The improvement of liposome-mediated transfection of pEGFP DNA into human prostate cancer cells by combining low-frequency and low-energy ultrasound with microbubbles

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Received August 25, 2011; Accepted September 30, 2011

DOI: 10.3892/or.2011.1510

Abstract. The aim of this study was to explore the use of a contrast agent to study the effects of exposure to ultrasound, in combination with microbubbles, on liposome-mediated transfection of genes into human prostate cancer cells. A contrast agent was used to study the effects of ultrasound exposure in combination with microbubbles on liposomes, which transfect genes into human prostate cancer cells. The human prostate cancer cell line PC-3 in suspension was exposed to ultrasound with a 20% duty cycle (i.e., 2 sec 'on' time and 8 sec 'off' time) lasting 5 min, with and without ultrasound contrast agent (SonoVue™) using a digital sonifier at a frequency of 21 kHz and an intensity of 4.6 mW/cm^2. Immediately after exposure to ultrasound, cell viability and membrane damage were measured. After exposure to ultrasound, the cell suspensions were put into 12-well plates and cultured for 24 h. Fluorescence microscopy and flow cytometry were used to detect pEGFP transfection efficiency. Exposure to ultrasound alone and ultrasound combined with microbubbles resulted in minimal cell death and induced negligible cell membrane damage. Ultrasound combined with microbubbles had a greater effect on cell membrane damage in all groups: the average cell membrane damage was 41.87%, and it was approximately 42-fold greater than in the control group. The average transfection efficiency of PC-3 cells was 20.30% for the liposome (Lipofectamine™)+pEGFP+ultrasound+ultrasound contrast agent (SonoVue) group; this was the highest rate of all groups measured and was approximately 81-fold greater than that of the control group. The use of low-frequency and low-energy ultrasound, in combination with microbubbles, could be a potent physical method for increasing liposome gene delivery efficiency. This technique is a promising non-viral approach that can be used in prostate cancer gene therapy.

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Key words: low-frequency ultrasound, low-energy ultrasound, liposome, microbubble, transfection, gene therapy

Introduction

Prostate cancer was ranked first in incidence rate of cancers among men in the US in 2010, and its mortality was ranked second, behind lung cancer (1). The main methods of therapy used to treat prostate cancer are surgery, radiation and hormone therapy (2,3). These methods have certain curative effects as therapies for cancer, but they still have some limitations, such as injury to surrounding tissues, drug resistance and recurrence (3-5).

Gene therapy is a promising method for the treatment of human diseases. Among many other anticancer treatments, gene therapy has gained attention in clinical trials for its low incidence of side effects, compared to chemotherapy and radiotherapy (6). Several methods have been developed for the delivery of DNA into cells. These methods include chemically facilitated, vector-mediated, mechanical (7) and electric pulse methods (8). In current clinical protocols for gene therapy, virus-derived vectors and non-virus-derived vectors have been used in most trials (9). Although viral vectors have high transfection efficiencies over a wide range of cell targets, they have major limitations, such as immune responses to viruses and insertional mutagenesis, when used as vectors in clinical trials (10). These unwanted side effects have drawn attention to non-viral methods of gene transfer. Non-viral vectors, such as liposome-mediated gene transfer, are attractive alternatives to viral vectors due to their safety, versatility and ease of preparation and scale-up. However, non-viral vectors generally suffer from relatively low transfection efficiencies (11,12). The combination of ultrasound with microbubbles is also a non-viral vector to mediate gene transfection and can possibly be applied for clinical use because it is considered easier and safer than other methods; also, it could strengthen the effect of the

non-viral methods of gene transfer. Collapsing microbubbles and the cavitation bubbles created by this collapse generate impulsive pressures, such as liquid jets and shock waves, that cause transient membrane permeability and allow exogenous molecules to enter cells. These pressures also affect neighboring cells. The shock-wave propagation distance from the center of a cavitation bubble that has the potential to damage the cell membrane is considerably greater than the maximum radius of the cavitation bubble (13).

