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● *Original Contribution*

ULTRASOUND ENHANCEMENT OF LIPOSOME-MEDIATED CELL TRANSFECTION IS CAUSED BY CAVITATION EFFECTS

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Abstract—Cationic liposomes (CL) are widely used vectors for gene transfer. Recently, ultrasound (US) was reported to enhance liposome-mediated gene transfer to eucaryotic cells in culture. The present study was aimed at studying the effects of 2-MHz pulsed Doppler US on malignant brain tumor cells transfection by cationic liposome/plasmid-DNA complexes (lipoplexes). Cationic liposomes consisting of DOSPA/DOPE were complexed with a plasmid carrying the cDNA encoding green autofluorescent protein (EGFP). Rodent (9L) and canine (J3T) glioma cells were exposed to pulsed US in the presence of EGFP-lipoplexes. A diagnostic transcranial Doppler device (MultiDop L) was used for insonation for 30, 60, and 90 s at 2 MHz/0.5 W/cm^2. To eliminate US reflection and cavitation, a custom-made absorption chamber was designed, where US is applied through a water tank before interacting with the cells and is fully absorbed after passing through the cell layer. Expression of the marker gene EGFP was quantified by FACS analysis and intravital fluorescent microscopy. Cell viability was accessed by Trypan Blue staining. US treatment of tumor cells on microplates for 60 s yielded a significant increase in transfection rates without damaging the cells, but 90-s treatment killed most of the cells. In the absorption chamber, no significant effects of US on transfection were noted. Additional experiments employed US contrast agent (Levovist®, Schering) which was able to significantly increase tumor cell transfection rate by enhancing cavitation effects, and also severely damaged most cells when applied at a concentration of 200 mg/mL. In conclusion, our results support the assumption that US effects on lipoplex transfection rates in brain tumor cells in culture are mediated by cavitation effects. © 2000 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Brain tumor, Cationic liposomes, Cavitation, DNA, Doppler, Gene therapy, Glioma, Ultrasound.**

INTRODUCTION

Nucleic acid transfer to eucaryotic cells by liposomal transfection became an essential technique for basic gene expression studies in cell culture and *in vivo*, and has already advanced to the stage of clinical trials (Gao and Huang 1995; Liu and Song 1998). Cationic liposomes (CL), a class of positively charged double-layered vesicles prepared from synthetic lipids, are nonviral delivery vectors with high transfection efficiency which, unlike that with retrovirus (RV) vectors, is independent of the cell cycle (Felgner et al. 1987). Moreover, CL are biologically safe because they do not carry any other genetic information than the plasmid DNA used for transfection, and are quite simple to prepare and use. Most CL used today for gene delivery are composed of a cationic lipid and a neutral helper lipid, and are termed lipoplexes when complexed with plasmid DNA (Nabel et al. 1993; Rainov et al. 1999). The first description of such a formulation was published by Felgner et al. (1987). These authors reported the use of lipoplexes consisting of DOTMA/DOPE for transfection of a reporter gene in cultured cells. It was shown that the transfection efficiency of CL-containing neutral lipids (DOPE) is higher than in such made with cationic lipids alone (Liu and Song 1998). Many CL preparations have been used in the last years and reported to be highly efficient in culture and *in vivo* (Gao and Huang 1995).

In addition to liposomes, ultrasound (US) seems to be another interesting tool for nonvirus gene transfer into eucaryotic cells. US is used in a broad variety of biomedical applications and passes readily through fluids, soft tissues, and bone and cartilage (Maresca et al. 1998). The earliest report of sonication-mediated DNA delivery

