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Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart

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Abstract

Naked plasmid DNA (pDNA) and short interfering RNA (siRNA) duplexes were transduced into adult murine heart by means of sonoporation using the third-generation microbubble, BR14. Plasmid DNAs carrying luciferase, β-galactosidase (β-gal), or enhanced green fluorescent protein (EGFP) reporter genes were mixed with BR14 and injected percutaneously into the left ventricular (LV) cavity of C57BL/6 mice while exposed to transthoracic ultrasound at 1 MHz for 60 s. Sonoporation at an output intensity of 2.0 W/cm^2 and a 50% pulse duty ratio resulted in the highest luciferase expression in the heart. Histological examinations revealed significant expression of the β-gal and EGFP reporters in the subendocardial myocardium of LV. Intraventricular co-injection of siRNA-GFP and BR14 with concomitant ultrasonic exposure resulted in substantial reduction in EGFP expression in the coronary artery in EGFP transgenic mice. The present method may be applicable to gain-of-function and loss-of-function genetic engineering in vivo of adult murine heart.

Keywords: Naked plasmid DNA; Heart; Sonoporation; Microbubble; Gene transfer; RNA interference; Ultrasound; Gene therapy; Molecular therapy

Genetic modification of the heart may provide a powerful molecular tool for improving our understanding of cardiac diseases. Moreover, a technology that enables safe and efficient gene delivery to the heart may provide a novel therapeutic modality to control heart disorders. Some studies recently demonstrated that efficient transfer of genes into murine heart can be achieved by intracoronary infusion of adenoviral or adeno-associated viral vectors followed by transient aortic occlusion [1,2]. Viral vector-mediated procedures, however, may induce complications associated with recombinant viruses, hindering clinical application of the systems to gene therapy for cardiovascular diseases. Although non-viral methods are free from virus-associated adverse effects, their transduction efficiency is low. For example, following direct intramyocardial injection of plasmid DNA into the heart of mice [3], rats [4,5], and hamsters [6], the transgenes were expressed only within a small area surrounding the injection site.

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A more efficient non-viral method of gene transfer is sonoporation of cells. In sonoporation, ultrasound is used to increase the porosity of the cell membrane [7]. The ultrasound induces the formation of cavitation bubbles that by mechanical action cause enough damage to the cell membrane to allow large molecules in the surrounding medium to enter the cell, but not so much damage that the cell cannot reseal the membrane and survive [8]. The extent of cavitation bubble formation and subsequent increase in membrane permeability can be enhanced by use of microbubble echo contrast agents. This method has been applied to skeletal muscle of mice [9], as well as myocardium of both rats [10] and dogs [11], in vivo, using various microbubble agents including Levovist, Hexabrix, and Optison [9–18]. It has been indicated that the transfection efficiency is dependent on both the ultrasound parameters and the formulation of microbubbles.

BR14 (Bracco Research SA, Geneva, Switzerland) is a recently developed ultrasound contrast agent, which has some advantages over other echo contrast agents [19,20], because it consists of stabilized relatively small microbubbles and is transiently retained within capillaries. These qualities mean that BR14 may be a better agent in sonoporation-mediated transfection. Indeed, recent studies have indicated that BR14 effectively enhanced sonoporation-based gene transfection into hepatic cancer implant in mice [21] as well as into saphenous vein graft in porcine [22].

RNA interference (RNAi) is a powerful means of analyzing the function of genes in basic researches, while the technology may also be quite useful in developing therapeutic molecular targeting strategies for treatment of diseases. RNAi was first discovered in the nematode *Caenorhabditis elegans* as a response to double-stranded RNA, which induced sequence-specific silencing of gene expression [23]. In mammalian cells, short interfering RNA (siRNA) 21–23 nucleotide pairs in length silences the gene with the homologous sequence [23,24]. The in vivo effectiveness of siRNA-mediated silencing was also studied in various organs, including the liver [25,26] and the skeletal muscle [27]. However, in vivo RNAi in cardiac tissues has not been reported so far.

In the present study, we investigated whether BR14 facilitates sonoporation-mediated transfection of naked plasmid DNA into the adult murine heart. Moreover, we applied this method to transfer synthetic siRNA duplex, to knock down targeted genes in the heart in vivo.

