Short interference RNA introduced into cultured cells with diagnostic ultrasound

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Abstract. Diagnostic ultrasound (US) is safe. Short interference (si) RNA of Frizzled (Fz)-9 suppresses proliferation of hepatocellular carcinoma cells. siRNA was introduced into Hep3B cells, a human hepatocellular carcinoma cell line, with transfection or US upward to culture plates at mechanical index (MI) = 0.4 or 0.8. siRNA of Fz9 was introduced for Western blot analysis and cell proliferation assay. siRNA of Fz9 suppressed cell proliferation to 32.8±13.6% at 200 nM (P<0.0001) with transfection. With US, cell proliferation decreased to 36.9±15.1% at 200 nM (P=0.039) at 0.4 of MI while 49.2±12.0% at 200 nM (P=0.041) at MI=0.8. Western blot analysis showed that 200 nM of siRNA decreased the expression of Fz9 with US at MI=0.4 and MI=0.8. No change was seen in cell proliferation between transfection and US. siRNA was introduced into cultured cells and functioned with US.

Introduction

Methods of introducing therapeutic genes are important to obtain satisfactory anti-cancer effects in the treatment of cancer. Viral vectors are potent in gene delivery but can be hazardous. Non-viral delivery is safe but its efficiency is disappointingly low in introducing genes. Plasmid DNA was successfully introduced into cultured cells with a sonicator (1). Irradiation of low output intensity ultrasound increases the effects of introduction of genes into tissues (2). The biological effects of ultrasound are categorized as thermal mechanism and non-thermal (3). Non-thermal effects are caused by cavitation, mechanical perturbation in the vicinity of bubbles, leading to membrane poration (4). This phenomenon, sonoporation, is a gene delivery method that is expected as a potential new method of gene therapy as evidenced in a murine model (5). Collapse of cavitation can generate small transient holes on a cell membrane and induces cell membrane permeabilization (6,7). Substrates, such as DNA, can pass through the hole in the cell membrane.

In addition to conventional treatment, molecular therapy to hepatocellular carcinoma (HCC) is currently under clinically investigation (8). To develop a new molecular therapy, analysis of signal transduction is essential (9). The Wnt signaling pathway is involved in abnormal cell growth and cancer (10). Fzizzled (Fz) is a cell surface receptor that mediates actions of Wnt ligands, and 10 members of the Frizzled family have been identified (11). Fz9 is not expressed in normal liver, but expressed in HCC and hepatoblastoma cell lines examined (12). Short interference (si) RNA of Fz9 suppresses proliferation of HCC cell lines decreasing the expression of cyclin D1 (12). Suppression of Fz9 would be an ideal target of a new molecular therapy of HCC since it would not cause any adverse events to surrounding normal liver tissues.

To increase the efficiency of gene delivery, cells can be damaged by intense standing waves and significant temperature increases (13). Safe methods of gene transfer would be desirable for treatment. Diagnostic US is relatively safe and capable of real-time monitoring of the irradiated fields. Thus, we used diagnostic ultrasound (US) to introduce siRNA to cultured HCC cells.

Materials and methods

Cell culture. Hep3B (RIKEN BioResource Center, Tsukuba, Japan), a hepatocellular carcinoma cell line, were cultured in Dulbecco's minimum essential medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) in 5% carbon dioxide at 37˚C. Twenty-four-well plates (Asahi Techno Glass, Funabashi, Japan) and 96-well plates (Asahi Techno Glass) were used for luciferase assay and Western blot analysis, and cell proliferation assay, respectively.

Irradiation with US. Cultured cells were irradiated with a linear probe at the frequency of 8 MHz at the mechanical index (MI) = 0.4 (the lowest in our machine) or 0.8 (the highest)

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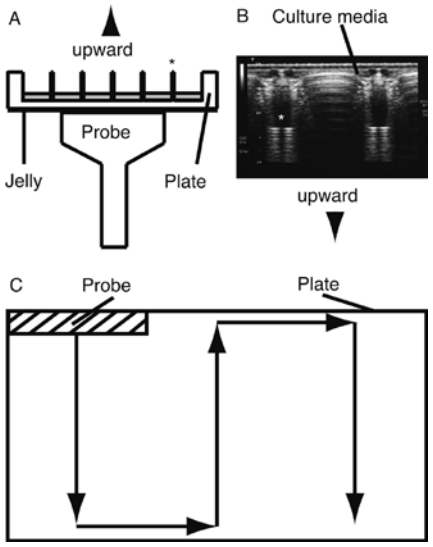

Figure 1. Methods of irradiation with US. A linear probe was touched upward to the surface of the bottom of a plate through ultrasound jelly (A). A representative image obtained on the US when aimed at a 24-well plate (B). (C) Depicts a schematic view from the beneath. The probe was moved slowly for 5 min. Gray box, culture media; hatched box, probe; arrowhead, direction to up; arrow, scanning direction; asterisk, plastic wall between wells.