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was the introduction of a plasmid carrying the Herpes simplex virus thymidine kinase (*HSV-tk*) gene into fibroblasts using pulsed US from a probe-type sonicator (Fechheimer et al. 1987). In the meantime, many studies have investigated this phenomenon (Bao et al. 1997; Kim et al. 1996; Lawrie et al. 1999; Unger et al. 1997). However, the detailed mechanisms by which US enhances gene transfer across cell membranes are still not completely understood, although there are several explanatory hypotheses. US is known to facilitate passage of small molecules across membranes (Pohl et al. 1993a; Pohl et al. 1993b), and it could also drive large biomolecules such as DNA or even lipoplexes through the lipid bilayer of the outer membrane into the cell. Because lipoplexes enter the cell by endocytosis and are trapped in endosomes, most of the DNA remains sequestered within the endosomes and is degraded (Zabner et al. 1995). It was contemplated that US energy may release endosomally trapped lipoplex DNA and force it into the nucleus (Unger et al. 1997) because low-energy US apparently does not cause major biologic damage to the cell (Pohl et al. 1995), but increases gene transfer and expression rates in cell culture (Lawrie et al. 1999; Bao et al. 1997). It was recently demonstrated that gases trapped in microbubbles may act as US contrast agents and amplify US reflection and biological effects on cells and tissues (Miller and Thomas 1995; Ward et al. 1999). US contrast agents (CA) based on protein microspheres (4–10 μm; Christiansen et al. 1994) or sugar microbubbles (2–5 μm; Schlief 1993a) are commercially available at present, but are approved in humans for diagnostic use only (Maresca et al. 1998). The introduction of CA propelled research on the previously known phenomenon of cavitation, which is generally believed responsible for transient cell membrane permeabilization or for cell damage (Miller et al. 1996; Tachibana et al. 1999; Williams et al., 1999).

The aims of our study were to investigate the possible use of pulsed Doppler US for enhancement of lipoplex transduction of brain tumor cells. Because pulsed 2-MHz US can penetrate the skull, we sought to establish a cell culture model for its therapeutic use in gene transfer and therapy of intracranial brain tumors. In addition, the effect of CA on transfection efficiency and brain tumor cell viability was studied.

MATERIALS AND METHODS

Plasmid DNA

The 4.7-kb eucaryotic expression plasmid pEGFP-N3 (Clontech GmbH, Heidelberg, Germany) was amplified in *E. coli*, isolated in sufficient quantities by the JETstar Maxiprep kit (Genomed GmbH, Bad Oeynhausen, Germany), and used as a reporter construct.

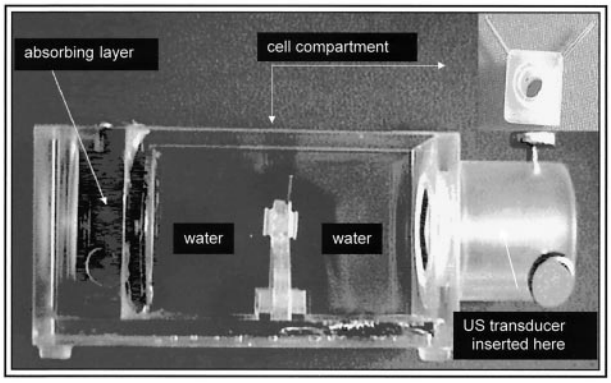

Fig. 1. Schematic view of the custom-built US absorption chamber with enlarget inset of the cell compartment where tumor cells are grown on polymer membranes (not shown). Castor oil was used as absorbing layer.

It carries the enhanced green fluorescent protein (EGFP) cDNA under the transcriptional control of the strong constitutive cytomegalovirus (CMV) promoter and, therefore, quantification of marker gene expression is possible in living cells without any further processing.

Lipoplexes

The commercially available CL preparation LipofectAmine (DOSPA/DOPE; Life Technologies, Karlsruhe, Germany) was complexed with plasmid DNA in 100 μL serum-free culture medium (Optimem, Life Technol.) at a ratio of 16:1 (lipid:DNA; w/w) and incubated at room temperature for 30 min before being diluted in Optimem. Final lipoplex volumes of 0.7 mL/well (for 12-well plates) and 0.2 mL/well (for absorption chamber) contained a total amount of 2 μg DNA each.

Cell culture studies

The rodent gliosarcoma cell line 9L was a gift from Dr. X. O. Breakefield (Massachusetts General Hospital, Boston, MA), and the dog glioblastoma cell line J3T was a gift from Dr. M. Berens (Barrow Neurological Institute, Phoenix, AZ). Both cell lines were grown in DMEM (Biochrom KG, Berlin, Germany) with 10% fetal calf serum (FCS; Biochrom KG) and 1% penicillin/streptomycin (Life Technology) at 37°C in a humid atmosphere containing 5% CO_2.

For transfection experiments, tumor cells were grown as monolayer in flat-bottom 12-well plates (Biochrom KG) or on polypropylene membranes (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in the cell compartment of a custom US absorption chamber (Fig. 1). After reaching 60–80% confluence, the cells were washed once, overlaid with lipoplexes in Optimem and insonated, and incubated for 5 h at 37°C before replacing lipoplexes with standard medium.

