfection rates (Lyerly and DiMaio 1993).

Many different techniques place foreign DNA, stably or transiently, into a target cell. These techniques can be divided into two broad categories—viral and nonviral. The two major forms of viral transfection are the use of

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retroviruses and adenoviruses. Retroviruses have proven to be a very effective means of stably delivering therapeutic nucleotides to cells. However, retroviruses have several shortcomings including the inability to infect nonproliferating cells, the random incorporation of the viral DNA into the genomic DNA of the cell causing the potential for insertional mutagenesis and malignancy, and the potential for replication competent viral particles to emerge, causing cell death (Isaka and Imai 1996; Sokol and Gewirtz 1996). Adenoviruses also have proven to be quite effective in the transfection of cells with therapeutic nucleotides, but they also have major shortfalls. Adenoviruses induce a short expression time of the delivered nucleotides, which is probably caused by the adenoviral delivery system itself, and also are not useful for re-treatment without major efficiency decrease due to immunological response, two potentially insurmountable problems for *in vivo* application (Sokol and Gewirtz 1996). Both adenoviral and retroviral methods of transfection also suffer from nonspecificity of delivery systems (Cosset and Russell 1996). If viral particles are not applied in anatomically isolated regions of the body such as the lungs, the particles will travel throughout the body transfecting nontarget cells, wasting large quantities of virus and increasing the possibility of deleterious side effects (Cosset and Russell 1996). This lack of site specificity is a major obstacle to establishing gene therapy as a common clinical modality for common, nonanatomically isolated disorders (Cosset and Russell 1996; Lyerly and DiMaio 1993).

Forms of nonviral transfection include electroporation, particle bombardment, lipofection and acoustically induced transfection. Electroporation refers to the utilization of high-intensity electric fields to open small pores in the membrane of a cell allowing for the diffusion of DNA into the cell (Chang and Reese 1990). Particle bombardment refers to the use of high-speed projectiles coated with DNA to introduce mechanically the coated DNA into cells (Daniell 1993). Lipofection refers to the use of cationic lipid microbubbles called liposomes to deliver foreign DNA to cells (Liu et al. 1995). Because of opposite electrical charges, the cationic lipid encircles and packages the anionic foreign DNA. When these lipid–DNA complexes are added to cells, the lipid fuses with the membrane of the cell and delivers the foreign DNA (Gershon et al. 1993). Compared to other methods, the liposomal method generally produces a high transfection rate with very little cell mortality (Gershon et al. 1993). In addition, of the mentioned nonviral methods of transfection, only lipofection has the potential for extensive *in vivo* use (Lyerly and DiMaio 1993), but, like viral methods, it also suffers from the lack of site specificity and various other application problems (Mahato et al. 1997).

Genetic therapy requires genetic alteration of cells with transfection *in vivo* or *ex vivo.* Generally, *ex vivo* methods involve harvesting a patient's affected cells, culturing them, transfecting the cells *in vitro* and reimplanting the genetically altered cells in the patient's body (Yang 1992). In this way, many transfection techniques that generally are not suited for *in vivo* use can be utilized, but unfortunately this complicated series of steps leads to many potential problems such as cell phenotype change (Lyerly and DiMaio 1993). DNA is transferred directly to the affected cells within the patient by means of *in vivo* transfection (Yang 1992). Esthetically, *in vivo* transfection is much more attractive than *ex vivo* methods because of the possibility of noninvasive, expedient gene therapy (Lyerly and DiMaio 1993). However, because *in vivo* target cells are much less accessible and not easily isolated from nontarget cells, many different *in vitro* transfection techniques are not extensively applicable *in vivo.* Also, as previously discussed, most *in vivo* techniques do not have a mechanism for constraining transfection both spatially and temporally. Therefore, there is a need for a transfection method that can place foreign genes into cells *in vivo* with well-controlled spatial localization and selectable transfection time.

The subject of this article—acoustically induced transfection—is a relatively recent development in gene transfer techniques that has been applied *in vitro* to both mammalian cells (Bao et al. 1997; Fechheimer et al. 1987; Kim et al. 1996; Tata et al. 1997) and plant cells (Joersbo and Brunstedt 1990; Joersbo and Brunstedt 1992), and it has the potential to be applied *in vivo* (Kim et al. 1996). This transfection technique utilizes ultrasound to permeabilize the membrane of cells allowing for the uptake of DNA through diffusion. The specific event that permeabilizes the cell membrane in acoustically induced transfection is thought to be cavitation (Bao et al. 1997; Kim et al. 1996; Lauer et al. 1997; Tata et al. 1997).