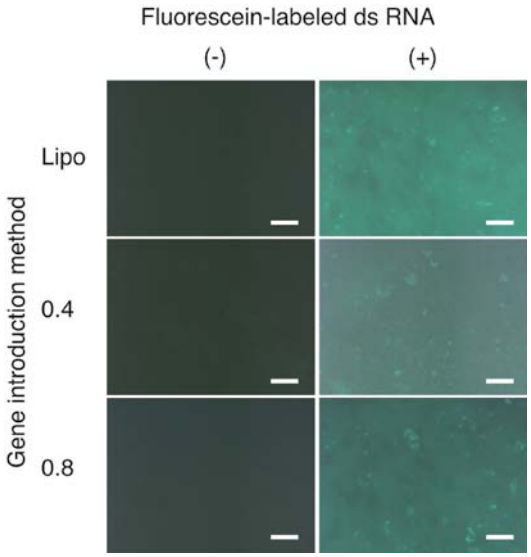

Figure 2. Introduction of fluorescein-labeled double-strand RNA with US. Fluorescein-labeled double-strand (ds)RNA was introduced into Hep3B cells with transfection with Lipofectamine 2000 (Lipo), or US at the mechanical index (MI)=0.4 (0.4) or 0.8 (0.8) with (+) or without (-) fluorescein-labeled dsRNA at 200 nM. Signals were observed in trasfected cells with Lipofectamine 2000 as well as irradiated with US. Lipo, tranctection with Lipofectamine 2000; 0.4, irradiation with US at the MI=0.4; and 0.8, irradiation with US at the MI=0.8. (-), without fluorescein-labeled dsRNA; (+), with fluorescein-labeled dsRNA. Original magnification, x20; scale bar, 100 μm.

using SSA-700A (Toshiba Medical Systems Corp., Ohtawara, Japan). The probe was touched upward to the bottom of a plate through ultrasound jelly (Fig. 1A). The irradiated field was observed through the display (Fig. 1B). The probe was scanned slowly to irradiate all fields for 5 min (Fig. 1C).

Introduction of fluorescein-labeled double-strand RNA. When cells reached 70% confluency fluorescein-labeled double-strand (ds)RNA (Invitrogen) was introduced into cells at the concentration of 200 nM by transfection with Lipofectamine 2000 (Invitrogen) or US. After 48 h, cells were observed under fluorescence microscope (Carl Zeiss Microimaging Japan, Tokyo, Japan).

Luciferase assay. When cells reached 70% confluency, they were transfected with 0.46 μg of pGL3-control (Promega, Madison, WI) together with 0.046 μg of pRL-TK (Promega) an internal control vector, with Lipofectin (Invitrogen) to obtain the same level of the luciferase activity. After 5 h, cells were transfected with siRNA of firefly luciferase (Nippon Gene Co., Ltd., Tokyo, Japan) using Lipofectamine 2000 or irradiated with US. After 48 h, the transcriptional activity was measured with a dual luciferase reporter assay system (Promega) Mock was applied as no siRNA in the media. Negative control siRNA (Invitrogen) was used as a negative control. The relative luciferase activity was calculated as firefly luciferase activity divided by renilla luciferase activity.

Cell proliferation assay. The next day after the cell spread at 1000/well, siRNA of Fz9 (Invitrogen) was introduced into cells with Lipofectamine 2000 or US. Mock was applied as no siRNA in the media. The negative control siRNA (Invitrogen) was used. After 72 h, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay was performed following the instructions (Promega).

Western blot analysis. Forty-eight hours after introduction of siRNA of Fz9 (Invitrogen) with transfection or US, protein was isolated and 10 μg samples were subjected to sodium dodecyl sulphate polyacylamide gel electrophoresis, and transferred to a nylon filter. The primary antibodies were rabbit polyclonal anti-Fz9 antibody (Bioworld Technology, Inc., St. Louis Park, MN), or mouse monoclonal anti-tubulin-α antibody (Lab Vision, Fremont, CA). The secondary antibodies were horseradish peroxidase (HRP)-linked anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK) or HRP-linked anti-mouse antibody (GE Healthcare). Dilutions were 1:500 for primary antibodies, and 1:1000 for secondary antibodies. The filter was reprobed with anti-tubulin-α antibody. The specific antigen-antibody complexes were visualized by enhanced chemiluminescence (GE Healthcare).