In this study, we evaluated the improvement of liposome-mediated transfection of green fluorescent protein (pEGFP) DNA into human prostate cancer cells by low-frequency and low-energy ultrasound combined with microbubbles. The aims of the study were to elucidate the mechanism by which ultrasound combined with microbubbles improves liposome-mediated gene transfer that might be used to treat prostate cancer.

Materials and methods

This study obtained permission from the ethics committee of the Shanghai Jiao Tong University Affiliated 6th People's Hospital and the Shanghai Institute of Ultrasound in Medicine.

Cell culture. In order to study solid prostate cancer tumor-cell transfection at the cellular level, we used a human prostate cancer cell line, PC-3, which was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37˚C in humidified air containing 5% CO_2. In the experiment, PC-3 cells were resuspended and counted for a density of $1x10^5$ cells/ml, and then they were put into 1.5-ml polystyrene sample test tubes, which showed no significant effect on the acoustic permeation ratio when exposed to ultrasound. Each tube contained a 1-ml suspension of PC-3 cells. The diameter of the tubes was 13 mm, and the tube bottoms were planar, which allowed them to be placed more closely to the ultrasound probe.

Ultrasound apparatus and microbubbles. Gene transfection was performed by FS-450 ultrasonic processing (Shanghai Institute of Ultrasound in Medicine, China) using a SonoVue™ microbubble echo-contrast agent (Bracco SpA, Milan, Italy). The FS-450 ultrasonic processor was equipped with a built-in digital timer, intensity regulator and duty factor controller; the duty cycle could range from 10 to 90%, and the probe frequency was fixed at 21 kHz. The peak acoustic amplitude in degassed water was measured using a calibrated poly-(vinylidene difluoride-trifluoroethylene) needle-type hydrophone, 0.5 mm in diameter (Toray Techno Co., Ltd., Japan), connected to a PC/AT-compatible computer and a digitizing oscilloscope (TDS3034, Tektronix Japan, Ltd., Japan). The spatial-average temporal average intensity (ISATA) and peak acoustic pressure corresponding to the reading output were 4.6 mW/cm^2 and 0.007 MPa, respectively. In all studies, ultrasound was generated by a 21-kHz ultrasound probe, and the duty cycle was 20% (i.e., 2 sec 'on' time and 8 sec 'off' time). The exposure time was 5 min. The shape of the probe was cylindrical, and the diameter of the ultrasound probe was 13 mm, which was the same as the diameter of the test tubes.

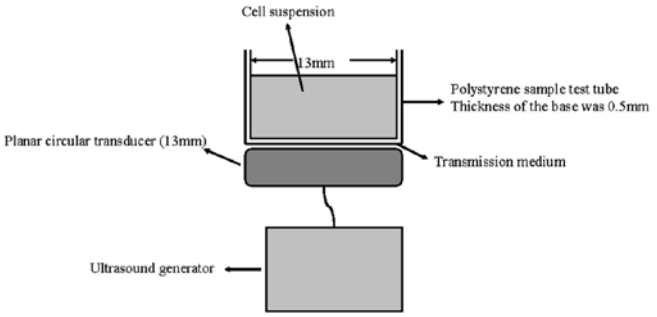

Figure 1. Experiment setup for ultrasound exposure.

In all experiments, the clamp was attached to a metal stand to keep the transducer facing directly upward. The tube was placed on the center of a transducer intermediated with gel (Fig. 1). This setup allowed for standing wave formation due to the reflection of ultrasound radiation at the water-air interface (14). We used a near acoustic field produced by the liquid-air interface and did not reduce standing waves because of the efficient occurrence of cavitation (15).

The SonoVue agent used was a lipid-shelled ultrasound contrast agent composed of microbubbles filled with sulfur hexafluoride gas. The microbubbles were 2.5-6.0 μm in diameter. Upon use, the SonoVue was reconstituted in 5 ml phosphate-buffered saline (PBS) that contained $2\text{-}5x10^8$ microbubbles/ml.