Cavitation refers to the formation and destruction of microbubbles of gas in acoustic fields. Cavitation begins as propagating pressure waves strike bubbles, which are preexisting or formed by the low-pressure portion of the acoustic wave as it passes through media rich in dissolved gases. These bubbles oscillate and gain gas through a process known as rectified diffusion. As the bubbles grow, they quickly reach resonant diameter and then are destroyed. This destruction can concentrate the intensity of an acoustic field up to 11 orders of magnitude in very small and localized volumes (Crum et al. 1992), which, hypothetically, increases cell membrane permeability and allows the uptake of foreign DNA (Kim

et al. 1996; Lauer et al. 1997; Tata et al. 1997). Because the mechanism of this form of ultrasound-mediated transfection is entirely governed by cavitation, which in turn is governed by ultrasound, it can be controlled both spatially and temporally through the exposure volume distribution and the application time of the ultrasound energy. The unique mechanism for external control makes this method potentially suitable for *in vivo*, site-specific transfection as a means of gene therapy (Lauer et al. 1997). However, ultrasound-mediated transfection currently achieves optimal transfection efficiencies of 2.4% using β-galactosidase reporter gene (Kim et al. 1996; Lauer et al. 1997) or 15% using green fluorescent protein (GFP) (Tata et al. 1997). The extremely low efficiencies make potential application *in vivo* improbable and even makes *in vitro* experiments difficult. Therefore, the intent of this research was to increase the transfection efficiency of acoustically induced transfection for possible application *in vivo* as an effective means of gene therapy. It was hypothesized that this goal of high transfection efficiency would be achieved by the addition of Albunex® microbubbles (Mallinckrodt Medical, Inc., St. Louis, MO, USA) to the exposure medium.

Albunex® is a commercially available ultrasound contrast agent consisting of human albumen that has been sonicated to produce a microbubble of gas encapsulated by a shell of albumen. These microbubbles have been shown to provide stable gas bodies that nucleate cavitational events (Miller and Thomas 1995) and even to increase mechanical cell damage done by ultrasound to erythrocytes (Miller and Brayman 1993). Therefore, the addition of Albunex® was expected to increase the number of cavitational events per unit volume, increasing the number of cells affected by cavitation and thus increasing transfection efficiency.

The hypothesis tested in this study was that increased cavitation, caused by the addition of artificial cavitation nuclei in the form of Albunex® to culture medium, would enhance the efficiency of the ultrasound transfection method. Furthermore, this study tested various protocol parameters for their effect and relationship to transfection efficiency and describes the results of experiments testing: 1) the effect of Albunex® concentration on transfection efficiency; 2) the effect of DNA concentration required for Albunex®-enhanced transfection; 3) the effect of ultrasound pulse pressure on Albunex®-enhanced transfection; and 4) the effect of multiple short bursts of ultrasound with replenished Albunex® on transfection efficiency. Liposomes were used to transfect cells to obtain a comparison of transfection rates with a widely used *in vitro* and *in vivo* method. Finally, the mechanism of this means of transfection was studied further through the use of Albunex® with fluorescent albumen shells.

METHODS AND PROCEDURE

The general methods used on all cells are described. Specific alterations to these general methods are noted in the Results section.

Reporter genes

To test the viability or efficiency of prospective methods of transfection, reporter genes are used. These genes have no therapeutic value but can be assayed easily because they produce proteins that can be measured very accurately and/or very conveniently. The proteins produced by reporter genes normally are not found in mammalian cells so that background levels of the protein are not taken as a false signal of transfection. Acoustic transfection in mammalian cells was investigated recently by Kim et al. (1996) using β-galactosidase as a reporter gene. Although β-galactosidase is a useful marker, it requires extra steps of color development for visualization and is not as sensitive as other reporter genes, such as the GFP used in these experiments. GFP fluoresces under ultraviolet light (Kain et al. 1995); therefore, transfected cells expressing GFP manufactured from the incorporated foreign DNA can be detected by their fluorescence. These fluorescent cells then can be counted by flow cytometry, a process that can quickly count thousands of cells and record their apparent size and intensity of luminescence (Galbraith et al. 1995), producing a large amount of quantitative and objective data on the number of cells that have taken up the foreign DNA.

Plasmid preparation

A relatively large amount of plasmid DNA was required to test various methods of transfection. The plasmid DNA from bacterial cultures was prepared with a Qiagen 2500 μg kit according to the company's protocol (Qiagen Inc., Chatsworth, CA, USA). Briefly to summarize, *E. coli* bacteria were made to express quantities of the targeted plasmid (5.0 kbp GFP construct GreenLantern-1 from Life Technologies, Gaithersburg, MD, USA). The *E. coli* transformants were grown to high densities, lysed, and the lysate was passed through the Qiagen column. A DNA-specific resin in the column isolated the plasmid DNA from the genomic DNA so that it could be collected separately. Finally, agarose gel electrophoresis was performed before and after restriction endonuclease digestion to verify the identity and purity of the plasmid DNA.

