



ACQUIRED DISEASES

RESEARCH ARTICLE

Ultrasound enhancement of cationic lipid-mediated gene transfer to primary tumors following systemic administration

K Anwer¹, G Kao¹, B Proctor¹, I Anscombe¹, V Florack¹, R Earls¹, E Wilson¹, T McCreery², E Unger², A Rolland¹ and SM Sullivan¹

¹Valentis, Inc, The Woodlands, TX; and ²ImaRx, Inc, Tucson, AZ, USA

The impact of a localized application of ultrasound on gene transfer to primary tumors following systemic administration of cationic lipid based transfection complexes was investigated. We have previously shown that systemic administration of DOTMA (N-[(1-(2–3-dioleoyloxy) propyl)]-N-N-N-trimethylammonium chloride):cholesterol-based transfection complexes to tumor-bearing mice resulted in expression in the tumor and other tissues, primarily the lungs. Application of ultrasound to the tumor before or after the injection resulted in a significant increase in gene transfer to the tumor with no increase observed in other tissues. The magnitude of increased expression ranged from three- to 270-fold depending upon the DNA dose. The following parameters were optimized for maximal increase: duration of

ultrasound application, the time interval between plasmid injection and sonoporation, and plasmid dose. A combination of plasmid quantitation and fluorescence microscopy showed that ultrasound increased tumor uptake of the plasmid and that uptake was limited to the tumor vasculature. Using an IL-12 expression plasmid, the combination of a single plasmid dose (10 µg) and ultrasound treatment produced significantly higher levels of IL-12 in tumor. This increased expression was sufficient to inhibit tumor growth compared with the control conditions. These data demonstrate the potential application of sonoporation as an effective method for enhancing the expression of systemically administered genes in tumor endothelium for cancer gene therapy. Gene Therapy (2000) 7, 1833–1839.

Keywords: tumor; cationic liposomes; plasmid; systemic gene delivery; ultrasound; IL-12

Introduction

Gene transfer strategies targeting tumor endothelium to provide sustained, high, and local concentrations of anti-angiogenesis mediators, cytokines, or cytotoxic proteins, thus minimizing potential systemic toxicity, have potential therapeutic value. Current gene delivery systems transfect cells *in vivo* in a manner largely determined by their colloidal and surface properties, blood flow and site of administration.^{1–3} The ability of these systems, however, to deliver therapeutic genes specifically to target cells *in vivo* is still somewhat limited. For example, cationic lipid/DNA complexes have been widely used for gene transfer to endothelial cells *in vivo*.^{3–8} The utility of current cationic lipid-based systems for delivery to distal tumors is limited due to passive targeting properties and relatively low *in vivo* gene transfer efficiency.⁹ Modification of liposome surfaces by covalent conjugation of monoclonal antibodies or other targeting moieties (eg specific peptides and lipids) has been proposed to improve tumor-specific gene delivery.^{10–14} Mechanical methods such as electroporation and jet injections have

also been described as useful external means to enhance gene transfer to target tissue.^{15,16}

Ultrasound-mediated delivery is potentially a powerful new method for enhancing the delivery of therapeutic compounds. Ultrasound-enhanced delivery to cells has been demonstrated *in vitro* by uptake of extracellular fluid, drugs, and DNA into cells.^{17–21} In the present study we describe the combination of an ultrasound method and systemic administration of cationic lipid/DNA complexes that provides for specific enhancement in gene transfer to primary tumors. The enhancement in gene transfer was restricted to sonoporated tumors and no effects were observed in non-sonoporated tissues. A combination of systemic administration and ultrasound treatment may prove to be a useful method for targeting anti-cancer genes to tumors.

Results

Enhancement of systemic gene transfer to subcutaneous tumors by sonoporation

Systemic administration of 15 µg pCMV-CAT complexed with DOTMA:CHOL (4:1 mol/mol) at 3:1 (+/–) lipid/DNA charge ratio into tail vein of SCCVII squamous cell s.c. tumor bearing mice yielded CAT expression in tumor. Ultrasound treatment (1.5 W/cm² for 5 min) of tumors following systemic administration of transfection

complexes increased the levels of CAT expression up to 270-fold (Figure 1a). The enhancement of tumor transfection at 15, 45 and 90 μg DNA dose was 270-, 40-, and six-fold over control, respectively. Increased gene expression following ultrasound application was restricted to the tumor with no increase in expression observed in other tissues, such as the lungs (Figure 1b).