Preparation of plasmid DNA. The pEGFP plasmid DNA (donated by Dr Cui-Xia Yang, Center Laboratory of Shanghai Jiao Tong University Affiliated 6th People's Hospital, China) was prepared with a special reagent (E.ZN.A Plasmid Miniprep kit II, Omega Bio-Tek Co., USA). Briefly, DH5α transformants of sufficiently high density that could express the target plasmid were made and lysed using the lysing solution in the kit. The plasmid DNA was isolated from the genomic DNA using the DNA-specific resin in the column and collected.

The purity of the extracted pEGFP plasmid DNA was more than adequate, given that the optical density value of 260/280 nm was 1.8, as measured on an ultraviolet spectrophotometer (DU800, Beckman Coulter, USA). pEGFP plasmid DNA was identified using a digestive enzyme (*Sal*I or *Xho*I) and subsequent electrophoresis. The map of pEGFP was analyzed and two restriction sites, *Sal*I and *Xho*I, were included to verify that the obtained plasmid was pEGFP.

Detection of cell death and membrane damage. Cells were randomly assigned into four groups: a control group, without exposure to ultrasound and without the addition of SonoVue; group A, without exposure to ultrasound and with the addition of SonoVue (200 μl/ml); group B, exposure to ultrasound without the addition of SonoVue; and group C, exposure to ultrasound with the addition of SonoVue (200 μl/ml). Each group consisted of six samples. Ultrasonically-induced cell damage was detected by using trypan blue dye (0.4%) immediately after 5 min of sonication (16). Cells stained with trypan blue were counted under an optical microscope using a hemocytometer. The cell death rate was obtained from the following

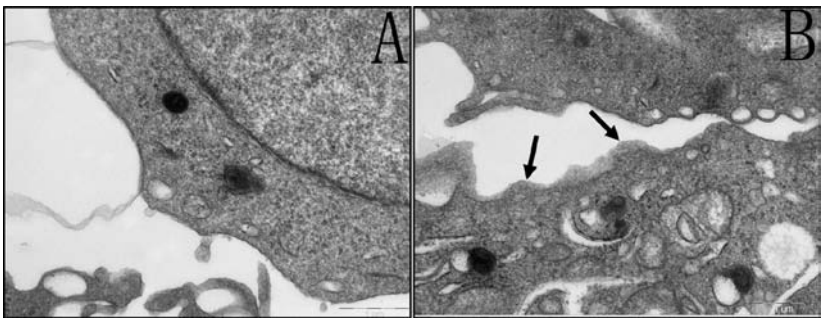

Figure 2. Representative transmission electron microscopy photomicrographs of PC-3 cell membrane damage in (A) the control group and group A and (B) in group B and C. Magnification, x24500.

equation: cell death rate = the number of stained cells/the total number of cells in the group x100%.

In order to confirm that membrane damage occurred, 50 μl of fluorescein isothiocyanate (FITC)-dextran (25 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to each sample. FITC-dextran is the conjugate of fluorescein and dextran, and has a molecular weight of 70000 Da, which makes it difficult to penetrate the cellular membrane under normal conditions (17). After 5 min of exposure, samples were immediately washed 3 times using PBS and the fraction of fluorescence-positive cells was measured (10000 cells in each well were detected) by flow cytometry (BD FACSAria, BD Biosciences, CA, USA), with an excitation wavelength of 492 nm and an emission wavelength of 518 nm (16).

Transmission electron microscopy observation. Immediately after 5 min of sonication, the cells were observed using transmission electron microscopy (TEM).