Cell preparation

Immortalized human chondrocytes [cell line CD4 C20-A4 (Goldring et al. 1994)] were either thawed (when starting from a frozen culture) or trypsinized and plated according to established protocols. Cells were plated at a concentration of 1.2×10^6 per six-well plate

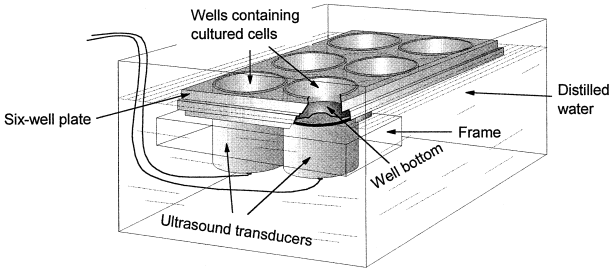

Fig. 1. Apparatus used to expose cells. A 35-mm-diameter 1.0-MHz transducer was placed 2 mm below each of two wells of a six-well culture plate. The experiment was conducted within a water bath maintained at 37°C. Water completed the acoustic coupling with the bottom of the culture plate. [Reproduced with permission from Kim et al. (1996)].

(35-mm diameter wells) (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and allowed to grow for about 48 h in Dulbecco's modified Eagle medium (DMEM), containing 10% fetal bovine serum (FBS), 10% sodium pyruvate, 10% L-glutamine and 1% penicillin–streptomycin in a 37°C humidified incubator (5% CO_2, 95% air) until the cells were 50–70% confluent. Cells were rinsed three times with Hank's balanced salt solution, and 1 mL of DMEM (without additives) with 40 μg of plasmid DNA was added. Negative controls were: cells not exposed to DNA but exposed to ultrasound; cells exposed to ultrasound but without DNA; and not experimentally manipulated. Control experiments also were performed which involved cells exposed to Albunex® and ultrasound but no DNA; Albunex® and DNA but no ultrasound; and DNA and ultrasound but no Albunex®. Unless specifically reported, these controls showed no significant increase in fluorescence over cells that were not experimentally manipulated.

Ultrasound calibration and application

Continuous-wave (CW) ultrasound of 1.0-MHz carrier frequency was delivered through the bottom of six-well plates using the same apparatus used in Kim et al. (1996). In brief, two 35-mm-diameter air-backed ultrasound transducers were fixed in a frame so that the bottoms of two adjacent wells of a six-well culture plate were aligned parallel with the transducers (Fig. 1). The frame was placed in a water bath filled with distilled, degassed water to 8 mm above the top of the transducers. Six-well culture plates were placed on this frame 3 mm above the top of the transducers and exposed to ultrasound that traveled through the water and plastic plate to the cells.

The ultrasound exposure was measured by placing a calibrated hydrophone with a 1.0-mm-diameter element embedded in a 80-mm diameter bilaminar shielded PVDF membrane (model Y-33-7611, GEC-Marconi Research Centre, Chelmsford, UK) on the top of the plastic bottom of a six-well plate in a water tank. Under mock-exposure circumstances, a 35-mm-diameter 1.0-MHz transducer was placed below the membrane hydrophone. The ultrasonic waves traveled through 3 mm of water, the well bottom, the hydrophone and the medium, and reflected off the air–media interface. This reflection set up standing waves in the near field that were identical to the experimental conditions. The reported values are the pressures measured at the position of the cells on the well bottom and in the same arrangement used for the experiments. The signals from the hydrophone were full-wave detected and low-pass filtered. The average and standard deviation of the signals were 0.41 ± 0.19 MPa, and 0.32 ± 0.16 MPa for the two main signal levels used in this study. The pressures reported are the spatial average pulse peak positive pressures and, because of the proximity of the cells to the transducers, the measured pressure peaks were found to be symmetrical and are therefore equivalent to the average peak negative pressures. The rippling water surface caused the near field of the transducer beam to average out, giving relatively flat average pressure values across the well.

Ultrasound exposure

Cells were exposed to ultrasound in a 37°C water bath. Two adjacent wells were exposed simultaneously to 1.0-MHz ultrasound at up to 0.41 MPa average peak pressure (unless otherwise specified) using two different 35-mm, air-backed transducers (Fig. 1). After exposure, cells were replaced into an incubator for 45 min, and then a solution containing twice the normal concentration of FBS (20% FBS, 80% DMEM with two times the normal concentration of antibiotics) was added. Cells were allowed to recover for 24 h (unless otherwise noted) and microphotographed before counting took place so that visual comparisons could be made. Cells then were trypsinized and analyzed.

