In vivo gene delivery by cationic tetraamino fullerene

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Application of nanotechnology to medical biology has brought remarkable success. Water-soluble fullerenes are molecules with great potential for biological use because they can endow unique characteristics of amphiphilic property and form a self-assembled structure by chemical modification. Effective gene delivery in vitro with tetra(piperazino)fullerene epoxide (TPFE) and its superiority to Lipofectin have been described in a previous report. For this study, we evaluated the efficacy of in vivo gene delivery by TPFE. Delivery of enhanced green fluorescent protein gene (EGFP) by TPFE on pregnant female ICR mice showed distinct organ selectivity compared with Lipofectin; moreover, higher gene expression by TPFE was found in liver and spleen, but not in the lung. No acute toxicity of TPFE was found for the liver and kidney, although Lipofectin significantly increased liver enzymes and blood urea nitrogen. In fetal tissues, neither TPFE nor Lipofectin induced EGFP gene expression. Delivery of insulin 2 gene to female C57BL6 mice increased plasma insulin levels and reduced blood glucose concentrations, indicating the potential of TPFE-based gene delivery for clinical application. In conclusion, this study demonstrated effective gene delivery in vivo for the first time using a water-soluble fullerene.

Results

Size and Stability of TPFE-DNA Complex. Characterization of the TPFE-DNA complex was performed to confirm the optimal pre-

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Gene Delivery Efficiency of TPFE-DNA Complex in Vivo. The TPFE-DNA complex, Lipofectin-DNA, and naked DNA were injected into the mouse tail vein. The in vivo biodistribution of injected DNA was evaluated by amplifying the EGFP gene encoded in the plasmid DNA. At 24 hr after injection, DNA samples were extracted from the organs of the lung, liver, kidney, and spleen. Comparable localizations of plasmid DNA delivered by TPFE and Lipofectin were found, although it is noteworthy that the amount of DNA in the TPFE group was approximately 2- to 3-fold higher than the Lipofectin group except lung (Fig. 2A). Injected plasmid DNA was not detected in the whole blood of any group. The naked DNA group showed virtually no amplification of the EGFP gene in any organ.

Gene expression of the EGFP gene was examined using real-time PCR for evaluating transfection efficacy and confocal microscopy image analysis for detailed localization of gene expression. In the TPFE and the Lipofectin group, mRNA expression was found in the lung, liver, and spleen at 24 hr after injection (Fig. 2B). In the TPFE group, the amount of expressed mRNA was higher than that of the Lipofectin group in the liver and spleen, but lower than that of the Lipofectin group in the lung. This tendency resembled that observed in plasmid DNA distribution described above (Fig. 2C). In accordance with these results, confocal microscopy image analysis showed EGFP signals in the lung, spleen, and liver of the TPFE and the Lipofectin group. Naked DNA showed no EGFP positive signal (Fig. 3). The similar observation was confirmed by immunohistochemical staining of anti-EGFP antibody (SI Text and Fig. S1). The efficacy of gene delivery to the fetus and fetal appendages was also examined. At 24 hr after injection of TPFE-DNA and Lipofectin-DNA to pregnant mice [8 days post coitum (d.p.c.)], fetuses were removed. Plasmid DNA was found in the placenta and yolk sac. The TPFE group showed a higher number of plasmid DNA copies in placenta than the Lipofectin group did (Fig. 4). The mRNA of EGFP group showed a higher number of plasmid DNA copies in placenta than the Lipofectin group did (Table 1).

Evaluation of TPFE Toxicity. Acute toxicity of the TPFE and the Lipofectin gene transfer systems was evaluated using serum biochemical examinations, which are often used in clinical practice. Blood specimens were collected 24 hr after injections. Liver injury was evaluated by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Increases of AST and ALT were found only in the Lipofectin group. The Lipofectin group showed modestly but significantly higher blood urea nitrogen (BUN) levels than the TPFE group did, possibly indicating that lipofection caused acute kidney injury (Table 2).

Delivery of Insulin 2 Gene for Therapeutic Application. We evaluated the potential application of gene delivery by TPFE with mouse insulin 2 gene (Ins2) expressing plasmid. As observed for the experiment of EGFP gene delivery, insulin expression was detected in the lung by immunohistochemistry. Modest insulin expression was also detected in the liver and spleen (Fig. 5A). At 12 hr after

**Fig. 1.** TPFE. (A) Structure of TPFE is shown. DLS measurements were done at 0, 10, 30, 60, and 120 min after preparation of TPFE-DNA complexes. (B) The size of TPFE-DNA complex was stable for 1 h (n = 4). *p < 0.05 versus 0 min. (C) Adding 10% serum increased the stability of the TPFE-DNA complex (n = 3). Error bars are SEM.