The enhancement of tumor transfection by ultrasound treatment was dependent on the duration of ultrasound treatment. The levels of CAT expression increased with

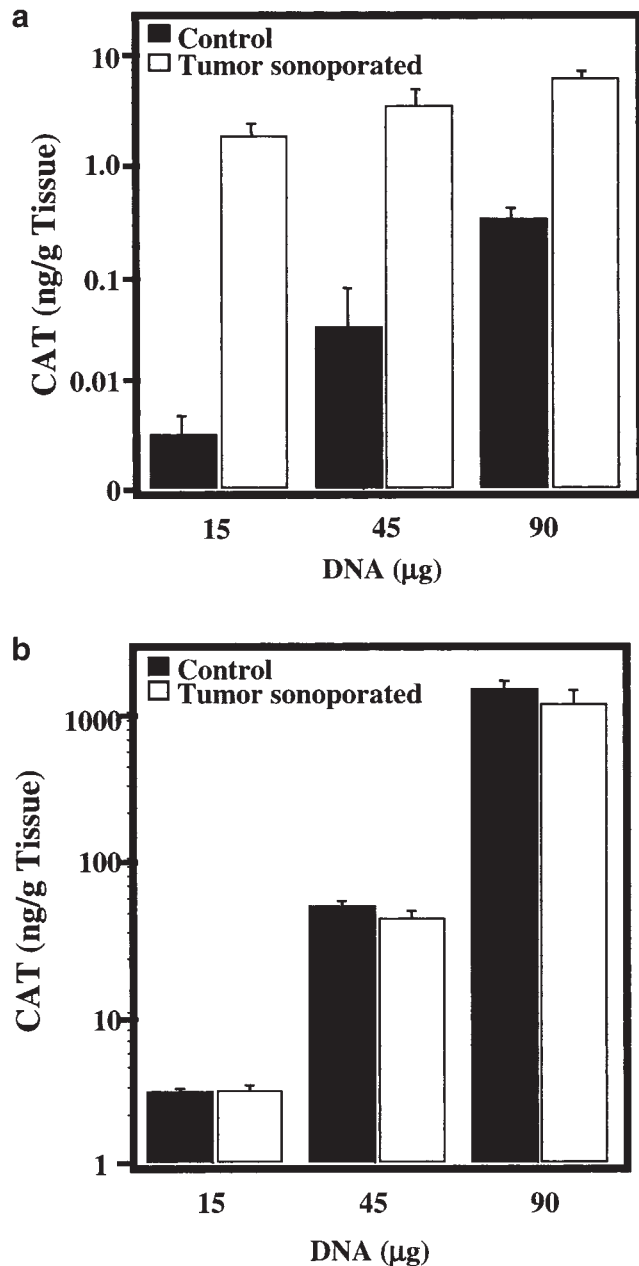


Figure 1 Effect of ultrasound treatment on cationic liposome-mediated systemic gene transfer to s.c. SCCVII tumor bearing mice. A 15, 45 or 90 μg plasmid dose of DOTMA:CHOL formulated CAT plasmid was administered by tail vein to SCCVII tumor-bearing mice. Tumors were sonoporated at 1.5 W/cm² for 5 min immediately after plasmid injection. CAT expression levels in tumor (a) and lung (b) were measured 18–20 h after DNA administration. The data is expressed as mean \pm s.d., n = 5.

sonoporation time (Figure 2). Maximal increase was observed by sonoporation for 5 min after plasmid injection. Increasing the sonoporation time to 15 min did not further enhance the transfection efficiency. The sonoporation efficiency was also sensitive to the time interval between plasmid administration and ultrasound treatment. As shown in Figure 3 maximal enhancement was achieved by applying ultrasound to tumors within 5 min after DNA injection. No enhancement in gene expression was observed when ultrasound treatment was given 30 min after DNA injection. The effect of ultrasound before plasmid administration was also examined. Application (1.5 W/cm² for 2 min) 5 min before DNA (90 μg) injection increased CAT expression from 0.28 \pm 0.09 ng/g to 0.93 \pm 0.21 ng/g tumor. The efficiency was also sensitive to the time interval between application of ultrasound and plasmid administration. Best results were obtained when tumors were treated within 5 min before DNA injection. Pretreatment of tumors (1.5 W/cm² for 2 min) 30 min before DNA administration did not change expression levels (control: 0.29 \pm 0.072; sonoporated 0.308 \pm 0.094 ng/g tumor). Modulation of energy output between 0.5 and 2 W/cm² did not significantly alter the levels of CAT expression in tumors (data not shown).