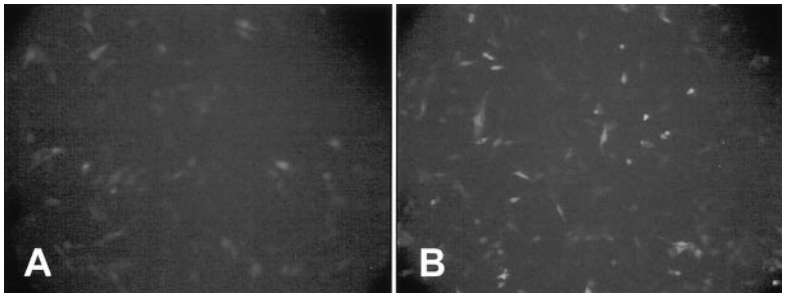

Fig. 2. Marker gene expression 24 h after lipoplex-mediated transfection of EGFP marker gene in 9L rat brain tumor cells grown on 12-well plates. (A) Fluorescence of living control cells without US treament; and (B) fluorescence of living cells treated for 60 s with US. Original magnification × 240.

US treatment

Spectral Doppler US was applied using a 2 MHz (I_{SATA} = 0.5 W/cm^2) pulsed clinical device for transcranial Doppler US diagnostics (MultiDop L, DWL, Sipplingen, Germany). Intensity calibration was performed by radiation force measurements using microbalances with a resolution of 1 μg (Sartorius, model M3P, Goettingen, Germany). Focus location and dimensions were determined by means of a membrane polyvinylidene fluoride hydrophone (NTR-Systems, Inc., Seattle, WA). One h after addition of lipoplexes, cells on 12-well plates were exposed to US for 30, 60, or 90 s The transducer was submerged in medium and held at a distance of approximately 1 cm above the bottom of the respective well. Cells grown in the absorption chamber were insonated through a water tank by two acoustical windows made of thin polyethylene membranes (Fig. 1). The chamber was positioned in the focus of the transducer under the control of a hydrophone. A sound absorber consisting of castor oil prevented the formation of standing waves in the water tank. Cells were grown on the inner face of the acoustic window membrane facing the absorber. As determined in control experiments without CA, this chamber was free of reflection and cavitation effects. A different exposure system was used consisting of an oscillator (HP Model 33120A, Hewlett Packard, Böblingen, Germany), an amplifier (Model 2100L, Electronic Navigation Industries, Inc.) and a focused transducer with a resonance frequency of 2 MHz (Panametrics, Hofheim, Germany). To exclude cavitation events during sound exposure, a hydrophone connected to a selective level meter (Anritsu, Tokyo, Japan) was used to detect subharmonic acoustic signals. The maximum peak pressure applied (0.6 MPa) did not yield cavitation. Because the peak pressure generated from the transcranial Doppler device was approximately 0.3 MPa, it was concluded that it is unable to induce cavitation, at least in the absence of nuclei. Experiments with CA were performed within the absorption chamber only. Levovist® (Schering AG, Berlin, Germany) in concentrations of 2 mg/mL, 20 mg/mL, and 200 mg/mL was pumped through the chamber by a syringe pump (Fresenius, Bad Homburg, Germany) at a continuous flow rate of 100 mL/h. The cell monolayer grown on the acoustical window remained immobile. US with the above characteristics was applied for 60 s.

Quantitation of marker gene expression

Fluorescence intensity was assessed visually 24 h after US treatment using an inverted fluorescence microscope (Axiovert 135, Zeiss GmbH, Oberkochen, Germany). For quantitation of EGFP expression, fluorescence intensity was measured by fluorescence-activated cell sorting (FACS; FACScan, Becton Dickinson, Heidelberg, Germany) 24 h after US treatment. Briefly, cells were rinsed with 1x phosphate-buffered saline (PBS, Life Technol.), detached with 1x trypsin-EDTA (Sigma, Deisenhofen, Germany), and resuspended in 0.5 mL PBS for FACS analysis of 10,000 events. Cell viability was assayed by Trypan Blue exclusion. Ten μL of cell suspension were mixed with the same volume of Trypan Blue dye (Sigma). Blue (dead) and white (living) cells were counted microscopically in a hemocytometer (Sigma). All data were statistically evaluated with the PC software package Microcal Origin® 5.0 for Windows® (Microcal Software, Inc., Northampton, MA).

