Plasmid DNA introduced into cultured cells with diagnostic ultrasound

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Abstract. The usefulness of diagnostic ultrasound in gene transfer was investigated. The hepatocellular carcinoma cell lines PRL/PRF/5 and Hep3B, and the pancreatic carcinoma Panc-1 cells were transfected with Lipofectin or irradiated with a linear probe with a frequency of 8 MHz at a mechanical index of 0.4 through the bottom of the plates for 5 min using diagnostic ultrasound (US) with pEGFP-N1 [green fluorescent protein (GFP) expression plasmid], and observed under fluorescence microscopy 48 h later. The cell lines were transfected or irradiated with US with pGL3 control (luciferase reporter plasmid), and a luciferase assay (48 h later) or an MTS assay (72 h later) were performed. Although signals of GFP were observed in cells with US, the transfection efficiencies of US were lower than those of transfection. Luciferase activities of cells with US were higher than those of non-irradiated or transfected cells, but lower than those of transfection. Cell viability with US did not change.

Introduction

Gene therapy shows promise for the treatment of inherited or acquired diseases, such as cancer. Genes can be transferred with viral or non-viral vectors. Adenovial vectors provoke severe systemic immune response (1). With plasmid DNA, on the other hand, induction of immune responses could be avoided, since this technique does not result in production of exogenous proteins, such as viral capsid proteins (2). Moreover, plasmid DNA rarely integrates with *in vivo* transfer (3). These characteristics make plasmid DNA ideal for gene transfer.

Plasmid DNA was successfully introduced into cultured cells with a sonicator (4). Irradiation of low output intensity ultrasound increases the effects of introduction of genes into tissues (5). The biological effects of ultrasound are categorized as thermal and non-thermal. Non-thermal effects are caused by cavitation, and mechanical perturbation in the vicinity of bubbles, leading to membrane poration (6). This phenomenon, sonoporation, is a potential new method of gene therapy as evidenced in a murine model (7).

Hepatocellular carinoma (HCC) arises from the liver after long term infection with hepatitis B virus or C virus (8). Pancreatic cancer is dismal since its prognosis is poor. To improve the prognosis of HCC and of pancreatic cancer, molecular therapy is currently under clinical investigation and promising results have been reported (9). Experimentally, novel approaches have been under investigation (10-12).

Problems arise regarding safety and accuracy when applying molecular therapy to HCC with gene transfer. Standing waves and temperature increases may damage cells to improve efficiency of gene delivery with sonoporators (13). It is impossible to monitor the fields to introduce genes with sonoporators since many of them do not have displays. Diagnostic ultrasound is safe enough for wide clinical use. It also enables irradiation of a target area with display and introduction of therapeutic genes to a tumor. We, thus, attempted to introduce plasmid DNA to cultured HCC and pancreatic cancer cells with a diagnostic ultrasound system.

Materials and methods

Cell culture, transfection and irradiation with diagnostic ultrasound. Hep3B and PLC/PRF/5, hepatocellular carcinoma cell lines, and Panc-1, a pancreatic cancer cell line, were purchased from Cell Bank (RIKEN BioResource Center, Tsukuba, Japan) and cultured in Dulbecco's minimum Essential medium (DMEM) (Sigma-Aldrich Japan K.K., Tokyo, Japan), and Rosewell Park Memorial Institute RPMI-1640 (Sigma Aldrich Japan K.K.), respectively, supplemented with 100 g/l of fetal bovine serum (FBS) (Life Technologies Japan, Ltd., Tokyo, Japan) in 5% carbon dioxide at 37˚C in a humidified chamber.