Materials and methods

Animals. Female C57BL/6 mice (16.8 ± 0.7 weeks old, weighing 24.0 ± 0.5 g) were purchased from Shimizu Laboratory Suppliers (Kyoto, Japan). Enhanced green fluorescent protein (EGFP) transgenic mice (TGM) (27.3 ± 4.8 weeks old, weighing 25.3 ± 1.1 g) were purchased from Charles River Japan (Yokohama, Japan). All the animal experiments were performed according to the approved guidelines of Kyoto Prefectural University of Medicine.

Plasmid vectors and siRNA. The plasmids pGEG.GL3 [28], pGEG.EGFP [29], and pGEG.β [30] carried GL3 firefly luciferase (Luc), EGFP, and *Escherichia coli* β-galactosidase (β-gal) genes, respectively, under the control of the CAG promoter. Each plasmid also contained Epstein–Barr virus (EBV) nuclear antigen 1 (EBNA1) gene and EBV oriP sequence [31]. Plasmids were purified using Qiagen MegaPrep Endo-free kits (Qiagen, Hilden, Germany). siRNA duplex targeting GFP (siRNA-GFP) was purchased from Dharmacon (Lafayette, CO, USA).

In vivo experiments. Plasmid DNA (500 μg) or siRNA (40 μg) was diluted in 400 μl PBS and mixed with 100 μl BR14 microbubble. Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 μg/g body weight), and tracheotomy was performed in the supine position to provide ventilation via a ventilator (MiniVent 845; Hugo Sachs Elektronik, March-Hugstetten, Germany) at the rate of 150 cycles per min. An incision was made in the greater and smaller pectoral muscles, and the microbubble/nucleic acid solution was injected over a period of about 10 s into the left ventricular (LV) cavity via the intercostal muscle using a 27-gauge needle. The same solution was injected into the tail vein (TV) of another group of mice. At the same time as the initiation of the injection, transthoracic ultrasound insonation (sonication) was performed through a 6-mm diameter probe with an input frequency of 1 MHz, an output intensity of 1.0–2.0 W/cm^2, a pulse duty ratio (PDR) of 10–50%, and a duration of 60 s. A Sonitron 2000 (Rich-Mar, Inola, OK, USA) was used to generate the ultrasound. Hydrodynamics-based transduction was performed as described previously [28]. Briefly, 40 μg siRNA-GFP was diluted in 1600 μl PBS and injected intravenously within 4s via the tail vein using a 27-gauge needle.

Echocardiography and ECG monitoring. Transthoracic echocardiography was performed using an ultrasound platform (Nemio 30, Toshiba Medical, Tokyo, Japan) equipped with a 13-MHz imaging transducer. Under anesthesia, a parasternal view was obtained and M-mode images of the LV were recorded. Electrocardiographic monitoring was performed by limb lead (II) during the sonoporation.

Luciferase assay. Biventricular muscle was minced with a pair of scissors and homogenized in 200 μl of reporter lysis buffer (Promega, Madison, WI, USA) using a sonicator. The extract was centrifuged at 14,000*g* for 15 min, and the Luc activity in the supernatant was measured as described [32]. Organs other than the heart were also treated as described above. Protein concentration in the extract was determined as described previously [28].

X-gal staining. The heart was fixed with 4% paraformaldehyde and dehydrated in sucrose solution. The specimens were then stained with X-gal solution (0.05% (v/v) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Nacalai Tesque, Kyoto, Japan), 1 mM $MgCl_2$, 150 mM NaCl, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, and 0.1% Triton X-100). After 12 h of incubation at 37 °C, the reaction was terminated by replacing the solution with PBS [33].

Fluorescence microscopic observation and immunohistochemistry. After perfusion with saline, the heart was fixed with 4% paraformaldehyde and dehydrated in sucrose solution. The specimens were embedded in OCT compound and immediately frozen at −80 °C. Serial sections 10 μm thick were cut on a cryostat and observed under a fluorescence microscope with excitation at 488 nm. The cryosections were stained with anti-GFP antibody (Molecular Probes, Leiden, The Netherlands) and visualized using the avidin/biotin/peroxidase method (Vector Laboratories, Burlingame, CA).

Statistical analysis. Differences among continuous variables were tested by Student's *t* test for paired and unpaired observations and by ANOVA with Fisher's PLSD correction for repeated comparisons. A value of $P < 0.05$ was considered to be statistically significant.