Plasmid levels in sonoporated tumors were measured to understand the mechanism of increased gene transfer by sonoporation. Measurement of plasmid DNA by qPCR showed three-fold higher plasmid levels in ultrasound treated tumors compared with control tumors (Figure 4). Plasmid delivery to lung was the same for both treated and untreated mice. The effect of ultrasound on intratumoral distribution of plasmid was determined

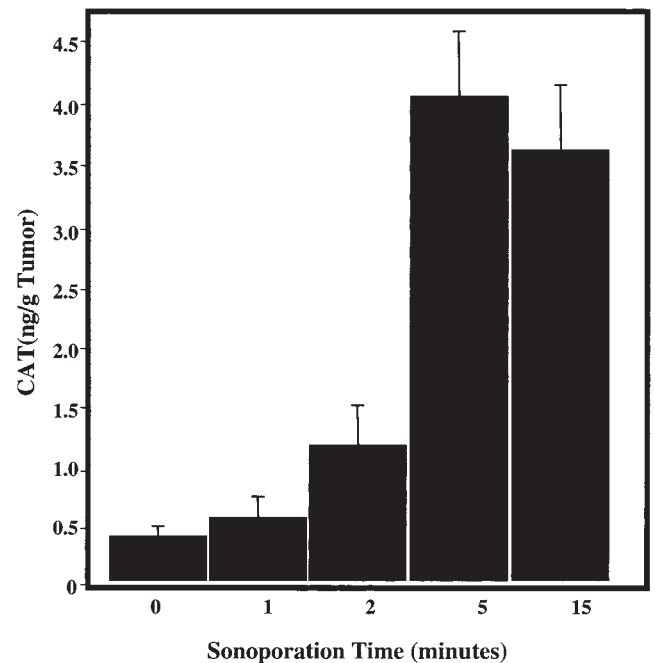


Figure 2 Influence of sonoporation time on CAT expression in mouse lung and s.c. SCCVII tumors after tail vein administration of DOTMA:CHOL/DNA complexes. pCMV-CAT was complexed with DOTMA:CHOL. A 90 μg DNA dose was administered to tumor-bearing mice by tail vein injection. Tumors were sonoporated at 1.5 W/cm² for 1, 2, 5 or 15 min immediately after transfection complex administration. Tumor and lung were collected 18–20 h later for measurement of CAT expression (mean \pm s.d., n = 5).

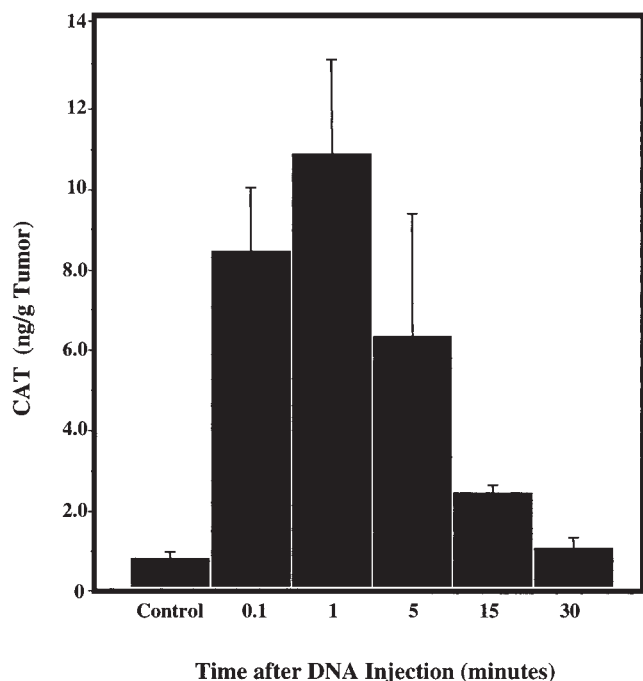


Figure 3 Effect of time interval between DNA administration and sonoporation on CAT expression in mouse lung and s.c. SCCVII tumors after tail vein administration of DOTMA:CHOL/DNA complexes. pCMV-CAT was complexed with DOTMA:CHOL. A 90 µg DNA dose was administered into tumor-bearing mice by tail vein injection. Tumors were sonoporated for 5 min (1.5 W/cm²) 0.1, 1, 5, 15 or 30 min after transfection complex administration. Tumors were collected 18–20 h later for measurement of CAT expression (mean ± s.d., n = 5).