Irradiation with diagnostic ultrasound. Cultured cells were irradiated with a linear probe at the frequency of 8 MHz, pulsed mode, at the mechanical index (MI) of 0.4 using SSA-700A

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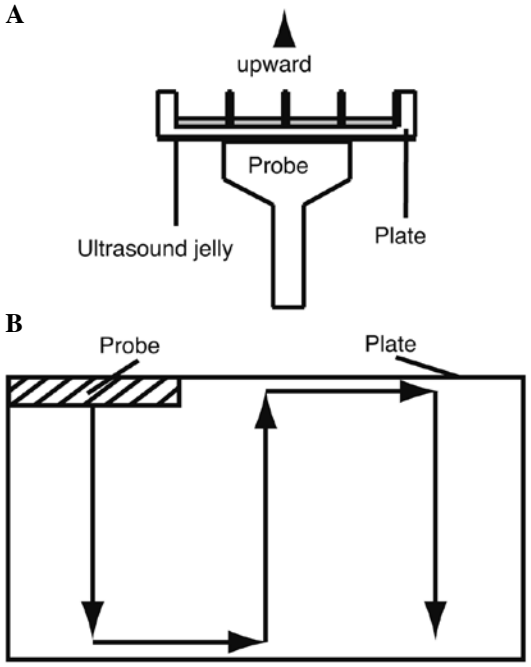

Figure 1. Methods of irradiation with diagnostic ultrasound. (A) A linear probe was touched upward to the surface of the bottom of a plate through ultrasound jelly (a coupling agent) and moved like scanning in clinical use. The actual orientation was vertically upward. (B) The image depicts a schematic view from the bottom. The probe was moved slowly for 5 min with care so that all the surface of the bottom was irradiated. Gray box, culture media; hatched box, probe; arrowhead, upward direction; arrow, scanning direction.

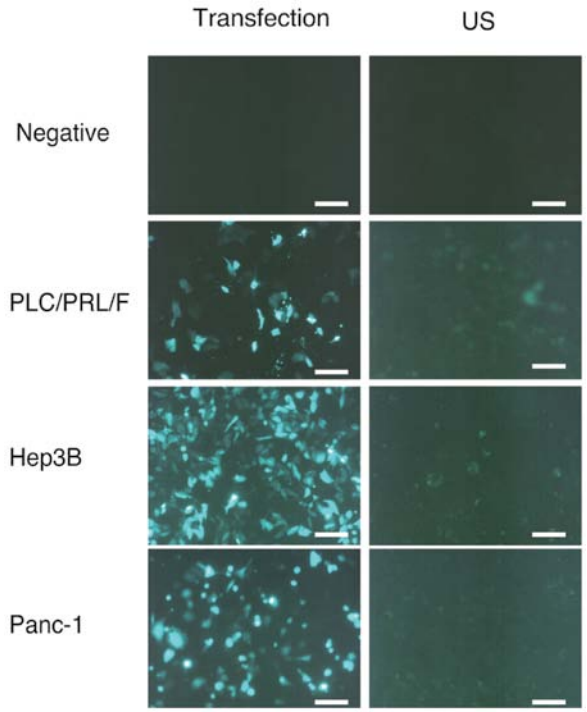

Figure 2. Introduction of GFP expression plasmids. Hep3G, PLC/PRF/5 and Panc-1 cells were cultured in media with pEGFP-N1 and transfected or irradiated with diagnostic ultrasound. Forty-eight hours later, the cells were observed under fluorescence microscopy. Transfection, transfection with Lipofectin; US, irradiation with diagnostic ultrasound; negative, no plasmid in the media of Hep3B as a representative. Original magnification, x20; scale bar, 100 μm.

(Toshiba Medical Systems Corporation, Ohtawara, Japan). The MI of 0.4 was the lowest value provided by our SSA-770A with the linear probe. The other probes, such as convex or sector, were not used since they had curved shape and did not fit the flat bottom of the plates. The probe was touched upward to the bottom of a plate through ultrasound jelly (Fig. 1A). Plates used were 96-well or 24-well plates (Asahi Techno Glass, Funabashi, Japan). The probe was scanned slowly with care to irradiate all fields for 5 min (Fig. 1B). It was thought that standing wave or increase of temperature of the media did not occur since the probe did not fix to a certain area.

Fluoroscence microscopy and transfection efficiency. When cells reached 70% confluency in 24-well plates, they were transfected or irradiated with diagnostic ultrasound with 0.46 μg of pEGFP-N1 (Clontech Laboratories Inc., Mountain View, CA, USA), a green fluorescent protein (GFP) expression plasmid, in each well. After 48 h, cells were observed under fluorescence microscopy. Photographs were taken from 5 different fields. Transfection efficiency was calculated as the number of fluorescence positive cells under a fluorescence microscope divided by the number of cells in the same field under light microscopy.