RESULTS

US treatment of 9L gliosarcoma cells grown in 12-well plates caused a significant increase of lipoplex-mediated transfection rates (Figs. 2 and 3). FACS analysis demonstrated mean values of 13.2%, 20.9%, and 32.7% transfected and US-treated cells (US treatment 30, 60 and 90 s, respectively) vs. 7.4% transfected control cells (Fig. 3). The differences between the groups were statistically significant ($p < 0.05$). Trypan Blue assays for cell viability revealed that US application for 90 s

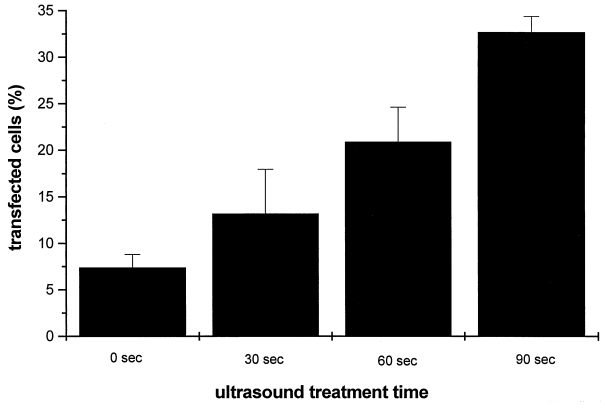

Fig. 3. Graph showing rate of transfection (percentage of transfected cells) after US treatment of lipoplex-incubated 9L cells grown on 12-well plates. The differences between the control group and the US treament groups are statistically significant ($p < 0.05$).

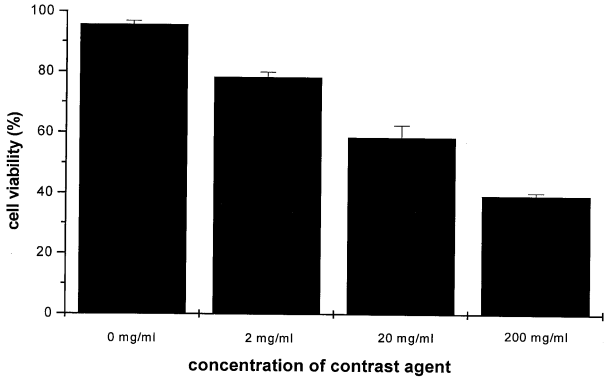

Fig. 5. Viability of J3T dog brain tumor cells grown in an absorption chamber and treated for 60 s with US in the presence of US contrast agent (Levovist®) in different concentrations. The differences between the control (0 mg/mL) and the 20 and 200 mg/mL groups are statistically significant ($p < 0.01$).

damaged most cells (more than 80%) in contrast to 30- and 60-s treatments (data not shown).

In the absorption chamber, 9L and J3T tumor cells were exposed to US (60 s) under conditions where US cavitation and reflection were excluded. This treatment had no significant effect on lipoplex transfection efficiency (Fig. 4). When CA (Levovist®, 2 mg/mL, 20 mg/mL and 200 mg/mL) was added to the lipoplex/medium mixture in the cell compartment of the chamber, and cells were exposed to US for 60 s, a significantly increased transfection rate was observed (Fig. 4). Control cells had 0.5% transgene expression, US-treated cells without CA 0.6%, with CA (2 mg/mL) 0.6%, with CA (20 mg/mL) 1.2% and with CA (200 mg/mL) 5.5% (Fig. 4). However, cell viability was impaired by CA application: 39.3% viable cells with 200 mg/mL CA, 58.5% with 20 mg/mL, and 78.3% with 2 mg/mL, compared to 95.7% without CA in the control group (Fig. 5).

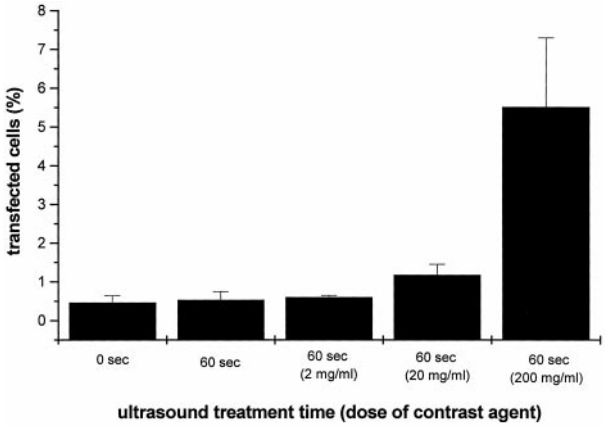

Fig. 4. Graph showing rate of transfection (percentage of transfected cells) after US treatment of lipoplex-incubated J3T dog brain tumor cells grown in an absorption chamber in the presence or absence of US contrast agent (Levovist®). Differences between the control groups (no US and 60-s US) and the 200 mg/mL group are statistically significant ($p < 0.01$).