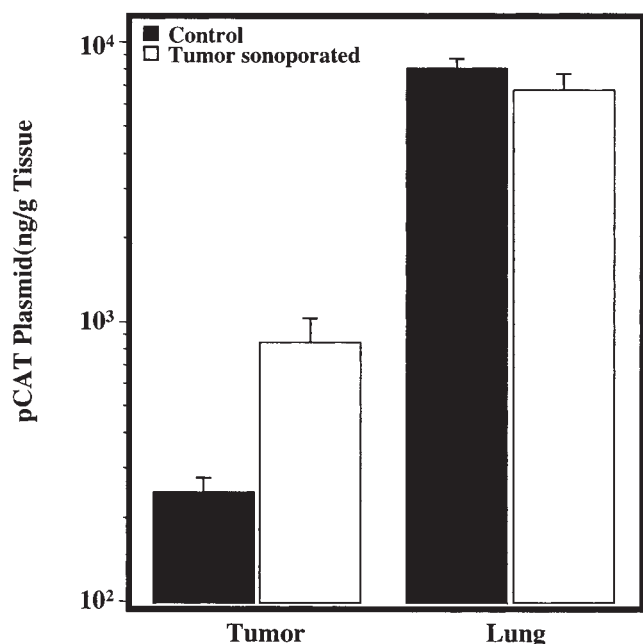


Figure 4 Effect of ultrasound on plasmid uptake by SCCVII primary tumors following tail vein administration of transfection complexes. A 90 µg plasmid dose of transfection complex was administered to SCCVII tumor-bearing mice by tail vein injection and tissues were collected 1 h later for quantitation of CAT plasmid (mean ± s.d., n = 5) by qPCR.

by forming transfection complexes with a psoralen-fluorescein labeled plasmid and administering a 90 µg plasmid dose intravenously. The intratumoral distribution of fluorescent plasmid is shown in Figure 5. The sections were counterstained with a rhodamine-labeled anti-CD31 endothelial cell marker. The fluorescent plasmid is in green and endothelial cells are in red. The fluorescence intensity and frequency of the labeled plasmid appeared to be greater in ultrasound treated tumors (Figure 5b) compared with untreated tumors (Figure 5a). Co-localization of the fluorescent plasmid and endothelial cells in ultrasound-treated tumors showed that increased uptake of transfection complexes in ultrasound-treated tumors was restricted to the tumor endothelium. This is more apparent from high magnification micrograph of the ultrasound-treated tumor.

Ultrasound treatment generated heat, which could increase tumor blood flow by vasodilation and consequently enhance plasmid delivery to tumor. To examine this, tumors were heated to 50–55°C for 5–10 min immediately after tail vein injection of transfection complexes. Heat treatment did not alter the transfection efficiency of intravenously administered DOTMA:CHOL transfection complexes (control: 1.48 ± 0.90 ng/g tumor, heat treated: 1.29 ± 0.70 ng/g tumor). Light microscopy showed increased vasodilation in heat-treated tumors compared with control tumors.

Expression of IL-12 gene and inhibition of tumor growth

The combination of ultrasound treatment and systemic delivery of DNA plasmid by liposomes was examined for expression and biological activity of an anti-cancer gene. Transfection complexes formed with an IL-12 expression plasmid were systemically administered into tumor bear-

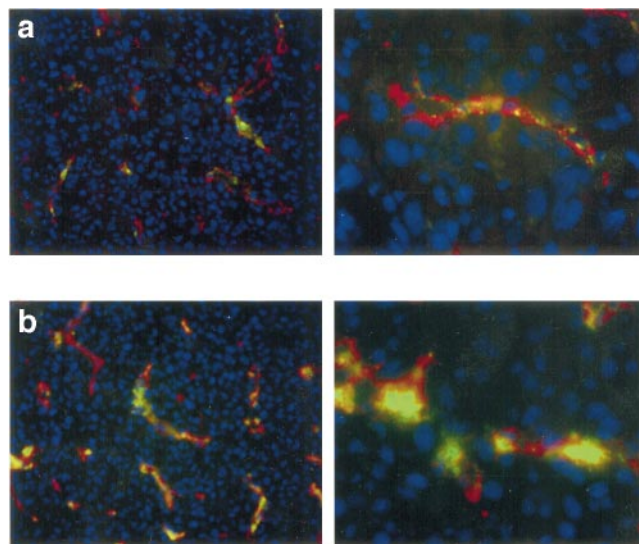


Figure 5 Intratumoral distribution and endothelial localization of intravenously administered fluorescent-labeled plasmid. Psoralen-fluorescein-labeled pCMV-CAT complexed with DOTMA:CHOL at 90 µg dose was administered into s.c. tumor-bearing mice by tail vein injection. Tumors were sonoporated at 1.5 W/cm² for 5 min immediately after plasmid injection. Control (a) and ultrasound treated (b) tumors were collected 15 min after plasmid injection, tissue cryosections (5 µm) were prepared for CD31 endothelial immunostaining and examined by fluorescence microscopy for fluorescent plasmid (green) and endothelial cell marker (red); magnification ×20 (left) and ×60 (right).