Luciferase assay. When cells reached 70% confluency in 24-well plates, they were transfected with Lipofectin (Life Science Technologies Japan K.K.) or irradiated with diagnostic ultrasound with 0.46 μg of pGL3-control vector and 0.046 μg of pRL-TK (Promega K.K., Tokyo, Japan) in the medium. The transcriptional activity was measured with a dual luciferase reporter assay system (Promega K.K.) using Gene Light (GL-200A) (Microtech Co., Ltd., Funabashi, Japan). Addition of plasmid in the media without transfection or US was used as the negative control.

Cell viability. Cells were split into 96-wells at the density of 1000 cells/well. After 24 h, cells were transfected or irradiated with diagnostic ultrasound with plasmid in the media. After 72 h, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed following the manufacturer's instructions (Promega K.K.). MTS was bioreduced by cells into a colored formazan product that reduces absorbance at 490 nm. Absorbance was analyzed with a multiple plate reader at a wavelength of 490 nm with iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. One-factor analysis of variance (ANOVA) was performed with JMP8.0 (SAS Institute Japan, Tokyo, Japan). $P<0.05$ was accepted as statistically significant.

Results

Signals of GFP were observed under fluorescence microscopy (Fig. 2). Cells transfected with Lipofectin showed significantly strong signals while those irradiated with diagnostic ultrasound (US) showed weak signals in all the cell lines, including PRL/PLC/5, Hep3B and Panc-1. No signal was detected in cells without plasmid in the media.

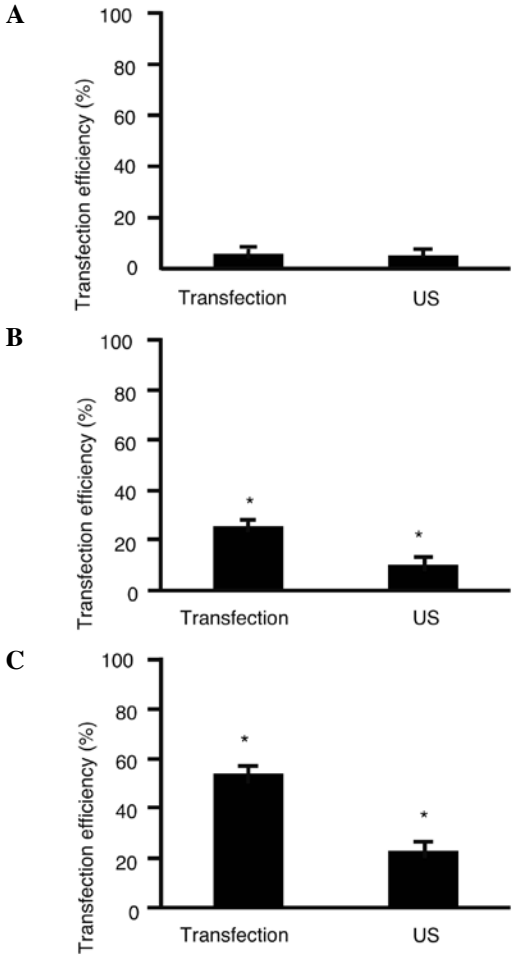

Figure 3. Transfection efficiency. The number of GFP-positive cells was counted and divided by the number of cells in the same field. No difference was found between transfection and irradiation with diagnostic ultrasound in (A) PLC/PRF/5 cells, while transfection efficiency was lower in US than with transfection (P<0.05) in (B) Hep3B and (C) Panc-1 cells. Transfection, transfection with Lipofectin; US, irradiation with diagnostic ultrasound. *P<0.05 (one-factor ANOVA). N=3.