DISCUSSION

Our results show that pulsed Doppler US (2 MHz, 0.5 W/cm^2) may significantly increase the rate of lipoplex-mediated transient gene expression in cultured brain tumor cells. It was shown that this phenomenon can be reversed by US treatment of cells in a custom-made absorption chamber, where US reflection and cavitation effects at a peak US pressure twice higher than the US pressure used with cell culture (0.3 MPa) could be excluded. Insonation of cells in the above chamber was unable to increase transfection rates. Addition of US contrast agent restored US effects on lipoplex-mediated tumor cell transfection. US treatment in the presence of CA was damaging to tumor cells, unlike US treatment in the absence of CA.

Using US for enhancement of lipoplex transfection offers several potential advantages in culture and *in vivo*. First, US is easy to apply in cell culture and the delivered energy can be measured, which allows a dose-response curve to be drawn for each particular cell type. In culture, serum-mediated inhibition of lipoplex transfection my be compensated for by the use of US (Bao et al. 1997). It was reported that transgene expression in cells that are difficult to transfect may be favorably influenced by US treatment (Lawrie et al. 1999). Moreover, US may be applied to a relatively small area and, thus, allow for

spatially regulatable gene transfer and expression, which is important for targeted gene therapy applications (Bednarski et al. 1997). Because Doppler US at 2 MHz penetrates bone and cartilage, it may be employed to insonate a small spot in the depth of an organ or tissue, which also may be monitored and guided by neuroimaging (Bednarski et al. 1997). This feature seems especially important in the CNS, where the skull severely limits noninvasive percutaneous treatments of the brain.

Liposomes are commonly used synthetic vectors for gene transfer. They are generally easy to produce in large quantities and do not carry the real risks implicit in virus vectors, such as insertional mutagenesis, oncogene activation or virus-mediated pathology (*e.g.*, inflammation and encephalitis) (Hug and Sleight 1991; Kramm et al. 1995). Liposomes have been shown to be highly effective in transfecting cultured cells, although different lipid formulations have different transfection efficiencies and are able to carry varying amounts of DNA (Felgner et al. 1994; San et al. 1993). On the other hand, liposomes have not yet been formulated to permit avoidance of degradation by cellular enzymes and nuclear transport with long-term high level transgene expression. One possible reason for these shortcomings may be the specific mode of action of lipid vesicles. Cationic liposomes probably fuse with cell membranes, releasing DNA into the cytoplasm (Hug and Sleight 1991), but the precise structures formed by lipids and DNA still remain somewhat controversial (Felgner et al. 1995).

After showing that, at least in the cell culture model we used, US cavitation and reflection are probably the main mechanisms responsible for the observed phenomenon of enhanced lipoplex-mediated transduction, the question was put if it is possible to induce cavitation in this setting by using artificial means. The clinically used and approved for diagnostics US contrast agent (Levovist®, Schering), a microbubble-based active carrier, was used in concentrations of 2 mg/mL, 20 mg/mL and 200 mg/mL. Our CA experiments demonstrated clear evidence for transfection enhancement because the group with the highest concentration of CA (200 mg/mL) and with 60-s insonation had an 11-fold higher transfection rate compared to the control cells with US, but without CA. A side effect of the induction of cavitation was dramatically reduced viability of tumor cells (39.3% with 200 mg/mL CA vs. 95.7% in controls, respectively). Although lower concentrations of CA did not significantly increase transfection rates (1.2% vs. 0.5%), cell viability was significantly reduced (58.5% viable cells with 20 mg/mL CA, and 78.3% with 2 mg/mL).