**Fig. 2.** Distribution of injected plasmid DNA and EGFP mRNA expression in each organ. (A) After 24 hr injection, plasmid DNA was detected at several organs in the TPFE and the Lipofectin group (n = 6 for each group). (B) mRNA expression of EGFP gene was analyzed using real-time-PCR at 24 hr after injection. mRNA was detected in the lung, liver, and spleen (n = 6 for each group). *p < 0.05 versus the Lipofectin group. Error bars are SEM.
the injection together with TPFE, plasma insulin levels in the \( \text{Ins}^2 \) group were significantly higher than the mock group (Fig. 5B). Lower fasting blood glucose levels were observed in the \( \text{Ins}^2 \) group until 24 hr after the injection (Fig. 5C).

**Discussion**

Tetraaminofullerene is a carbon nanomaterial that has an amphiphilic character. In fact, TPFE can form a small aggregate with double-strand DNA, protect nucleic acid from nucleases, and penetrate into the cells. These factors enable us to use this material for gene delivery. In this study, we demonstrated the fact that tetraaminofullerene is applicable as an in vivo gene delivery system. Moreover, we compared the efficiency and toxicity of TPFE with a liposome-based system (Lipofectin). Results show that TPFE can deliver a gene more efficiently in the liver and spleen and showed no acute toxicities to liver or kidney tissues, although Lipofectin induces liver and kidney injury. Finally, we successfully delivered insulin 2 gene by the TPFE system and demonstrated the effect of insulin 2 gene delivery on blood glucose levels.

The specific molecular ratio of DNA to TPFE (\( R = 5 \)) allowed an assembly particle size of 50–100 nm, which is suitably sized for endocytosis. Based on our previous studies, we reported that several amphiphilic compounds showed different optimal \( R \) values (18). Results of the present study demonstrated that TPFE can form a more stable complex with DNA under coincubation with serum. In vitro optimization is indispensable to set up effective delivery systems in vivo.

The in vivo biodistribution of TPFE-DNA and Lipofectin complexes showed similar patterns. However, levels of reporter gene expression varied remarkably. The TPFE achieved 5- to 10-fold higher gene expression than Lipofectin in the liver and spleen. Reportedly, lipid-based gene delivery can transfer genes to the lung effectively because cationic liposome can be trapped well at the lung immediately after injection from the tail vein (9, 10, 21). Therefore, many studies have been undertaken to deliver genes to the lung and to treat lung diseases using liposomal gene transfer systems (8, 11, 12). In the TPFE system, the TPFE-DNA complex structure would differ significantly from those of lipid-DNA complex, possibly because of the \( \pi-\pi \) interaction between the fullerene surface and nucleobases, which might be the origin of the observed organ selectivity. The TPFE will be applicable to other organ diseases including those of the liver and spleen.

Transfection and expression of a foreign gene in embryos and fetuses at desired stages will overcome problems in transgenic animals such as embryonic lethality and unpredictable regulatory effects of transgene integration. In 1995, Tsukamoto et al. reported that cationic liposome efficiently introduces a foreign gene into pregnant mice (22). They showed that a single intravenous injection of foreign gene-cationic liposome complexes into pregnant mice led to effective gene delivery to the fetus and placenta. In this study, we demonstrated that TPFE can be a promising gene delivery system for in vivo use.

**Table 1. Number of animals showing positive signals of mRNA expression by real-time PCR**

<table>
<thead>
<tr>
<th></th>
<th>Fetus</th>
<th>Placenta</th>
<th>York sac</th>
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</thead>
<tbody>
<tr>
<td>Naked DNA</td>
<td>0/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>Lipofectin</td>
<td>0/7</td>
<td>0/7</td>
<td>1/7</td>
</tr>
<tr>
<td>TPFE</td>
<td>1/8</td>
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Liver and kidney injuries were evaluated using AST, ALT, and BUN measurements (\( n = 6 \)).

\( ^* p < 0.05 \) versus the TPFE group.
gene transfer into pregnant mice and their fetuses, the reported efficiency was insufficient and far from the level necessary for clinical use (24). We evaluated the efficiency of gene delivery to fetuses, the yolk sac, and placenta. The DNA-TPFE complex was trapped at lungs to a lesser degree than Lipofectin-DNA was. Therefore, it was expected that the TPFE system can deliver DNA more efficiently to the fetus. However, virtually no signal was detected in fetuses in either the TPFE or Lipofectin group. The placenta might play a barrier function for these two gene delivery systems.