ing mice. Ultrasound was applied to the tumors for 2 min at 0.5 W/cm² or 1.5 W/cm² following administration of a 10 μg plasmid dose. Expression of IL-12 in tumor was measured 18–20 h after plasmid injection. As shown in Figure 6, tail vein injection of DOTMA:CHOL/pCMV-IL-12 complexes produced IL-12 expression in tumors. The IL-12 levels in ultrasound treated tumors at 0.5 W/cm² and 1.5 W/cm² were 0.44 ± 0.18 ng/g and 0.52 ± 0.31 ng/g tumor, respectively. IL-12 levels in non-sonoporated tumors were 0.064 ± 0.066 ng/g. A concern arose that increased gene transfer may be the result of physically manipulating the tumor and not due to the application of ultrasound. An experiment was designed to address this concern in which transfection complexes were administered intravenously and the gel and ultrasound device were applied without administration of an ultrasound pulse. Comparisons were made with control and ultrasound-treated groups. Only the tumors to which ultrasound was applied showed increased gene expression compared with untreated tumors and tumors that were manipulated with gel and the ultrasound probe (results not shown).

The effect of IL-12 protein levels on primary tumor growth was determined. The tumors were sonoporated for 2 min at 1.5 W/cm² followed by a single tail vein injection of DOTMA:CHOL transfection complexes containing 10 μg IL-12 plasmid. Tumor growth was measured 14 days after DNA injection. As shown in Figure 7, the combination of systemic administration of IL-12 transfection complexes and ultrasound treatment produced a significant inhibition of tumor growth. The combination of IL-12 transfection complex systemic administration and ultrasound treatment yielded a 65% reduction in tumor growth rate compared with the lactose control

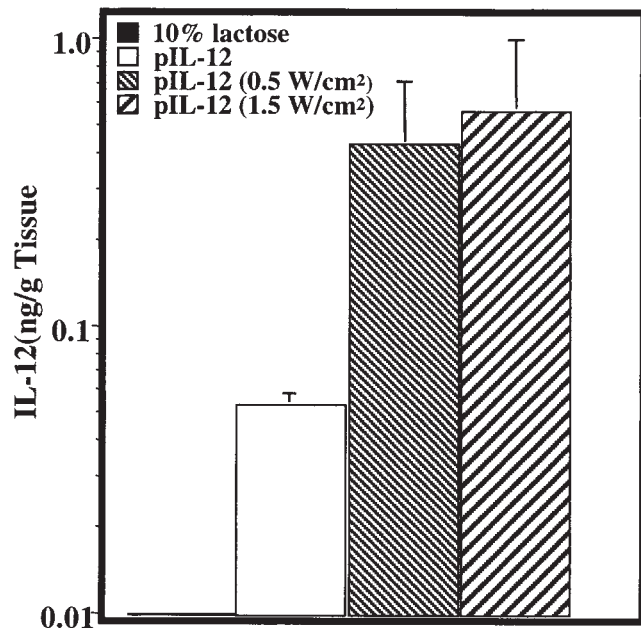


Figure 6 Effect of ultrasound treatment on IL-12 expression in s.c. tumors by tail vein injection of IL-12 plasmid complexed with DOTMA:CHOL liposomes. A 10 μg plasmid dose of cationic lipid formulated IL-12 plasmid was administered by tail vein injection into SCCVII tumor bearing mice. Tumors were sonoporated for 5 min at 0.5 W/cm² or 1.5 W/cm² immediately after the DNA administration. Tissues were collected 18–20 h later for measurement of IL-12 (mean ± s.d., n = 5).

