Effect of microRNA-145 to prevent vein graft disease in rabbits by regulation of smooth muscle cell phenotype

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Objective: Because microRNA-145 (miR-145) is a specific mediator in the regulation of the proliferation and differentiation of smooth muscle cells, we investigated the effect of miR-145 on the intimal hyperplasia in the rabbit model of vein graft disease using electroporation-mediated gene transfer.

Methods: The right jugular vein of male Japanese white rabbits was harvested and transduced with miR-145–encoding plasmids using an electroporator and then interposed in the carotid artery. At 2 or 4 weeks postoperatively, the venous graft was explanted, and the intimal thickness and intima/media area ratio were evaluated. Furthermore, 3 days after implantation, the myocardin and serum response factors were measured using real-time polymerase chain reaction. At 2 weeks after implantation, immunohistochemical investigations using mature smooth muscle markers, myosin heavy chain smooth muscle-1 and -2, and proliferation marker Ki-67 were performed.

Results: MiR-145 transduction significantly reduced the neointimal thickness at both 2 and 4 weeks (2 weeks, 52.1 ± 15.7 vs 113.2 ± 26.9 μm, $P < .05$, $n = 6$; 4 weeks, 42.4 ± 4.8 vs 136.5 ± 38.3 μm, $P < .05$, $n = 8$), and it also significantly reduced the intima/media area ratio at 4 weeks (0.22 ± 0.04 vs 1.13 ± 0.23, $P < .01$, $n = 8$). Additionally, it upregulated the mRNA expression level of myocardin compared with that in the grafts that did not receive gene transfer. Smooth muscle-2 and Ki-67 expression revealed that miR-145 transduced grafts contained more smooth muscle-2–positive mature smooth muscle cells and fewer Ki-67–positive proliferating cells.

Conclusions: Nonviral transduction of miR-145 into the bypass graft could be a novel option for preventing intimal hyperplasia in vein graft disease. (J Thorac Cardiovasc Surg 2014;148:676-82)

Supplemental material is available online.

The autologous vein graft has been widely used for revascularization in coronary artery bypass grafting since its introduction in 1969.[1] Nevertheless, owing to accelerated atherosclerosis, these vein grafts have had a lower patency rate than those of other arterial grafts.[2] Thrombotic occlusion followed by intimal hyperplasia is the major cause of vein graft disease, and vascular smooth muscle cell (SMC) proliferation and migration into the intima are the key mechanisms in this process. Chamley-Campbell and colleagues[3] first described the phenotypic modulation of SMCs from the contractile state to the synthetic state. Phenotypic alternations of vascular SMCs have been identified as playing an important role in the response to atherosclerosis, arterial injury, and bypass vein grafting.[4-10] We have previously demonstrated that C-type natriuretic peptide suppressed neointimal lesion formation in the arterialized rabbit vein graft model using adenovirus-mediated gene transfer.[11] We also reported that overexpression of C-type natriuretic peptide in SMCs induced growth inhibition of proliferating SMCs, with alteration of the phenotype from the synthetic state to the contractile state.[12]

MicroRNAs are a new class of small (~22 nucleotides) noncoding RNA. In most cases, microRNAs negatively regulate expression of protein-coding genes by promoting degradation or suppressing translation of target mRNAs and modulating various biologic functions.[13] Currently, microRNAs are under investigation as a new modality of gene therapy for ischemic heart disease and vascular diseases.[14] Recent studies have reported that microRNA-145 (miR-145) is a specific mediator in the regulation of proliferation, differentiation, and phenotype of SMCs and is necessary for maintaining a differentiated SMC phenotype.[4-7]

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Abbreviations and Acronyms

GFP = green fluorescent protein
miR-145 = microRNA-145
SMC = smooth muscle cell
SM-1 = smooth muscle-1
SM-2 = smooth muscle-2
SRF = serum response factor

In recent years, electroporation-mediated gene transfer has emerged in the in vivo study for future clinical applications designed at overcoming virus-related problems.[15] Therefore, in the present study, we investigated the effect of miR-145 on intimal hyperplasia in the rabbit model of vein graft disease using electroporation-mediated gene transfer.

METHODS

Animals

A total of 50 male Japanese white rabbits weighing 2.5 to 3.5 kg were used. The rabbits were maintained with a 12-hour light/dark schedule and fed a regular rabbit chow diet. The rabbits were randomly divided into 2 groups: a control group (n = 25) without electroporation-mediated gene transfer and a miR-145 electroporation group (n = 25). Three days after the operation, 4 rabbits were killed for green fluorescent protein (GFP) immunostaining and 5 rabbits for reverse transcriptase polymerase chain reaction (PCR) study in each group. Another 8 rabbits from each group were killed at 2 and 4 weeks after the initial histologic examination. The Kyoto University animal experiment review board approved the present experiment (approval reference no. MedKyo 12185). All the rabbits were used in accordance with the guidelines for animal experiments of Kyoto University, which conforms to the law of the "Guide for the Care and Use of Laboratory Animals" in Japan.