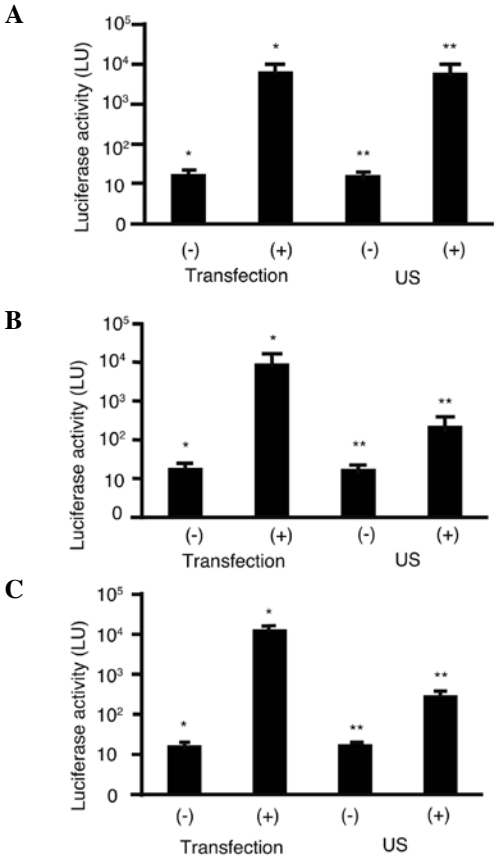

Figure 4. Luciferase assay. Hep3B, PLC/PRF/5 and Panc-1 cells were transfected or irradiated with diagnostic ultrasound with pGL3 control in the media. Forty-eight hours later, the cells were subjected to luciferase assay. The Y-axis is on a logarithmic scale. (A) PLC/PRF/5, (B) Hep3B and (C) Panc-1. LU, light unit; transfection, transfection with Lipofectin; US, irradiation with diagnostic ultrasound; (-) and (+), plasmid in the media without or with transfection or irradiation of diagnostic ultrasound, respectively. *P<0.05, **P<0.05 (one-factor ANOVA). N=3.

The transfection efficiency was calculated to compare the efficiency of introduction of the plasmid to cells with US versus that with transfection with Lipofectin. The transfection efficiency of PLC/PRF/5 cells was 5.9±1.8% (average ± standard deviation) by transfection and 5.2±1.2% with US (Fig. 3A). The transfection efficiency of Hep3B cells was 25.7±1.7%, by transfection and 10.2±1.1% with US (Fig. 3B). The transfection efficiency of Panc-1 cells, was 54.1±9.0% by transfection and 23.0±5.1% with US (Fig. 3C). No difference was seen in PLC/PRF/5 cells, while the transfection efficiency was lower in Hep3B and Panc-1 cells by US (P<0.05). Specifically, the transfection efficiency of US was 89.1% of that with transfection in PRL/PRF/5 cells, 39.7% in Hep3B and 42.5% in Panc-1 cells. There was a tendency that the transfection efficiency was largest in Panc-1 cells with transfection as well as US and lowest in PLC/PRF/5 cells.

Next, luciferase activity was analyzed to reveal the efficiency of expression of genes introduced into cells with plasmid. The luciferase activities without or with transfection of PLC/PRF/5 cells was 18.0±2.0 (average ± standard deviation) and $6.6x10^3$±$1.5x10^3$ (P<0.05), respectively, while those of without or with US were 17±1.1 and $6.3x10^3$±$4.0x10^3$ (P<0.05), respectively (Fig. 4A). Likewise, in Hep3B cells, the respective luciferase activities were 19±2.0% and $9.4x10^3$±$6.6x10^3$ (P<0.05), and 18±3.0 vs. $2.3x10^2$±$1.4x10^2$ (P<0.05) (Fig. 4B). In Panc-1 cells, the luciferase activities were 17.0±2.0 vs. $1.3x10^4$±$5.8x10^2$ (P<0.05) with transfection, and 18.0±1.0 vs. $3.0x10^2$±24 (P<0.05) with US, respectively (Fig. 4C). The luciferase activity of US was 94.6% of transfection in PLC/PRF/5, 2.4% in Hep3B and 2.3% in Panc-1 cells.

Cell viability was assayed to analyze cell damage with US (Fig. 5). Compared with transfection, cell viability of US were 111±16% (PLC/PRF/5), 101±25% (Hep3B) and 93±2.7% (Panc-1), respectively.