Statistical analysis. One-factor analysis of variance (ANOVA) was applied as statistical analysis with JMP5.0J (SAS Institute Japan, Tokyo, Japan). $P<0.05$ was accepted as statistically significant.

Results

After transfection or irradiation with US, the signal was observed under fluorescence microscope (Fig. 2). Although the numbers were smaller than transfection, the signals were clearly seen in cells irradiated with US at both MI=0.4 and 0.8.

siRNA of firefly luciferase was introduced into Hep3B cells. With transfection, relative luciferase activity decreased

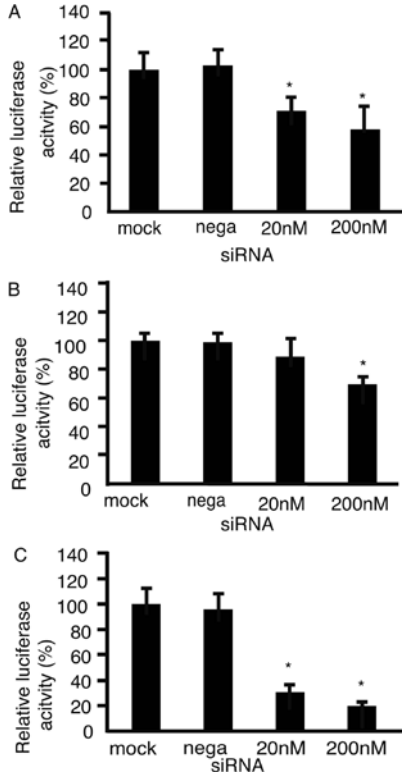

Figure 3. Luciferase assay. siRNA of firefly luciferase were introduced into Hep3 cells with transfection with Lipofectamine 2000 (A) or irradiation with US at mechanical index = 0.4 (B) or 0.8 (C), 5 h after transfection of pGL3 control vector. After 48 h, they were subjected to luciferase assay. Mock, no addition of siRNA; nega, 200 nM of negative control siRNA. *P<0.05 (one-factor ANOVA), n=3.

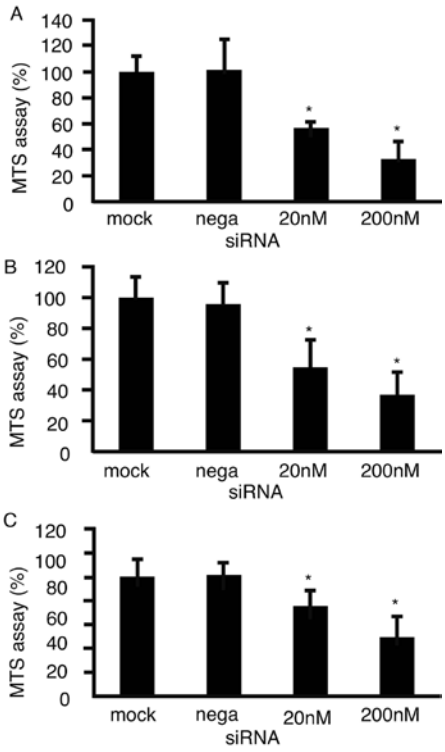

Figure 4. Cell proliferation assay. Cell proliferation was suppressed with transfection (A). With irradiation with US, cell proliferation was suppressed at both the MI = 0.4 (B) and 0.8 (C). Mock, without siRNA; nega, negative control of siRNA; 20 nM, addition of siRNA of Fz9 at 20 nM; 200 nM, addition of siRNA of Fz9 at 200 nM. *P<0.05 (one-factor ANOVA), n=3.

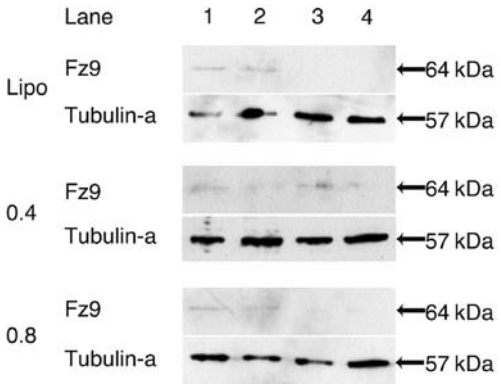

Figure 5. Western blot analysis. Protein was isolated after transfection or irradiation with US, and subjected to Western blot analysis. Lane 1, mock (without siRNA); 2, negative control of siRNA; 3, addition of siRNA of Fz9 at 20 nM; 4, addition of siRNA of Fz9 at 200 nM. Lipo, transfection with Lipofectamine 2000; 0.4 and 0.8, mechanical index = 0.4 and 0.8.