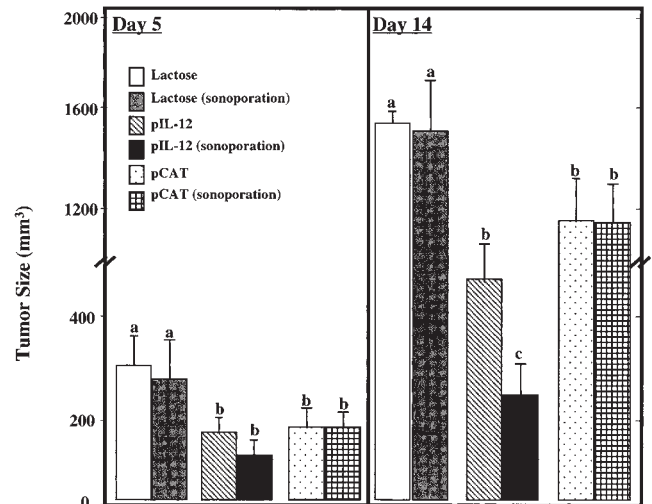


Figure 7 Effect of ultrasound treatment on inhibition of tumor growth following systemic administration of IL-12 transfection complexes. SCCVII tumor-bearing mice were sonoporated for 2 min at 1.5 W/cm² followed by a single tail vein injection of DOTMA:CHOL transfection complexes containing 10 μg IL-12 plasmid or CAT plasmid. Control animals received 10% lactose. Tumor size was measured 14 days after plasmid injection. Bars with different superscripts are statistically different (P < 0.05, mean ± s.d., n = 5) as determined by one-way ANOVA and Duncan's multiple range test.

group. Tumor inhibition from combination treatment was significantly higher than that obtained with IL-12 gene treatment alone. The effect of ultrasound treatment on tumor growth was specific to IL-12 gene since no effect of ultrasound treatment was observed in lactose or CAT gene injected group.

The ultrasound treatment was well tolerated by mice since no obvious sign of discomfort was noted. Gross examination of sonoporation site and major organs including lung, liver, heart, spleen, and guts showed no adverse effects of sonoporation.

Discussion

Cationic lipid-based formulations have been widely described to achieve gene transfer into normal endothelial tissue by systemic administration.^{3–6} Previously, the *in vivo* gene properties of the DOTMA:CHOL/plasmid formulation was characterized with regard to gene transfer to endothelial cells of primary and metastatic tumors following systemic administration.⁹ Transgene expression was clearly demonstrated in tumors. However, significant expression was also detected in nontumor tissues. In this report, a combination of ultrasound treatment and systemic administration of the same DOTMA:CHOL/plasmid formulation yields high levels of gene expression and biological activity of an anti-cancer gene in mouse primary tumors. The enhancement of gene transfer was restricted to tumors treated with ultrasound and did not affect gene expression in nontreated tissues. Gross tissue examination and general physical assessment of the ultrasound-treated animals did not show any signs of adverse effects.

Ultrasound has been a well established diagnostic and therapeutic tool in medicine^{22–25} with specific applications for delivery of drugs including peptides, genes,

steroids and other macromolecules has been recently reported.^{17–21} Ultrasound treatment has been used to introduce DNA vectors into yeast, plant cells, and mammalian cells *in vitro*.^{19–21} In this study, we have demonstrated the application of ultrasound treatment for gene transfer to primary tumors in mice. More than a two-log increase in systemic gene transfer to primary tumor by sonoporation without any effect on gene expression in nontumor tissue demonstrates the usefulness of ultrasound for systemic gene targeting to primary tumors. The differences were greater at low DNA doses. A higher increase in the tumor/normal tissue expression ratio by ultrasound at low plasmid doses showed that the therapeutic index of the cationic lipid delivery system can be significantly increased by combining it with ultrasound. Ultrasound could be effectively used in combination with tumor-targeted delivery systems or expression systems administered systemically to achieve high levels of tumor-specific gene transfer. The magnitude of ultrasound enhancement of tumor gene transfer was affected by the sonoporation exposure time, which indicates that selection of appropriate duration of ultrasound exposure is important to achieve gene transfer at desirable level. In contrast, the observation that enhancement of gene transfer depends only weakly on ultrasound energy over the observed range is useful information, since it permits greater flexibility in designing ultrasound protocols. The effect of ultrasound was inversely related to the time interval between DNA administration and sonoporation application suggesting sonoporation effect is acute and in all likelihood does not involve new protein synthesis.

Higher DNA levels in sonoporated tumors compared with non-sonoporated tumors, as determined by PCR and fluorescent microscopy, suggest that the sonoporation enhancement of gene expression in tumors could be associated with increased DNA accumulation. Colocalization of DNA plasmid with CD31 endothelial cell marker indicates that tumor endothelial cells are the primary sites of DNA uptake in sonoporated tumors. The ultrasound treatment of tumor for longer duration (>5 min) generated heat at the treatment site. To examine if increased DNA accumulation in ultrasound treated tumors results from heat-induced tumor vasodilation, the tumors were heated to 50–55°C immediately after DNA injection. As described in Results, tumor heating resulted in marked vasodilation of the tumor but did not affect the transfection efficiency of the lipid/DNA complexes, suggesting that the increase transfection from sonoporation is not associated with heat-induced tumor vasodilation.

