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PRE-CLINICAL RESEARCH

Ultrasound-Microbubble–Mediated Intercellular Adhesion Molecule-1 Small Interfering Ribonucleic Acid Transfection Attenuates Neointimal Formation After Arterial Injury in Mice

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Objectives The purpose of this study was to investigate the efficiency of small interfering ribonucleic acid (siRNA) in murine arteries. We transfected it using a nonviral ultrasound-microbubble–mediated in vivo gene delivery system.

Background siRNA is an effective methodology to suppress gene function. The siRNA can be synthesized easily; however, a major obstacle in the use of siRNA as therapeutics is the difficulty involved in effective in vivo delivery.

Methods To investigate the efficiency of nonviral ultrasound-microbubble–mediated in vivo siRNA delivery, we used a fluorescein-labeled siRNA, green fluorescent protein (GFP) siRNA, and intercellular adhesion molecule (ICAM)-1 siRNA in murine arteries. Murine femoral arteries were injured using flexible wires to establish arterial injury.

Results The fluorescein-labeled siRNA and GFP siRNA showed that this nonviral approach could deliver siRNA into target arteries effectively without any tissue damage and systemic adverse effects. ICAM-1 siRNA transfection into murine injured arteries significantly suppressed the development of neointimal formation in comparison to those in the control group. Immunohistochemistry revealed that accumulation of T cells and adhesion molecule positive cells was observed in nontreated injured arteries, whereas siRNA suppressed accumulation.

Conclusions The nonviral ultrasound-microbubble delivery of siRNA ensures effective transfection into target arteries. ICAM-1 siRNA has the potential to suppress arterial neointimal formation. Transfection of siRNA can be beneficial for the clinical treatment of cardiovascular and other inflammatory diseases. (J Am Coll Cardiol 2010;55:904–13)

The most effective strategy for evaluating gene function is using ribozymes and ribonucleic acid (RNA) interference (1). Small interfering ribonucleic acids (siRNA) are powerful tools that suppress gene expression in cells (2). However, the therapeutic application of siRNA is dependent upon the development of delivery vehicles (3). In addition, such a delivery vehicle should be administered efficiently, safely, and repeatedly. However, there are only a few reports on effective and safe in vivo transfection of siRNA into target organs. Although viral vector systems are efficient for transfection of genes, such as short hairpin RNAs and molecular derivatives of siRNAs, there is concern that these systems may adversely affect clinical utility (4). As therapeutic ultrasound increases cell membrane permeabilization (5), the nonviral ultrasound method increases the transfection efficiency of decoy in vivo into arteries (6), and other organs. Although there are a few papers that demonstrate sonoporation using microbubble-promoted plasmid deoxyribonucleic acid/siRNA transduction to murine hearts (7) and to joint synovium (8), there have been few reports of the transfection of siRNA into target arteries using ultrasound-microbubble methods.

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Recently, we reported decoy oligonucleotide transfection into the arteries using this methodology (9). Thus, we have used the fluorescein isothiocyanate (FITC)-labeled siRNA and a murine arterial injury model to study the transfection efficiency of siRNA using an ultrasound microbubble method into target arteries. Because intercellular adhesion molecule (ICAM)-1 plays a critical role for progression of inflammation and arterial neointimal formation (10), we examined the effects of ICAM-1 siRNA for prevention of neointimal formation after arterial injury using the ultrasound-microbubble–mediated transfection.

This is the first paper to demonstrate that the ultrasound microbubble method significantly increased siRNA transfection efficiency into arteries. We revealed that ICAM-1 siRNA transfection using this method has potential to prevent inflammatory-related cardiovascular diseases.

Methods

Preparation of siRNA-microbubble complexes. The 1,2 dioleoyl-3-trimethylammonium-propane (DOTAP) was purchased from Roche Diagnostics (Alameda, California). For each artery, 20-μg siRNAs in 90-μl transfection buffer was transferred into a sterile Eppendorf tube. In a separate sterile polystyrene tube, 50-μg DOTAP was mixed with 90-μl transfection buffer with 10-μl microbubble (Optison solution [human albumin microspheres], Mallinckrodt, Hazelwood, Missouri), and then the siRNA mixture was transferred to the polystyrene tube containing the DOTAP and incubated at room temperature for 30 min. The mixture should have turned cloudy, but no precipitates or aggregation were visible. Charge ratios of DOTAP and siRNAs varied from 1:2 to 2:1.

