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● *Original Contribution*

INSONATION FACILITATES PLASMID DNA TRANSFECTION INTO THE CENTRAL NERVOUS SYSTEM AND MICROBUBBLES ENHANCE THE EFFECT

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Abstract—Many of the diseases which affect the central nervous system are intractable to conventional therapies and therefore require alternative treatments such as gene therapy. Therapy requires safety, since the central nervous system is a critical organ. Choice of nonviral vectors such as naked plasmid DNA may have merit. However, transfection efficiencies of these vectors are low. We have investigated the use of 210.4 kHz ultrasound and found that 5.0 W/cm^2 of insonation for 5 s most effectively transfected a plasmid DNA into culture slices of mouse brain (147.68-fold increase compared with 0 W/cm^2 of insonation for 5 s). The effect was reinforced by combination with echo contrast agent, Levovist. One hundred fifty mg/mL of Levovist significantly increased gene transfection by ultrasound (5.23-fold when insonated at 5.0 W/cm^2 for 5 s). When DNA was intracranially injected, Levovist also enhanced gene transfection in newborn mice (4.49-fold increase when insonated at 5.0 W/cm^2 for 5 s). Since ultrasound successfully transfected naked plasmid DNA into the neural tissue and Levovist enhanced the effect, this approach may have a significant role in gene transfer to the central nervous system. (E-mail: manome@jikei.ac.jp)

Key Words: **Ultrasound, Plasmid, DNA, Gene therapy, Microbubbles, Central nervous system.**

INTRODUCTION

Merit exists for gene therapy in the treatment of many congenital and acquired diseases that are resistant to conventional therapies. Recent advancement of molecular biology in the medical field allows for the practical application of gene therapy with various candidate genes. While the development of therapeutic genes has progressed remarkably, present delivery systems have potential limitations to their clinical application (Nathwani et al. 2004). In principle, two major kinds of vectors are available; one is viral and the other is nonviral. The viral vector has the advantage of efficiency of gene delivery and is used for most clinical trials. However, despite its superior efficiency in gene transfer both *in vitro* and *in vivo*, the viral vector arouses concern in terms of invoking strong adverse effects, such as immune responses, inflammation and/or carcinogenesis (Kang and Tisdale 2004). On the contrary, the nonviral vector is less hazardous. It lacks proteins that cause immune responses in the host. In addition, the vector itself is less toxic to the host, due to the lack of cytopathic effects attributable to viral infection. The nonviral vector also does not contain unnecessary genes, such as the viral structure genome. Furthermore, it is easier to prepare, has no gene–size limitations that affect delivery and can deliver genes into quiescent cells (Coonrod et al. 1997). Thus, the nonviral vector has several advantages. Among nonviral approaches, naked DNA transfer has recently been highlighted, since progress has been made in delivery to muscle in combination with electroporation or to the liver with intravascular delivery (Hartikka et al. 2001; Herweijer et al. 2001; Maruyama et al. 2002; Somiari et al. 2000). It also has a strong potential for use in gene therapy, since this approach becomes more significant

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when combined with a better strategy for treatment, such as combination with ribozyme, antisense or RNA interference (Pardridge 2004; Yu et al. 2002). Nevertheless, naked plasmid DNA gene transfer has been used mainly for genetic immunization studies, due to its low transfection efficiency (Herweijer and Wolff 2003).

We have utilized ultrasound as a mechanical force and reported that ultrasound irradiation, called insonation, increased the efficiency of transfection into colon carcinoma cells both *in vitro* and *in vivo* (Manome et al. 2000). Since insonation achieved efficient *in vivo* transfection of naked plasmid DNA, the method will prove beneficial in gene therapy, especially for critical organs such as the central nervous system or the cardiovascular system. In this study, we attempted to evaluate the efficiency of gene transfer by ultrasound on the central nervous system cells. Recent studies demonstrated that microbubbles of echo contrast material enhanced gene transfection by ultrasound (Bekeredjian et al. 2003; Christiansen et al. 2003; Li et al. 2003; Lu et al. 2003; Pislaru et al. 2003). Therefore, we also attempted to evaluate the effect of microbubbles on gene transfection to the nervous system.