Plasmid Preparation

Expression vector for human miR-145 (pMIW-cGFP-miR-145) was obtained from B-Bridge Co, Ltd (Tokyo, Japan). In the construction of this vector, a GFP expression site was included to confirm successful gene transduction after electroporation (Appendix Figure 1).

Electric Pulse Delivery for Gene Electroporation

Electric pulses were delivered using an electric pulse generator (Super Electroporator NEPA21, Nepa Gene Co, Ltd, Chiba, Japan). Figure 1, *A*, shows a schematic representation of the electroporation device. Freshly isolated vein grafts were suspended in balanced salt saline (140 mM NaCl, 5.4 mM KCl, and 10 mM HCL, pH 7.6) with 500 μg/mL of vector and placed in an electrode chamber, as reported previously.[16,17] First, to confirm the validity of the electroporation method, we performed a preliminary in vitro study using the DsRed expression vector in which several electric pulse parameters were tested ("poring pulse": voltage 25, 50, and 75 V; pulse duration, 5 and 10 ms; interval, 95 and 90 ms; number of pulses, 2, 4, and 6; "transfer pulse": voltage, 10 V; pulse duration, 5 and 10 ms; interval, 95 and 90 ms; number of pulses, 5 and 10; Appendix Table 1). After electroporation, the grafts were suspended in α-minimal essential medium (Invitrogen, Carlsbad, Calif) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen), and cultured in standard plastic dishes for 2 days. The grafts were observed using fluorescence microscopy and by immunostaining using monoclonal antibodies against DsRed. Second, to decide on the optimal conditions for electroporation, several electric pulse parameters were tested using the miR-145 expression vector with a GFP expression site. These variables were chosen on the basis of the results from our experiment using the DsRed expression vector and earlier well-documented reports that used the electroporation method.[16-18] Finally, 2 electric poring pulses (25 V, 5-ms duration, 95-ms interval) followed by 8 transfer pulses (10 V, 5-ms duration, 95-ms interval) were adopted according to the results of both DsRed and GFP apparent expression shown in Appendix Figure 2 and Figure 1, *C*.

Arterialized Rabbit Vein Graft Model

The operative procedure was performed under aseptic conditions. Anesthesia was achieved by administration of pentobarbital sodium (25 mg/kg intravenously) and lidocaine hydrochloride (50 mg/kg subcutaneously). After a longitudinal neck incision, a 15- to 20-mm segment of the right jugular vein was exposed, and all the branches of the vein were ligated. A 2F Fogarty balloon catheter was inserted. After the balloon was inflated with 0.2 mL of air, the intima of the vein was denuded by 3 passages of the catheter. The vein was removed and kept moistened in heparinized saline (10 IU/mL) at room temperature. After the rabbits had been given intravenous heparin (200 IU/kg), a 15- to 20-mm segment of the common carotid artery was meticulously isolated with vascular forceps and then removed. The prepared vein graft was interposed in the carotid artery in a reversed end-to-end fashion. Anastomoses were created with 10-0 nylon continuous suture under ×10 magnification using an operative microscope. To facilitate the intimal hyperplasia, the internal carotid artery and one of the branches of the external carotid artery were ligated with 6-0 silk suture. The wound was closed layer to layer. Cefazolin sodium was given intravenously as a prophylactic measure.

Intimal Hyperplasia Assessment

At 2 or 4 weeks after the operation, the vein grafts were harvested under general and local anesthesia, and the rabbits were killed by an overdose of pentobarbital sodium. The harvested vein grafts were fixed with 10% phosphate-buffered formalin at a pressure of 100 mm Hg for 20 minutes. The perfused vein graft was immersed in the same fixative overnight and subjected to histologic examination. At least 4 sections were obtained from each vein graft and stained with both hematoxylin and eosin and elastica van Gieson. The neointima was defined as the area from the inner surface to the internal elastic lamina. The cross-sectional intimal thickness was measured at 6 randomly selected views per section. The average of the 6 values was used to represent intimal hyperplasia. The cross-sectional intima/media area ratio was also calculated.

Immunohistochemical Staining

At 3 days postoperatively, the vein grafts were explanted, and GFP immunostaining was performed to confirm the gene transduction of miR-145 using the electroporation method. At 2 weeks postoperatively, the vein grafts were explanted and analyzed by immunostaining using monoclonal antibodies against myosin heavy chain smooth muscle-1 (SM-1) and smooth muscle-2 (SM-2; Yamasa Corp, Tokyo, Japan). SM-1 is constitutively expressed in all types of adult SMCs, but SM-2 exists only in mature differentiated SMCs.[8-10] Additionally, immunohistochemical staining with Ki-67 monoclonal antibody (DAKO Cytomation, Glostrup, Denmark) was performed to identify the proliferative cells in the neointima. The quantitative analysis was performed as follows. The total and Ki-67–positive cells were counted in 8 randomly selected high-power fields (original magnification, ×400) per section, and the number of Ki-67–positive cells/total number of cells counted was defined as the Ki-67 index.

Quantitative Real-Time PCR Analysis

At 3 days after implantation, the vein grafts were explanted and preserved immediately in liquid nitrogen. Total RNA was then extracted from the vein using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and reverse transcribed with the SuperScript III First-Strand Synthesis

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