Counting of cells

Cell counting was conducted on the live cells in a flow cytometer (FACScan or FACS Vantage from Becton Dickinson, San Jose, CA, USA). The GFP used in this experiment has maximum excitation of 490 nm and emits light in the 520-nm range (according to the manufacturer). Through the flow cytometer, the cells were exposed to 488-nm light and were detected at 530 ± 15 nm. The background level of fluorescence was determined by assaying cells that had not been experimentally manipulated and setting a threshold of fluorescence above which cells were defined to be transfected ($< 0.5\%$ of control cells). This threshold was used to determine the transfection rate of living

cells. In most cases, 10,000 cells were counted. After flow cytometric analysis, the cells were preserved with 2% formalin.

Acoustically induced transfection in the presence of Albunex® micronuclei

Albunex® was tested for its potential to enhance acoustically induced transfection. Albunex® was added to DMEM, containing DNA and cells, immediately before exposure at concentrations of 5, 50 and 250 $\times$ 10^6 microbubbles/mL. This experiment was repeated at concentrations of 25, 50 and 100 $\times$ 10^6 microbubbles/mL. The exposure was continuous-wave 1.0-MHz ultrasound at 0.41 and 0.32 MPa average peak pressure for 20 s. These are the same ultrasound conditions previously reported without artificial micronuclei (Kim et al. 1996).

Cells were treated with DNA at concentrations of 100, 25, 10, 5 and 2 μg per well and exposed to 0.41 or 0.32 MPa, 1.0-MHz ultrasound for 20 s in the presence of 50 $\times$ 10^6 microbubbles/mL.

Albunex® was tested for enhancement of acoustically induced transfection at 0.41, 0.32, 0.28, 0.22, 0.20, 0.16, 0.14 and 0.11 MPa average peak pressure, 20 s of exposure, 40 μg/mL of DNA and 50 $\times$ 10^6 microbubbles/mL. In a separate experiment, repetitive 1.0-s exposures to 0.41 and 0.32 MPa ultrasound also were investigated in which fresh Albunex® (50 $\times$ 10^6 microbubbles/mL) was added to the medium before reexposure to ultrasound (the 40 μg/mL of DNA was not replenished prior to reexposure).

Liposomal transfection

As a positive control, liposome transfection (using LipofectAmine® from Gibco BRL, Gaithersburg, MD, USA) also was performed for comparison to acoustically induced transfection in the same cell type.