MATERIALS AND METHODS

Insonation

Ultrasound was generated by a wave synthesizer, wave factory WF 1943 (NF electronic instruments, Yokohama, Japan). The amplifier and ultrasound probe were designed by one of the authors (H.F.) and constructed by Honda Electric Inc., Toyohashi, Japan. Transducer is made of piezoceramics and the diameter of the probe was 5 mm. This apparatus could emit continuous wave of 210.4 kHz ultrasound up to 5.08 W/cm^2 measured by force balance method in water using ultrasound power meter (UPM-DT-1, Ohmic Instruments Co., Easton, MD, U.S.A.). In this setting, ultrasound intensity of 5 W/cm^2 corresponds to 0.274 MPa. The insonation method to the culture slice is shown in Fig. 1. The distance between probe tip and culture slice was 2 mm and the slice was insonated passing through phosphate-buffered saline, with or without plasmid. Since transducer-generated plane wave and ultrasound was unfocused, the culture slice could be uniformly insonated. Insonation was performed at 23°C. This insonation at 5.0 W/cm^2 for 5 s increased temperature of 4.19 ± 0.79°C (n = 12) in the inner dish.

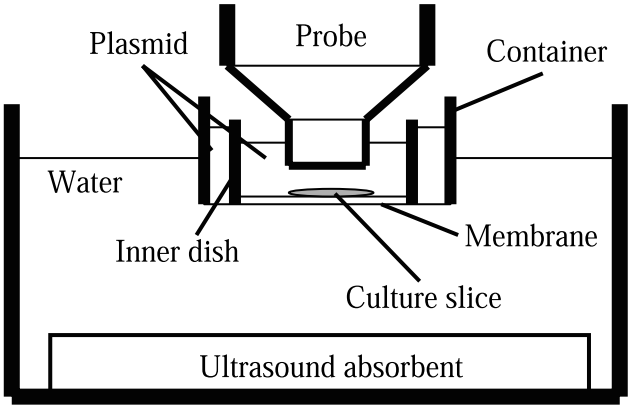

Fig. 1. Schematic diagram of insonation to culture slice. Culture slice was directly incubated in plasmid DNA solution with or without microbubbles and insonated from the top of the inner dish of the culture insert. After insonation, the culture slice on the inner dish was returned to the six-well plate and further cultivated.

Plasmid used for transfection

The pCAG-Luc plasmid was provided by Kiyotsugu Yoshida (Tokyo Medical and Dental University, Tokyo, Japan). In this vector, CMV-IE enhancer was coupled with chicken β-actin promoter (CAG promoter) which drives the firefly luciferase (Luc) gene. The plasmid was prepared with alkali-SDS method with double cesium ultracentrifugation. Since the amounts of luciferase protein could be easily determined, this plasmid was used for the quantitative analysis of transgene expression. Expression of the luciferase gene was measured by the luciferase assay (Stratagene, La Jolla, CA. U.S.A.) with the luminometer, Lumat LB9501 (Berthold Japan, Tokyo). In contrast, the CMV-GFP plasmid (provided by Michiko Watanabe, Jikei University School of Medicine, Tokyo, Japan), in which the CMV promoter drives the modified green fluorescent protein gene, was used for histologic determination of the transfected cell type, since transfected cells can be identified by the confocal laser microscope (LSM510, Zeiss, Gottingen, Germany).

Animal studies

The ICR/Jcl mice were purchased from Clea Japan (Tokyo, Japan). All the animal experiments were performed under the guidelines of the Animal Care Facility in Jikei University School of Medicine. To obtain culture slices of the mouse brain, 1 to 4–d–old ICR/Jcl mice were decapitated and the cerebra were quickly isolated under antiseptic conditions and sliced by a microslicer (DTK-1000, D. EM, Kyoto, Japan) at a thickness of 300 μm. After preparation, cerebral slices were cultured on semiporous membranes (Millicell-CM, 60 mm, Millipore, Bedford, MA, USA) (Stoppini et al. 1991) and placed in six-well culture dishes with 1 mL of culture medium composed of 25% heat-inactivated horse serum, 25% Hanks's balanced salt solution (HBSS) and 50% minimum essential medium (MEM) without glutamate (Invitrogen Japan, Tokyo, Japan) (Kudo 2000). We cul-