Detection of gene transfection efficiency. To prepare the reagent of transfection according to the protocol of the Lipofectamine™ 2000 kit (Invitrogen), the ratio of plasmid DNA (μg) to liposome (μl) was 1:2. Before ultrasound irradiation, the reagent was added to the suspension of PC-3 cells in each sample. The PC-3 cells, at a density of $1x10^5$ cells/ml, were resuspended in polystyrene sample test tubes. Diluted DNA in 100 μl of DMEM without serum was mixed gently. Lipofectamine 2000 was mixed gently before use and then the appropriate amount was diluted in 100 μl of DMEM without serum, followed by incubation for 5 min at room temperature. After 5 min, the diluted DNA was combined with the diluted Lipofectamine 2000, mixed gently and incubated for 20 min at room temperature. Cells were divided into eight groups, each consisting of six samples: the control group (pEGFP), group A (SonoVue+pEGFP), group B (ultrasound+pEGFP), group C (SonoVue+ultrasound+pEGFP), group D (Lipofectamine+pEGFP+SonoVue), group E (Lipofectamine+pEGFP), group F (Lipofectamine+pEGFP+ultrasound) and group G (Lipofectamine+pEGFP+ultrasound +SonoVue). In groups A, C and D, 200 μl of SonoVue was added into each tube in each group before exposure. After 5 min of exposure, the cell suspensions were plated into 12-well plates, and after 4 h, the serum-free medium was replaced by medium containing 10% fetal bovine serum. If the pEGFP DNA transfected into the cytoplasm, the cells would express green fluorescence and could be identified by flow cytometry. After culturing the cells for 24 h, the pEGFP transfection efficiency was detected with fluorescence microscopy and flow cytometry.

Table I. Cell death and membrane damage after ultrasound exposure (mean ± SD).

Group	Cell death (%)	Cell membrane damage (%)
Control group	0.45±0.19	1.13±0.12
A (SonoVue)	0.47±0.33	1.28±0.15
B (ultrasound)	4.98±1.23[a,b]	23.02±2.54[a,b]
C (ultrasound+SonoVue)	6.27±0.81[a-c]	41.87±2.55[a-c]

[a]P<0.05 vs. control group; [b]P<0.05 vs. group A (SonoVue); [c]P<0.05 vs. group B (ultrasound).

Statistical analysis. ANOVA was used to analyze the differences in cell death and membrane damage among the four groups and the differences in gene transfection efficiency among the eight groups. P<0.05 was considered statistically significant.

Results

Cell death and membrane damage. The results of cell death and membrane damage are presented in Table I. To assess cell death and membrane damage, the cells were examined immediately after sonication. The cell death rates induced in group B and group C were significantly greater than those of the control group and group A. There was no difference between the control group and group A.

Cell membrane damage was evaluated with flow cytometry. The cell membrane damage rate in group C was the highest of the four groups, and it was approximately 42-fold greater than the control group. The cell membrane damage rate in group B was also greater than those of the control group and group A.

Transmission electron microscopy observation. Immediately after ultrasound treatment, the cells were observed using TEM. In groups B and C, there was some cell membrane damage, cell membrane discontinuity, and there were gaps in the cell

Table II. Gene transfection efficiency of PC-3 cells after ultrasound exposure (mean ± SD).

Group	Gene transfection efficiency (%)
Control (pEGFP)	0.25±0.39
A (SonoVue+pEGFP)	0.32±0.55
B (ultrasound+pEGFP)	1.50±0.51[a,b]
C (SonoVue+ultrasound+pEGFP)	3.85±0.55[a-c]
D (Lipofectamine+pEGFP+SonoVue)	8.87±0.94[a-d]
E (Lipofectamine+pEGFP)	9.65±1.20[a-d]
F (Lipofectamine+pEGFP+ultrasound)	14.00±0.77[a-f]
G (Lipofectamine+pEGFP+ultrasound+SonoVue)	20.30±1.17[a-g]

[a]P<0.05 vs. control group; [b]P<0.05 vs. group A (SonoVue+pEGFP); [c]P<0.05 vs. group B (ultrasound+pEGFP); [d]P<0.05 vs. group C (SonoVue+ultrasound+pEGFP); [e]P<0.05 vs. group D (Lipofectamine+pEGFP+SonoVue); [f]P<0.05 vs. group E (Lipofectamine+pEGFP); [g]P<0.05 vs. group F (Lipofectamine+pEGFP+ultrasound).