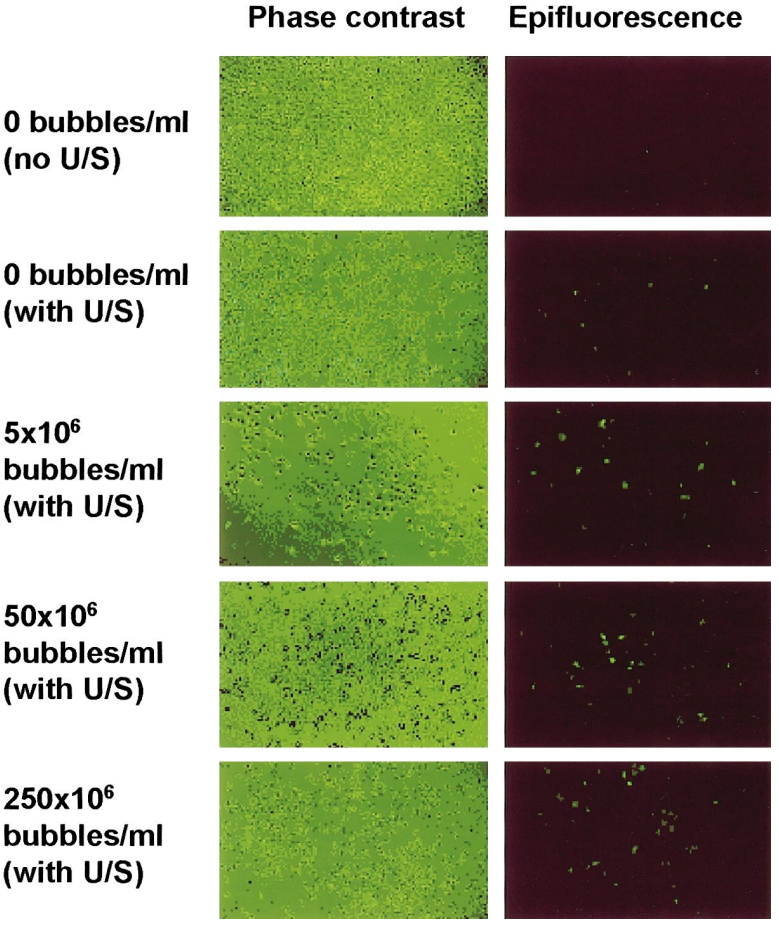

Fig. 2. Comparison of cells imaged in phase contrast (left) with the same cells imaged with epifluorescence showing transfected cells expressing GFP (right) after exposure to 0.32-MPa, 1.0-MHz ultrasound, 40 μg/ml of DNA, and the indicated concentrations of artificial micronuclei (microbubbles/mL of Albunex®). Magnification $\times$200.

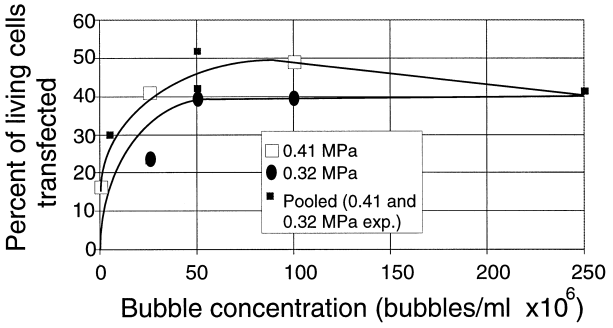

Fig. 3. Transfection rate of living cells after exposure plotted against Albunex® concentration at the time of exposure. Exposure time is 20 s at the indicated average peak pressure. Pooled data were from a separate experiment. Continuous lines are hand drawn.

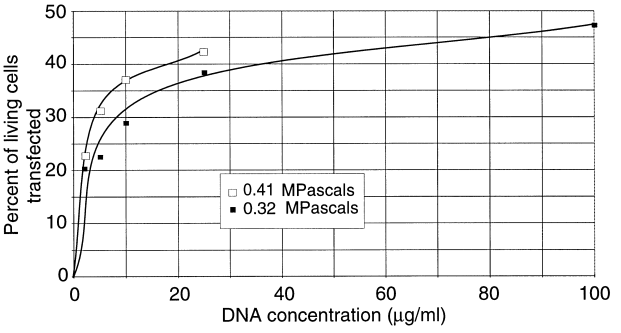

Fig. 4. Transfection rate after 20-s exposure to 1.0-MHz ultrasound at indicated average peak pressure with a microbubble concentration of 50×10^6 bubbles/mL prior to exposure. Graph shows relationship between transfection efficiency and DNA concentration.

Exploration of the effect of destruction of Albunex® on cells

Cells were treated to experimental quantities (50×10^6 microbubbles/mL) of fluorescently labelled Albunex® but no DNA and then exposed to 1 s of 0.41, 0.32 or 0.0 MPa, 1.0-MHz ultrasound. About 24 h after exposure, the cells were rinsed three times with Hank's balanced salt solution, then microphotographed.

RESULTS

To determine the concentration of Albunex® required to obtain maximum transfection efficiency, various concentrations of microbubbles at two fixed ultrasound pressures and one DNA concentration were investigated. Figure 2 shows photomicrographs of selected regions of the ultrasonically exposed cells using an inverted microscope and epifluorescence attachments. The increase in transfection efficiency with addition of microbubbles is apparent in the images showing GFP fluorescence. The ratio of fluorescent cells to nonfluorescent cells, as determined by flow cytometric analysis, was taken as the percent of living transfected cells for the various experimental conditions of this study. The relationship between the concentration of artificial microbubbles and the transfection efficiency of immortalized chondrocytes is shown in Fig. 3. Overall, the efficiency is seen to reach a plateau near 10% Albunex® by volume (about 50×10^6 bubbles/mL). The efficiency of 0.41-MPa ultrasound is slightly higher than that of 0.32 MPa.

Because the concentration of DNA is known to affect the efficiency of most transfection methods, a range of DNA concentrations was tested with the micronuclei concentration of 50×10^6 microbubbles/mL. Data describing the effect of DNA concentration on the enhancement of transfection by Albunex® microbubbles are shown in Fig. 4. The curve changes slope at about 8–10 μg/mL of DNA.

Because a pattern of increased transfection with increased intensity was detected in previous experiments, the relationship between ultrasonic pressure and transfection efficiency at fixed DNA and microbubble concentrations was investigated. The results of this experiment, presented in Fig. 5, show a linear relationship between transfection efficiency and average peak ultrasound pressure, with an apparent threshold of about 0.12 MPa.