The exact mechanism by which ultrasound treatment enhances gene transfer to tumor is unknown. Increased permeability of cells to macromolecules by ultrasound treatment is thought to be mediated by cavitation, a process of creation and oscillation of gas bubbles in membrane environment.^{25,26} Membrane lipid disordering from cavitation process forming a transport channel in the disordered lipid region has been proposed as the mechanism of transdermal delivery by sonoporation.¹⁸

The biological activity of the ultrasound mediated increase in gene transfer was investigated by substituting an IL-12 expression plasmid for the reporter plasmid. IL-12 is a potent immunostimulatory cytokine^{27–29} with anti-angiogenic activity.^{29,30} The observation that expression levels of IL-12 plasmid were significantly

enhanced by ultrasound treatment demonstrated the potential for ultrasound in anti-cancer gene therapy. We have recently demonstrated expression and anti-tumor activity of IL-12 gene following repeated intravenous administration of DOTMA:CHOL/pCMV-IL-12 (15 µg) complexes in mice.³¹ The data in Figure 7 showed that the combination of a single dose of IL-12 plasmid (10 µg) and ultrasound treatment was more effective in inhibiting tumor growth than the injection of IL-12 transfection complexes alone. This suggests that the sonoporation can be applied to shrink solid tumors that are externally accessible for ultrasound treatment and hence may provide an alternative to surgical ablation that often results in metastases of residual tumors to distal organs through traumatized blood vessels. Another alternative is that ultrasound probe technology can be developed to treat nonexternally accessible tumors.³²

The ultrasound treatment appeared to be well tolerated by the animals during and after the treatment as determined by animal survival, mobility and general well-being. In human cadaver skin, ultrasound enhanced transdermal drug delivery without causing damage to the skin or underlying tissue or altering the permeability properties of the epidermis.¹⁶

In summary, ultrasound is a promising new and safe method for enhancing the efficiency of systemic gene delivery to tumor endothelium. The ultrasound technology used in this study was off-the-shelf technology with no special power supplies or specially designed probes. Using this first generation technology allowed a proof of concept to be demonstrated showing that the combination of ultrasound and systemic administration of a plasmid gene delivery system could achieve selective gene transfer to tumors and yield not only selective expression but inhibition of tumor growth. Minimizing delivery to normal tissue is critical for the development of safe and effective systemic gene therapy methods for treatment of cancer. Further studies are needed to understand the sonoporation mechanism and evaluate its application for other tumor types and tumor lesions that are not accessible without surgery.

Materials and methods

Plasmid construction and preparation

The expression plasmids pCMV-CAT and pCMV-IL-12 were isolated and purified from *Escherichia coli* as described previously.^{6,33} The purity of pDNA preparations was determined by 1% agarose gel electrophoresis followed by SYBR Green (Molecular Probes, Eugene, OR, USA) staining. The DNA concentration was measured by UV absorption at 260 nm. The percentage of supercoiled pDNA and OD_{260/280} ratios was in the range of 70–95% and 1.8–1.9, respectively. Endotoxin levels of pDNA preparations were determined using the chromogenic limulus amoebocyte lysate assay (Chromogenic End-Point, LAL BioWhittaker, Walkersville, MD, USA) and were <50 endotoxin units (EU)/mg.

Preparation of plasmid/cationic lipid complexes

Liposomes containing N-[(1-(2–3-dioleoyloxy)propyl)]-N-N-N-trimethylammonium chloride (DOTMA) and cholesterol (CHOL) (Avanti Polar Lipids, Alabaster, AL, USA) were prepared at 4:1 DOTMA:CHOL molar ratio.

Briefly, lipids were mixed in chloroform and evaporated to a thin film in a 50 ml round bottom flask using a rotary evaporator. The film was hydrated in sterile water, probe-sonicated, centrifuged at 32 000 g for 30 min, and sterile filtered to obtain small unilamellar vesicles (SUV). DOTMA:CHOL/DNA complexes were formed in 10% lactose by controlled mixing of liposomes with DNA plasmid at 3:1 (+/-) charge ratio. The DNA concentration in the formulation for 90 µg dose was 300 µg/ml. For 10 µg, 15 µg, and 45 µg doses, serial dilutions with 10% lactose were made to obtain DNA concentrations of 33 µg/ml, 50 µg/ml, and 150 µg/ml, respectively.