Discussion

Up until December 2009, there were 1,579 gene therapy clinical trials, of which about 25% utilized non-viral vectors (14). While viral vectors dominate clinical gene therapy due to greater efficiency of gene delivery, non-viral vectors would be preferable regarding safety. The major problems of cationic agents as a gene delivery system are that their interaction with blood and their transfection efficiency is lowered (15). Continuous-wave irradiation improves introduction of plasmid

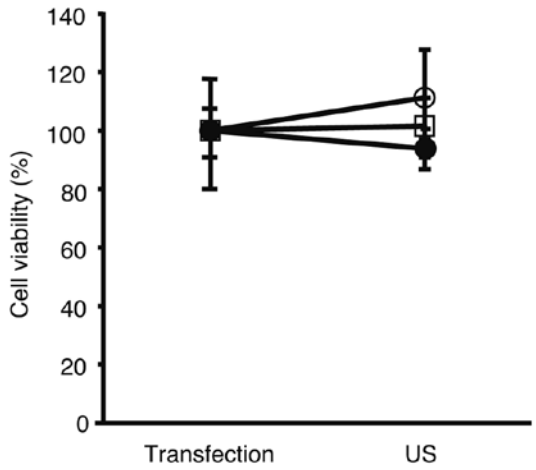

Figure 5. Cell viability. Cell viability was analyzed with the MTS assay. Transfection, transfection with Lipofectine; US, irradiated with diagnostic ultrasound. No difference was found in cell viability. ○, PLC/PRF/5; □, Hep3B; ●, Panc-1.

into cells (16). The transfection efficiency of continuous wave was 2.4% for primary chondrocytes and 3.7% for epidermoid cells (17,18). In our experiments, the transfection efficiency was 5.2% in PRL/PRF/5, 10.2% in Hep3B, and 23.0% in Panc-1. These data indicated that diagnostic ultrasound might be more efficient for gene transfer than sonoporation although the transfection efficiency greatly depended on the cell lines. In contrast, luciferase activity was significantly lower in cells irradiated with diagnostic ultrasound as compared to those transfected with Lipofectin (94% in PLC/PRF/5, 2.4% in Hep3B, and 2.2% in Panc-1, respectively). Lawrie *et al* reports that luciferase activity of utrasound irradiation is 11.0% of that with transfection when endothelial cells were irradiated with 1 MHz for 60 sec (19). Apparently, the expression of introduced genes is much less than expected (20). One possible explanation of this phenomenon was that copy numbers introduced into cells were significantly lower in cells irradiated with ultrasound than those transfected. Another speculation is the efficiency of nuclear transport. Plasmid DNA needs to be transported into the nucleus for gene expression. A complex of cationic agent and plasmid DNA may be easily transported into the nucleus while plasmid alone may have difficulty.

With continuous-wave, cell viability decreases to 80% for attaching cells and 30% for suspended cells at 2 W/cm^2 of acoustic power (21). In our experiments with pulsed-wave ultrasound, cell proliferation did not decrease with US as compared with transfection even after 5-min irradiation. Our results clearly showed that diagnostic ultrasound was safe for cells.

The mechanism of biophysical effects of ultrasound is categorized as cavitation, radiation and acoustic microstreaming (22). Among them, cavitation has mostly been under investigation. It appears that cavitation is unlikely to occur at lower than 0.7 of MI (23). Ultrasound, theoretically, did not introduce plasmid into cells at 0.4 of MI but our data clearly showed that it did. Currently, it is not fully known how molecules enter cells. Based on the literature, there are three candidate mechanisms for molecular uptake by ultrasound: active transport, passive transport and uptake through actively repairable wounds (22). The first is via endocytosis by surface receptors that could be upregulated. The second is through nanometer pores in the membrane similar to those caused by electroporation. The third is through wounds in the membrane similar to those caused by physical stresses (24).

The efficiency of gene introduction was low in the present study. Microbubbles are used to improve gene transfer with ultrasound due to shear stress or physical interaction with the membrane (25,26). Our next step would be the use of microbubble reagent or contrast media to improve the efficiency of introduction of plasmid DNA into cultured cells.

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