In the previous experiment, it was observed that the microbubbles were destroyed immediately after exposure to ultrasound. It was, therefore, hypothesized that short exposures to bursts of ultrasound, with renewed nucleation through the addition of fresh microbubbles prior to each exposure, should result in sequential increases in transfected cells. Results presented in Fig. 6 show that a sequence of short exposures of ultrasound to media with about 50×10^6 bubbles/mL transfects cells with increasing efficiency.

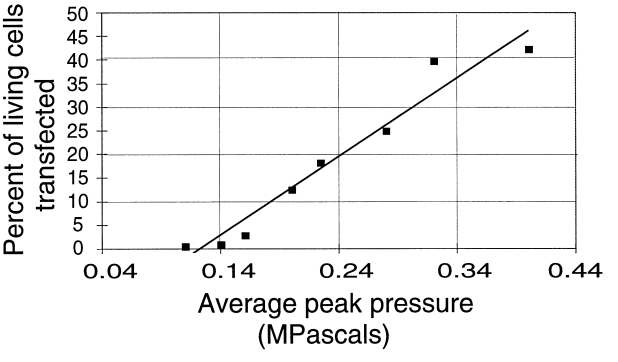

Fig. 5. Transfection rate of living cells after ultrasound exposure as a function of average peak pressure of the 20-s burst of 1.0-MHz ultrasound. The DNA concentration was 40 μg/mL and the Albunex® concentration was 50×10^6 bubbles/mL prior to exposure. Continuous line is best linear fit.

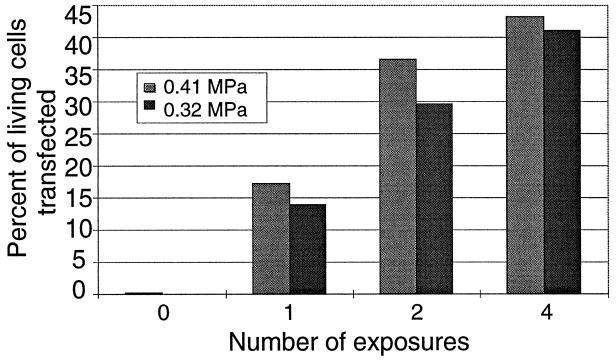

Fig. 6. Transfection rate of live cells after repeated 1-s exposures to ultrasound at the indicated average peak pressure. Prior to each exposure, 50×10^6 microbubbles were added to the 1 mL of media in each well.

To compare this method to another more widely used method, various concentrations of liposomes were evaluated for transfection efficiency using the lipofection method as a positive control. Liposomes were applied to the cells according to the manufacturer's protocol. The optimal concentration of liposomes was 8–12 μg/mL with 2 μg/mL of DNA, as shown in Fig. 7. This concentration transfected 65% of living cells.

The hypothesis that, after sonication, particles of Albunex® might have been driven into the cells was tested using fluorescently labelled Albunex®. The remains of the destroyed microspheres were shown to embed themselves either in the membrane of the cells or inside the cell itself. Digitally enhanced images of these experimentally manipulated cells are presented in Fig. 8 and show treated cells to be fluorescent.

DISCUSSION

Previous results from our laboratory reported in Kim et al. (1996) were obtained using β-galactosidase in primary chondrocytes and found a transfection efficiency of about 2.4% of surviving cells by visually counting cells expressing the reporter gene using an inverted microscope. The current protocol uses: a different reporter gene, GFP; a different cell line, immortalized chondrocytes; and flow cytometry for automated counting of fluorescent cells. This new protocol, using the same ultrasound exposures as those of Kim et al. (1996) resulted in a transfection rate of around 15% (Fig. 3) without the addition of Albunex®. These increased transfection rates could result from several differences between the two protocols: the reporter gene vectors produce different strengths of signal; the flow cytometer is better at counting positive cells; and/or immortalized chondrocytes are more amenable to this form of transfection. Overall, enhancements, including the addition of Albunex®, presented in this study improved the optimal transfection efficiency from 2.4% as reported in Kim et al. (1996) to about 50%, or about 20fold.

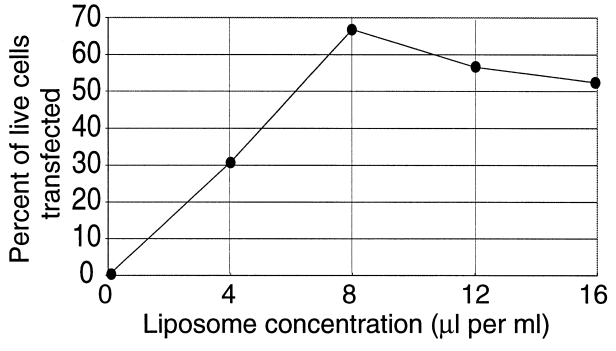

Fig. 7. Transfection rate of living cells after lipofection as a function of liposome concentration.