In vivo gene transfer and sonoporation procedure

Solid tumors were created on the flank of 6–8-week-old female C3H mice (20–22 g) (Charles River Laboratories, Raleigh, NC, USA) by s.c. injection of 4×10^5 squamous carcinoma cells. The average tumor size before DNA injection was typically in the range of 30–40 mm³ (6–7 days after implantation). pCMV-CAT was complexed with DOTMA:CHOL (4:1, mol/mol) at a cationic lipid to DNA ratio of 3:1 (+/-) and administered intravenously into the tail vein of mice. Immediately after the injection of transfection complexes, tumors were covered with ultrasound conducting gel Spectrogel 50 (Parker Laboratories, Fairfield, NJ, USA) and sonoporated for 1, 2, 5 or 15 min at 1000 Hz input frequency and 0.5 W/cm² or 1.5 W/cm² output intensity using a sonoporation device (ImaRx, Tucson, AZ, USA). Tumors and lungs were harvested 18–20 h after DNA administration, immediately frozen in liquid N₂, and stored at -80°C.

Assay for CAT and IL-12 expression in mouse tissue

Tumor and lung were homogenized in 0.35 ml and 1 ml TENT (Tris 10 mM, EDTA 1 mM, NaCl 0.1 M, Triton X-100 0.5%) buffer, respectively. The tissue homogenate was centrifuged at 10 000 g for 15 min and supernatant was assayed for CAT or IL-12 using respective ELISA assays (Boehringer Mannheim, Indianapolis, IN, USA).

Distribution of lipid/DNA complexes in tumor

Psoralen-fluorescein-labeled pCMV-CAT⁶ was complexed with DOTMA:CHOL (4:1, mol/mol) at 3:1 (+/-) lipid/DNA charge ratio and injected into s.c. tumor bearing mice via tail vein at a dose of 90 µg DNA per mouse. The tumors were sonoporated at 1.5 W/cm² for 5 min immediately after DNA injection. Fifteen minutes after DNA injection, animals were anesthetized by intraperitoneal administration of a mixture of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and acepromazine (1.4 mg/ml) at a dose of 0.5–0.7 ml/kg, and whole-body perfusion was performed with 1% BSA/PBS solution to clear entrapped blood. Tumors were removed and embedded in OCT embedding medium. Tissue cryosections (5 µm) were examined for plasmid fluorescence with an Olympus BX-60 fluorescence microscope (Melville, NY, USA) and photographed using Montague spot camera (Leica, Buffalo, NY, USA).

CD31 immunostaining of tumor endothelium

Five µm tumor paraffin cryosections (JUNG CM3000) were first incubated with a rat anti-mouse CD31 antibody (Pharmingen, San Diego, CA, USA), and then with a rabbit anti-rat IgG labeled with Texas Red fluorophore (Vector Laboratories, Burlingame, CA, USA). The tumor

sections were fixed in formaldehyde and counterstained with Vectashield mounting medium containing DAPI to highlight nuclei. Controls included unstained sections and incubation with secondary antibody alone. Tissue sections were viewed using an Olympus BX-60 fluorescence microscope and photographed using a Montague spot camera.

Plasmid isolation and quantification

Tissues were digested by incubation with digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS, and proteinase K (0.1 mg/ml) at 50°C. The samples were extracted with an equal volume of Tris-buffered phenol (pH 8.0), followed by extraction with chloroform:isoamyl alcohol (24:1, v/v) and ethanol precipitation. The DNA precipitates were dissolved in TE buffer (10 mM Tris (pH 7.5), 1 mM EDTA), and DNA concentration was measured by UV absorption at 260 nm. The amount of plasmid DNA associated with the tissue was quantified by a polymerase chain reaction (PCR) assay using Taqman PCR (Perkin-Elmer, Foster City, CA, USA). The primers used in the reaction were a forward primer, 5'-TGA CCT CCA TAG AAG ACA CCG GGA C-3' (Genosys Biotechnologies, The Woodlands, TX, USA), which primes in the CMV 5' untranslated region (UTR), and a reverse primer, 5' AGG CCG TAA TAT CCA GCT GAACG-3', which primes in the CAT coding region. The probe sequence was 5'-CCA GCC TCC GGA CTC TAG AGG A-3'. The initial copy numbers of unknown samples were determined by using an Applied Biosystem 7700 sequence detector to compare them with a standard curve generated from purified pCMV-CAT of known initial copy numbers.

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