Our data from this and previous reports indicate several potential advantages of a TPFE-based gene delivery system. First, TPFE can form stable aggregates with DNA, with less than 100-nm diameter with serum containing buffer. In contrast, liposome-based transfection agents are often impeded by plasma proteins (25). Second, TPFE has been demonstrated to have protective ability of DNA against enzymatic degradation (18). Third, TPFE can further penetrate into the nuclear region partly, and the amino-group of TPFE can be removed or neutralized with an amide reaction, by which DNA is released into the nuclear space (18). On the other hand, the mechanisms of cationic liposome penetrating into the cells and nucleus remain unclear.

Cationic liposome-based gene delivery is the most popular technique for in vivo animal experiments. However, the ineffectiveness and toxicity of this method has been described (26). Some reports in the literature describe in vivo gene delivery by Lipofectin, although it induced gene expression found only in lung tissues (27). One report described that expression was under the detection limit in all organs by Lipofectin (20). Song et al. evaluated several different cationic liposomes and found that they needed more than 100 μg of plasmid DNA and a large amount of lipids that were a nearly a lethal dose for mice (19, 20). The present study found virtually the same efficiency of gene delivery with 24 μg of plasmid DNA for the TPFE group with no lethal damage to the animals.

Results of this study demonstrated the advantage of fullerene-based transfection systems in terms of toxicity and possible therapeutic application. No acute toxicity was found in the TPFE group, although the Lipofectin group showed acute organ toxicity in the liver and kidney. The two liver enzymes (AST and ALT) are present in hepatocytes and are released rapidly into the bloodstream when hepatocytes are damaged by toxins and/or acute inflammation (28). An increase of BUN indicates dysfunction of the kidney as a filtrating organ. Although these injury markers demonstrate the organ injury and its severity, further investigation is necessary to clarify the mechanism of toxicity by Lipofectin. Several previous reports described no evidence of toxicity by fullerene (29–31). On the other hand, cationic liposome is reported to have toxicity (9, 26, 32). Filion and Phillips showed that cationic liposome can cause remarkable immunological response in vivo (26). Further evaluations of long-term toxicity must be undertaken to apply a fullerene-based gene delivery system to humans. Finally, we demonstrated that insulin gene delivery by TPFE can reduce blood glucose levels. Insulin gene delivery by liposomal gene transfer or hydrodynamics-based system has been reported (33, 34). When TPFE gene delivery system is combined literally with hemagglutinating virus of Japan (HVJ)-liposome-based system, the reduction of blood glucose levels of normal mice is virtually the same degree (20–25 mg/dL). The HVJ-liposome system was also demonstrated to reduce blood glucose in diabetic mice (34). Further evaluation is necessary to clarify the potential application of TPFE gene delivery to animal models of disease. Our data related to safety and therapeutic effects indicate the potential of fullerene-based gene delivery systems for clinical applications.

In conclusion, we demonstrated that a water-soluble fullerene TPFE can deliver genes as efficiently as a cationic liposome-based system in vivo. In fact, TPFE can produce a stable complex.
with DNA and show no acute organ toxicity. Moreover, insulin gene delivery by TPFE could reduce blood glucose levels. These data demonstrated successful in vivo gene delivery by carbon nanomaterials.

Materials and Methods

Preparation of TPFE-Plasmid DNA Complex. TPFE transfection reagent was prepared as reported previously (17). Plasmid pCAGGS EGFp was kindly provided by the RIKEN Bioresource Center Cell Bank. This plasmid DNA encoding green fluorescence protein (GFP) was amplified in Escherichia coli (DH5α; Toyobo Co.) and was purified using a commercially available kit (Plasmid GIGA Kit; Qiagen Inc.). For insulin 2 gene (Ins2) delivery experiments, we used the pcdNA3 plasmid containing mouse Ins2 cDNA. Details of this plasmid were described in an earlier report (35).

The TPFE dissolved in 2 mM potassium chloride solution and plasmid DNA dissolved in Tris buffered saline (TBS) were mixed to obtain the reagent-to-base pair ratio (R) of 5, which showed the highest transfection efficiency in vitro in our previous report (18). The R value was calculated by dividing the nitrogen-to-phosphorus (N/P) ratio by two. The mixture was incubated at room temperature for 5 min before injection.

DLS Analysis. The size of TPFE-plasmid DNA complex was measured at 0, 10, 30, 60, and 120 min after complex formation. Dynamic laser light scattering analysis was performed to measure the particle size distribution and average particle sizes using a Zetasizer Nano ZS machine (Malvern Instruments Ltd.).