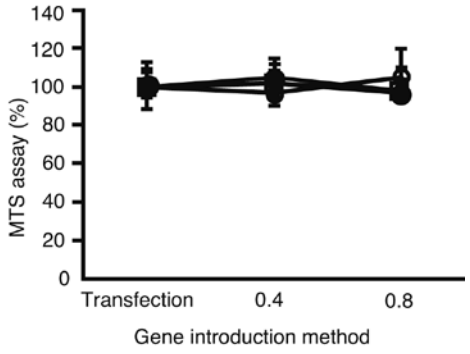

Figure 6. Cell proliferation assay. To analyze the damage to the cultured cells with US, cell proliferation was analyzed after 72 h culture with cells in media only (○), mock (without siRNA) (□) or 200 nM of negative control of siRNA (●). 0.4 and 0.8, mechanical index = 0.4 and 0.8..

to 71±9.6% (mean ± standard deviation) (P=0.015) at 20 nM and 57.9±17% (P=0.02) at 200 nM (Fig. 3A). With US at MI=0.4, relative luciferase activity decreased to 69.6±5.1% at 200 nM (P=0.014) (Fig. 3B). At MI=0.8, relative luciferase activity significantly decreased to 30.1±5.9% (P=0.021) at 20 nM and 19.2±3.1% (P=0.0055) at 200 nM (Fig. 3C).

SiRNA of Fz9 suppressed cell proliferation when transfected with Lipofectamine 2000 (Fig. 4A) to 56.8±4.8% (P=0.013) at 20 nM and 32.8±13.6% at 200 nM (P<0.0001). When irradiated with US, cell proliferation was significantly suppressed by siRNA of Fz9 at MI=0.4 to 54.8±17.8% (P=0.022) at 20 nM and 36.9±15.1% at 200 nM (P=0.039) (Fig. 4B) as well as 0.8 to 75±4.8 at 20 nM (P=0.047) and 49.2±12.0% at 200 nM (P=0.041) (Fig. 4C).

When transfected with siRNA of Fz9, the expression of Fz9 decreased at 20 and 200 nM (Fig. 5). The expression of Fz9 decreased when irradiated with 200 nM of siRNA of Fz9 at MI=0.4. At MI=0.8, the expression of Fz9 decreased at 20 as well as 200 nM. These results clearly indicated that siRNA suppressed protein expression introduced into cells when irradiated with ultrasound.

The effect of US on cell proliferation was analyzed (Fig. 6). No change in cell proliferation was seen among transfection,

US at MI=0.4, or MI=0.8 in cells under media only. The same trend was shown in mock and negative control. These data clearly indicated that US did not damage cells.

Discussion

siRNA is a novel tool for treatment of human diseases. Regarding clinical trials, siRNA has been applied to inherited skin disorder and cancer (14,15). Because it is hard for naked siRNA to cross the cell membrane, special delivery methods have been developed, such as physical methods, hydrodynamic injection, mechanical massage, and electroporation (16). Hydrodynamic injection is based on rapid injection of much siRNA (17). Due to invasive nature, hydrodynamic injection and electroporation are mostly used for animal experiments. siRNA has been introduced into cells with sonoporation, a non-invasive method, and silenced the expression of green fluorescent protein (GFP) (18). In our expreriments, siRNA was successfully introduced into cultured cells with US. Our data, moreover, clearly showed that siRNA of Fz9 suppressed proliferation of Hep3B cells with US. Our study first presented anti-cancer effects with therapeutic siRNA introduced with US. Interestingly, siRNA to GFP silences the signal after intravenous injection to mice bearing GFP-expressing tumor cells (19). siRNA accumulates in the liver when administered *in vivo* (20). Taken together, application of siRNA would be one of suitable methods of molecular therapy to HCC. It would be expected that HCC could be introduced with therapeutic siRNA precisely through real-time monitoring. Moreover, US was safe since no cells were damaged as shown in Fig. 6. It would be safe to conclude that US is one of the potential methods for gene therapy with siRNA.

The mechanism of biophysical effects of ultrasound is categorized as cavitation, radiation and acoustic microstreaming (3). Among them, cavitation mostly has been under investigation. However, cavitation is unlikely to occur at lower than 0.7 of MI (21). In our experiments, siRNA was introduced and functioned at MI=0.8. It was speculated that our MI was strong enough for cavitation. On the other hand, siRNA of luciferase silenced the expression of luciferase marginally at MI=0.4 while suppressed the expression of Fz9 and showed significant anti-cancer effect. The mechanism of introduction of siRNA at MI=0.4 was not clear. In fact, 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 (3). The first is via endocytosis by surface receptors that could be up-regulated. 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 (22).

Our next step would be the application of siRNA with US in an animal model of cancer to validate the concept of using US in gene transfer *in vivo*.

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