Arterial injury models. Male wild-type mice and transgenic mice constitutively overexpressing enhanced green fluorescent protein (GFP [C57BL/6, age 4 to 6 weeks, weight 20 to 25 g] 006567, Japan Charles River Laboratories, Atsugi, Kanagawa, Japan) were used in this study. We made an arterial injury model that was modified from the previous reports (11). Briefly, the femoral artery was looped and tied off with 6-0 silk sutures for temporary vascular control during the procedure. A transverse arteriotomy was made, and a flexible angioplasty guidewire (a curved 350-μm polished copper wire) was introduced and advanced 1 cm. Endothelial denudation injury of the artery was performed by use of wire withdrawal injury, and 3 passes were made along the artery. A sham operation (no wire injury) was also performed. The animals were fed a standard diet and water and were maintained in compliance with animal welfare guidelines of the Institute of Experimental Animals, Tokyo Medical and Dental University. The investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Transfection of FITC-labeled siRNA into injured arteries of wild-type mice. To study the in vivo transfection efficiency of siRNA, we used the FITC-labeled siRNA and the wild-type murine arterial injury model. FITC-labeled siRNA (siRNA Alexa Fluor 488) was purchased from Qiagen K.K. (Tokyo, Japan). Immediately after injury of right femoral arteries, 90 μl of the FITC-labeled siRNA (20 μg) plus microbubble (10 μl) mixture was incubated within the arterial lumen. The ultrasound transducer was held by hand, and it was attached directly to the artery. The ultrasound was irradiated for 20 s (1 MHz, 0.5 W/cm^2, duty 50%) to the artery. The siRNA transfection was performed while the vessel was being ligated (n = 6). For control studies of injured arteries: 1) no siRNA, microbubble and ultrasound (n = 2); 2) non–FITC-labeled siRNA without microbubble and ultrasound (n = 4); 3) FITC-labeled siRNA without microbubble and ultrasound (n = 4); and 4) FITC-labeled siRNA with microbubble but no ultrasound irradiation (n = 6) were performed. Additionally, we compared the FITC siRNA transfection efficiency between de-endothelialized vessels (n = 6) and non–de-endothelialized vessels (n = 6) using the same ultrasound-microbubble method. The arteries and other organs were harvested 8 h after transfection; the transfection efficiency was judged using fluorescent microscopy. Systemic adverse effects were defined as death or significant body weight loss during the observation period.

Transfection of GFP siRNA into injured arteries of GFP transgenic mice. To study the chronic efficiency of siRNA, we used the GFP siRNA (AM4626, Ambion, Foster City, California) and the GFP transgenic murine arterial injury model. Immediately after injury of right femoral arteries of GFP transgenic mice, 90-μl of GFP siRNA (20 μg) plus microbubble (10 μl) mixture was incubated within the arterial lumen. The siRNA was simply mixed with the microbubbles. The flow in the femoral artery was temporarily disrupted during the ultrasound-microbubble destruction. Thus, the flow was similarly disrupted during the control experiments. The ultrasound was irradiated for 20 s (1 MHz, 0.5 W/cm^2, duty 50%) to the artery (n = 4). For control studies: 1) no siRNA, microbubble and ultrasound (n = 2); 2) GFP siRNA without microbubble and ultrasound (n = 4); and 3) GFP siRNA with microbubble but no ultrasound irradiation (n = 4) were performed. Additionally, we compared GFP expression between GFP siRNA transfected vessels (n = 4) and remote vessels (n = 4) of GFP-transgenic mice. The arteries were harvested 7 days

Abbreviations and Acronyms

- **cDNA** = complementary deoxyribonucleic acid
- **DOTAP** = 1,2 dioleoyl-3-trimethylammonium-propane
- **FITC** = fluorescein isothiocyanate
- **GFP** = green fluorescent protein
- **ICAM** = intercellular adhesion molecule
- **MLR** = mixed lymphocyte reaction
- **mRNA** = messenger ribonucleic acid
- **RT-PCR** = real-time polymerase chain reaction
- **siRNA** = small interfering ribonucleic acid
- **VCAM** = vascular cell adhesion molecule