These data support recent reports on CA-mediated US bioeffects. Due to the presence of CA, an enhancement of US-induced cell destruction and sonoporation was demonstrated in suspensions of cervical cancer cells with low cell concentrations (Ward et al. 1999), and in human whole blood samples (Poliachik et al. 1999). Miller et al. (1996) stated that the primary effect of US application to cells in culture is membrane damage. The threshold reported for US CA to nucleate inertial cavitation *in vitro* was about 0.4 MPa at 2 MHz (Miller and Thomas 1995). If the sensitivity of the isoluminol method used by these authors to detect H_2O_2 is considered, cell damage at 0.3 MPa used in our study seems likely. Accordingly, contrast agent-induced hemolysis (Miller et al. 1997) as well as cervical cancer cell (HeLa S3) damage (Ward et al. 1999) were found at pressure levels of 0.2 MPa at the same frequency. Along with cavitation, other mechanisms may contribute to cell membrane poration and cell death. Energy transfer to the liquid and bubble streaming are likely to occur at higher concentrations of CA.

Usually, little cell damage is observed unless the US exposure tube is rotated (Miller and Thomas 1995; Ward et al. 1999). The rotation effect is explained by bubble recycling from the rear wall, where the US beam exits the tube, backs again to the front where the bubbles may then renucleate the suspension, and thereby promotes cavitation bioeffects (Miller et al. 1999; Miller and Williams 1989). With the cells growing attached to an immobile membrane in the chamber we used, rotating seems not required for cavitational effects to occur (Figs. 2 and 3).

Enhancement of lipoplex-mediated transfection may only be a concomitant phenomenon of glioma cell damage caused by US. However, it should be possible to employ this selective cell damage also *in vivo* and to kill tumor cells by systemic or local application of CA and focused insonation of the area. Because Levovist® can be injected in humans in doses of 200–400 mg/mL and in total volumes of up to 20 mL (Schlief 1993b), an extrapolated blood concentration of 1–2 mg/mL may be reached in an average adult. This CA agent consists of stabilized galactose microbubbles, as opposed to other CA consisting of stabilized microbubbles produced by albumin sonication with or without octafluopropane gas (Albunex® or Optison®, respectively; MBI, San Diego, CA). The later agents, however, are apparently more efficient in producing US signal enhancement and also in mediating cellular effects of US (Christiansen et al. 1994; Schlief 1993a, 1993b). Miller and Thomas (1995) measured hydrogen peroxide concentration after insonation in a noncavitating rotating tube system, adding Albunex® or Levovist® at different concentrations. These authors demonstrated significant H_2O_2 production for 2-MHz US at 0.41 MPa. Their system, however, is not comparable to our assay, which is based on attached cells, or to assays using floating (nonattached) hematopoetic cells (Poliachik et al. 1999; Brayman and Miller

1999). Greenleaf et al. (1998) were able to increase significantly the efficiency of US-mediated DNA transfection of human chondrocytes by adding to the system artificial cavitation nuclei (Albunex®). Cells in 6-well culture plates were exposed to 1 MHz US with or without Albunex®, and transfection efficiency increased linearly with increased US energy. Because the DNA concentration used in their study was 40 mg/mL, which is much higher that the total amounts of 2.85 μg or 10 μg DNA we used for plate- or chamber-based transfections, respectively, no comparisons between the Greenleaf et al. study and the present study are possible in respect to CA concentration or cell transfection efficiency. Moreover, cell membranes in different cell types have different biophysical characteristics as related to transfection and physical stability (Pagano and Weinstein 1978), which prohibits comparisons between human hematopoetic or connective tissue cells on the one hand, and rat or dog brain tumor cells on the other hand.

US-induced cavitation is a phenomenon observed mainly in cell culture, and US-enhanced gene transfer in vivo seems feasible only if CA is given prior to insonation. In the absence of CA, low-intensity US signals, similar to that employed in clinical therapy, were found to mediate differential gene transfer in human cancer cell lines only under standing wave conditions (Tata et al. 1997) that are hardly achievable *in vivo*. Cell damage, which is commonly described in the presence of CA, can be mediated both by microstreaming around CA bubbles as well as by bubble collapse similar to that seen with cavitation bubbles. The phenomenological similarity of results obtained in our study with microplates and with the absorption chamber, however, suggests that cavitation may be involved in both cases, the cavitation nuclei being provided by the gas content of fresh medium in the first and by CA in the second case.

Further investigations are underway to clarify the role of US enhancement with and without CA on lipoplex transfection in the brain of living animals with transplanted brain tumors.

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