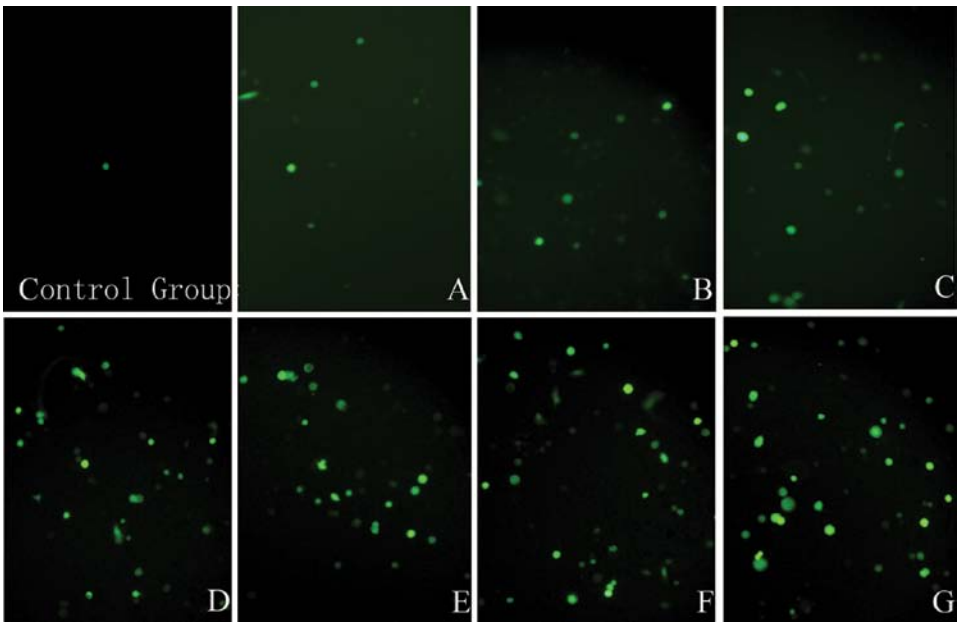

Figure 3. pEGFP expression in PC-3 cells of the control group and of groups A-G examined by fluorescence micrographs. Magnification, x20.

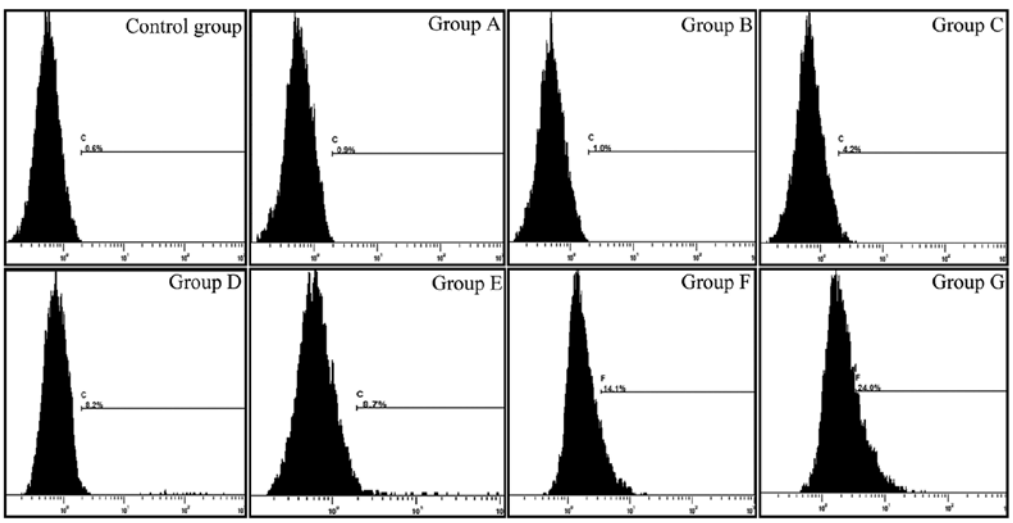

Figure 4. pEGFP expression in PC-3 cells of the control group and of groups A-G examined by flow cytometry.

membranes (Fig. 2B). In the control group and group A, the cell membranes were continuous (Fig. 2A) and there was no cell membrane damage.