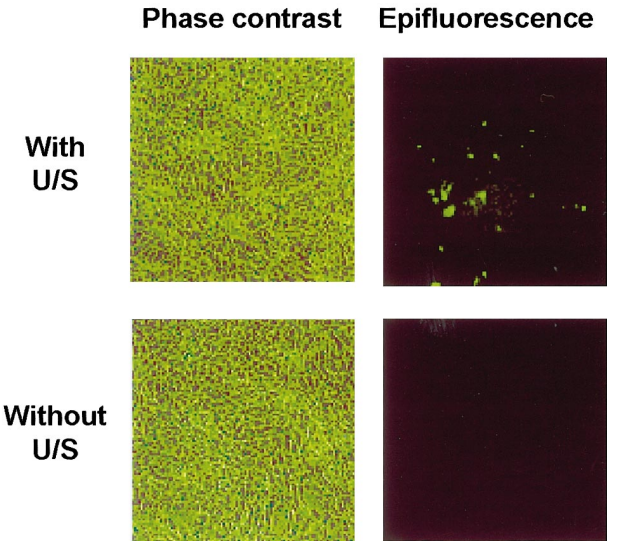

Fig. 8. Microphotographs of cells exposed to 50×10^6 microbubble/mL of fluorescent Albunex® and either 1 s of 0.41 MPa, 1.0-MHz ultrasound or no ultrasound. After exposure, cells were thoroughly rinsed to ensure that fluorescent fragments of Albunex® were firmly attached or enveloped by the cell. Both epifluorescence images have been identically electronically enhanced to give a better indication of location of the fluorescent fragments (or lack thereof). Magnification ×200.

The transfection rate did not increase linearly with concentration of microbubbles (Fig. 3), probably because the attenuation of ultrasound increases greatly with the concentration of Albunex® (Marsh et al. 1997). The optimal concentration of Albunex® was about 50×10^6 microbubbles/mL.

These experiments suggest that DNA concentration and transfection efficiency are related, but are not linearly proportional. As DNA concentration is increased, the trans-

fection efficiency also is increased but eventually plateaus, correlating to a logarithmic relationship (Fig. 4). This relationship implies that a DNA saturation point is reached with high DNA concentrations implying permeability-dependent transfection. This dependence of transfection on ultrasound-mediated membrane permeabilization is supported by the repetitive exposure experiments.

The high transfection rate from cumulative, 1-s exposures of ultrasound with fresh cavitation nuclei implies that permeabilization of the membrane occurs coincident with destruction of microbubbles within 1 s and does not require 20 s of exposures (Fig. 6), a conclusion that also is supported by visual observations (Fig. 8). Ultrasound at 0.32 or 0.41 MPa destroys Albunex® within a fraction of a second (observed), implying that the transfection, or at least increased permeability, is triggered by the destruction of the microbubbles. These data also show that the ultrasound exposure time can be reduced from more than 20–30 s as described in Kim et al. (1996) and Tata et al. (1997) to 4 s cumulatively, while increasing the transfection from 15% to 43%. This cumulative exposure method significantly decreases the overall amount of energy from the acoustic field to which tissue would be exposed during this ultrasound treatment. Lauer et al. (1997) exposed cells to very high pressure (80 MPa) in a lithotripter and achieved only 0.04% transfection after 250 shocks, indicating that there is a limit to shortening the pulse and increasing the pressure. Tata et al. (1997) obtained 20% transfection at about 100-Hz pulse rate and 120-s exposure in different cell types and without artificial microbubbles.

The relationship between the average peak pressure and the transfection efficiency in media containing 50×10^6 microbubbles/mL Albunex® may be related to the violence of the microbubble destruction. Even at lower pressures, the microbubbles are ruptured (this also was visually and microscopically verified). Apparently, rupturing of the albumen microbubbles at low ultrasound intensities does not necessarily equate with transfection. One interpretation of the results of these experiments is that, at high intensities, the microbubbles burst violently with enough energy to permeabilize the cell and result in transfection, while, at lower intensities, the microbubbles may rupture more sedately and have little effect on the cell. Hence, the violence with which the Albunex® ruptures and, therefore, the intensity of cavitation, can be controlled directly by the variation of ultrasound pulse pressure. The transfection threshold found here (0.12 MPa) is very similar to that found by Bao et al. (1997) who reported amplitude pressure thresholds for transfection around 0.11 MPa peak positive pressure.