Administration of TPFE/EGFP-Expressing Plasmid DNA Complex in Vivo. Pregnant ICR mice purchased from Saitama Experimental Animals Supply Co. Ltd. were kept at 23 ± 2°C, moisture at 55 ± 15% during experiments. Animals were maintained at a 12-hr day and 12-hr night cycle. They ate a regular mouse diet and had free access to water. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (36).

Pregnant ICR mice (B d.p.c.) were assigned randomly to one of three groups: the TPFE group, the Lipofectin group, or the naked DNA group. For the TPFE group, TPFE-plasmid DNA complex containing 24 μg plasmid DNA with 300 μg total RNA from liver, kidney, lung, and spleen were collected and snap-frozen using liquid nitrogen. From liver, kidney, lung, and spleen were collected and snap-frozen using liquid nitrogen. A 96 well-plate reader (SpectraMax; Molecular Devices Corp.). For immunohistochemical analysis, the tissues were collected at 24 hr after injection and kept in –80°C before use. Total RNA was extracted using a kit (QuickGene RNA tissue kit SII and QuickGene-810; Fujifilm). To obtain cDNA of transcripts, reverse transcription was performed with 1 μg total RNA (High Capacity CDNA Reverse Transcription Kit, Applied Biosystems). Subsequent real-time PCR was performed with the synthesized cDNA and a set of primers for GFP (forward, 5′-CAACCTACAA-CAGGCACAAGGGTC-3′, reverse, 5′-GGTGTGGTCTGCTGGATGGTCG-3′) and for internal control (Taqman Eukaryotic 18S rRNA; Applied Biosystems). Subsequently, PCR amplification was performed using a TaqMan universal PCR master mix (Applied Biosystems) under the following conditions (PRISM 7000; Applied Biosystems): denaturation (95°C for 30 s), annealing (61°C for 30 s), and extension (72°C for 30 s) for 40 cycles.

RNA Extraction and Real-Time PCR Analysis. Tissues from the organs were collected at 24 hr after injection and kept in –80°C before use. Total RNA was extracted using a kit (QuickGene RNA tissue kit SII and QuickGene-810; Fujifilm). To obtain cDNA of transcripts, reverse transcriptase reaction was performed with 1 μg total RNA (High Capacity CDNA Reverse Transcription Kit, Applied Biosystems). Subsequent real-time PCR was performed with the synthesized cDNA and a set of primers for GFP (forward, 5′-CAACCTACAA-CAGGCACAAGGGTC-3′, reverse, 5′-GGTGTGGTCTGCTGGATGGTCG-3′) and for internal control (Taqman Eukaryotic 18S rRNA; Applied Biosystems). Subsequently, PCR amplification was performed using a TaqMan universal PCR master mix (Applied Biosystems) under the following conditions (PRISM 7000; Applied Biosystems): denaturation (95°C for 30 s), annealing (61°C for 30 s), and extension (72°C for 30 s) for 40 cycles.

Measurement of Blood Chemistry. BUN was measured using the urease-indophenol method with Urea NB (Wako Pure Chemical Industries Ltd.), AST and ALT were measured using pyruvate oxidase method with Transamnase CII (Wako Pure Chemical Industries Ltd.).

Statistical Analysis. Differences among the experimental groups were detected using Student’s t-test or Wilcoxon’s rank test. Values are expressed as means ± SEM, p < 0.05 was considered significant.

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36. Committee on Care and Use of Laboratory Animals (1985) Guide for the Care and Use of Laboratory Animals (Natl Inst Health, Bethesda), DHHS Publ No (NIH) 85-23.
Immunohistochemical staining for enhanced green fluorescent protein gene (EGFP) on 10-μm frozen sections was performed using indirect biotin avidin technique according to the manufacturer’s protocols (ABC Kit–Rabbit; Vector Laboratories Inc.). The cold acetone fixation sections were preincubated with 0.3% hydrogen peroxide for 30 min and incubated with a primary antibody (rabbit anti-GFP antibody; MBL Co., Ltd.) overnight at 4 °C, followed by incubation with biotin-conjugated anti-rabbit IgG and avidin-conjugated horseradish peroxidase. Diaminobenzidine tetrahydrochloride (Nichirei Corp.) was used for the substrate-chromogen reaction. Counterstaining was performed with hematoxylin. Control sections were subjected to secondary antibody only. Mounted preparations were examined under a light microscope (E600; Nikon Corp.). Images were captured using a CCD camera (DM-1200; Nikon Corp.).

**Fig. S1.** Immunohistchemical analysis of organ tissue sections. EGFP expression was indicated with brown staining (arrow head). Hematoxylin eosin was used as a counterstain.