Detection of gene transfection efficiency. The results for gene transfection efficiency by flow cytometry are presented in Table II and Fig. 4. In group G (Lipofectamine+pEGFP+ultrasound+SonoVue), fluorescence microscopy revealed that the PC-3 cells transfected by the pEGFP gene had a detectable green fluorescence in the cytoplasm, while the cells in the other groups had less coloration (Fig. 3). Flow cytometry also showed that group G of the PC-3 cells had higher pEGFP expression than the other groups, and the transfection efficiency of group G was ~81-fold greater than that of the control group.

Discussion

In this study, PC-3 cells containing FITC-dextran were treated with ultrasound alone and with ultrasound and SonoVue and the results showed that ultrasound exposure alone induced permeation of FITC-dextran (23.82%) into PC-3 cells, as detected by flow cytometry. The proportion of FITC-dextran-positive PC-3 cells increased to 41.87% after the addition of SonoVue. Using transmission electron microscopy, we also found cavitation of the cells after ultrasound exposure, with and without SonoVue. In this study, we used regular cell media that were neither degassed nor air-saturated, to avoid any changes in cells produced by these procedures. The cell media may have contained air bubbles that could produce cavitation (18). Under ultrasound exposure, microbubbles suspended in liquid can be collapsed intentionally by insonation; this collapse creates a mechanical force on the cell membrane and destroys the integrity of the adjoining cellular membrane. When microbubbles are added to the cell suspension, the cavitation effect is greater than when ultrasound is used alone. The mechanical and physical forces of ultrasound exposure will destroy cells if the energy and exposure time are beyond the tolerance limit of the culture, and these forces can even induce cell lysis, leading to death (19,20). Our data indicate that ultrasound combined with SonoVue has a minimal effect on the viability of PC-3 cells. Our results imply that ultrasound combined with SonoVue is a promising microbubble-based technique for gene delivery.

In this study, we found that ultrasound combined with microbubbles improved the gene transfection efficiency of liposomes, and the efficiency was greater in group G than in groups A, B, C, D, E or F or the control group. The molecular weight of pEGFP is 27000 Da, which is less than that of FITC-dextran. The amount of genetic material that entered the cells through the cavitations that were induced by ultrasound combined with microbubbles may be greater than the amount of FITC-dextran that entered under similar conditions.

In our study, we did not find that ultrasound or ultrasound combined with microbubbles had high gene transfection efficiency (21,22). We believe there are various reasons for this finding. Firstly, different microbubbles have different characteristics. The nature of the shell and parcel of the gas affect the transfer capacity of the cell and different microbubbles, used with the same experimental cells and tissues, will produce different results (23,24). In past studies, the microbubbles used were Levivost (25), Optison (7) and SonoVue (26). Secondly, ultrasound combined with microbubbles improved gene transfection efficiency, not only because of the induced cavitation, but also because of the influence of the microbubbles and because of DNA or RNA interactions (27,28). Thus, some of our results differ from those of past studies. In the past studies, it was shown that lipid-based contrast agents are more suitable for ultrasound-mediated gene transfection than no contrast agents at all (29). Therefore, in this study, we chose SonoVue, which contains phospholipids.

In past studies, the authors usually used ultrasound frequencies between 1-3 MHz (10,21). In this study, we used low-frequency and low-energy ultrasound, which has advantages such as easy penetration of the organism, less tissue absorption and less induction of tissue injury. The effects induced by low-frequency ultrasound are mainly mechanical effects and cavitation effects and the temperature increase through the thermal effect is virtually negligible (30). Given these advantages, low-frequency and low-energy ultrasound shows promise for future use in cancer therapy.

Our study showed that sonoporation, in the presence of microbubbles, is a promising technique that improved the liposome transfer of genes into prostate cancer cells, and may provide an experimental model for clinical gene therapy. Additionally, low-frequency and low-energy ultrasound that induced the destruction of microbubbles and is combined with liposomes is a feasible and efficient method of gene delivery into prostate cancer cells. Although the exact mechanisms underlying efficient gene transfection remain incompletely understood, the rapid collapse of microbubbles during sonoporation is considered to play a major role in gene delivery into cells.

Acknowledgements

This study was supported by the major infrastructure projects of Shanghai Science and Technology under grant No. 10JC1412600.

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