Fluorescently tagged Albunex® was used to show that, when the Albunex® is destroyed with a 1-s pulse of ultrasound, shards of the albumen protein shell actually embed themselves in the cell (Fig. 8). This observation provides novel, persuasive evidence that the destruction of these bubbles, cavitation, is the mechanism of this transfection process and that these protein shells actually enter the cell. In addition, this finding graphically illustrates that the microbubbles are violently destroyed within the first second of ultrasound exposure.

Unfortunately, an accurate determination of *in vitro* mortality was not possible. However, because of the great difference in cell mechanics that cells enjoy *in vivo*, it is doubtful that this mortality information would be applicable *in vivo*.

Comparison to other methods and applications

With enhancements described in this article, the acoustically induced transfection method is fairly comparable to other high performance techniques of transfection, such as lipofection (Fig. 7). Because the technique can be performed through the walls of plastic culture dishes, it reduces the possibility of contamination compared to many other methods *in vitro.* This form of transfection also could be used to introduce foreign DNA into plant cells. Because of the intense mechanical energy used to increase membrane permeability, the ultrasound method should work on cells with tough extracellular matrixes or cell walls, perhaps favorably competing with other purely mechanical techniques such as particle bombardment described by Daniell (1993). Potentially, the intensity of the ultrasound can be increased so that destruction of the cavitation nuclei occurs with enough violence to open the plant cell wall and allow uptake of foreign DNA (Fig. 5).

All data recorded in this study were from cells assayed 1 day after transfection. Therefore, no reliable data have been collected on the stability of gene expression. The stability of gene expression could be an area of further study.

One possible drawback to this procedure is the relatively large amounts of plasmid DNA needed to obtain competitive transfection rates. Because the DNA, like the microbubbles, is distributed uniformly throughout the nutrient medium, it has a fairly low effective concentration. Other transfection methods, such as lipofection and calcium precipitation, carry the DNA directly to the cells by gravity or electric charge. Therefore, the concentration of DNA near the cells in these methods is much greater. However, plasmid DNA is comparatively inexpensive, and the possible site-specific, *in vivo* applications of this method generally outweigh this small disadvantage.

With the enhancement that microbubble nucleation of cavitation brings to this procedure and the potential for further enhancement with DNA attachment to the microbubbles, acoustically induced transfection is more likely to be applied *in vivo. In vivo* application is most

likely feasible because other, more technically difficult methods of transfection such as particle bombardment (Yang et al. 1990) have been successfully applied *in vivo.* However, it is likely that extensive experiments will be required to determine the precise experimental protocol for use of this method *in vivo.*

Because of its potential for efficient, site-specific, *in vivo* gene therapy, acoustically induced transfection could be applied to a variety of diseases. One such application is transfection of endothelium of blood vessels. A treatment procedure could potentially consist of injection of DNA and Albunex® and exposure to ultrasound either by means of a small transducer on the tip of a catheter or by means of an external transducer. Inserted genes then could cause the cells locally to produce proteins for control of, among other things, vasoconstriction, atherosclerosis or vascular tissue growth. Other *in vivo* applications could include transfection of striated muscle or, more broadly, gene therapy of nearly any nonanatomically isolated region of the body.

CONCLUSION

Significant enhancement of acoustically induced transfection was accomplished using cavitation nuclei in the form of the contrast agent Albunex® and various other protocol changes. Optimal exposure procedures, enhanced with cavitation nuclei, resulted in ultrasound-mediated transfection of upwards of 50% of living cells after exposure. In addition, transfection efficiencies were shown to be directly proportional to ultrasound intensities and, therefore, the violence with which the microbubbles are destroyed was shown to have an effect on transfection. Repetitive, short ultrasound exposures were seen to have a nearly cumulative effect on transfection and, with four repetitive exposures, transfected 43% of the cells with much shorter total exposure times than were formerly required. Finally, it was shown that energy from the cavitational event embeds debris from the Albunex® microbubble shell into the cell.

The addition of microbubble cavitation nuclei, along with other procedural changes, significantly enhanced acoustically induced transfection nearly 20fold over what has been previously reported and makes this method comparable in efficiency to other methods such as lipofection. The increased efficiency along with other possible enhancements, such as the construction of DNA–Albunex® complexes, opens the door for applications *in vivo.*

Acknowledgements—Randall R. Kinnick designed and constructed the transducers and their holder and calibrated them. This research was supported by the Mayo Clinic and Foundation, specifically the Orthopedic Research Laboratory.

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