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Tissue-engineered acellular small diameter long-bypass grafts with neointima-inducing activity

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ABSTRACT

Researchers have attempted to develop efficient antithrombogenic surfaces, and yet small-caliber artificial vascular grafts are still unavailable. Here, we demonstrate the excellent patency of tissue-engineered small-caliber long-bypass grafts measuring 20–30 cm in length and having a 2-mm inner diameter. The inner surface of an acellular ostrich carotid artery was modified with a novel heterobifunctional peptide composed of a collagen-binding region and the integrin α4β1 ligand, REDV. Six grafts were transplanted in the femoral–femoral artery crossover bypass method. Animals were observed for 20 days and received no anticoagulant medication. No thrombogenesis was observed on the luminal surface and five cases were patent. In contrast, all unmodified grafts became occluded, and severe thrombosis was observed. The vascular grafts reported here are the first successful demonstrations of short-term patency at clinically applicable sizes.

1. Introduction

Synthetic artificial vascular grafts with inner diameters (IDs) larger than 6 mm are frequently used for bypass grafts or replacements, and they provide good, long-term clinical patency [1]. However, small-caliber vascular grafts with IDs smaller than 4 mm cannot achieve patency due to acute thrombogenicity on the luminal surface and occlusion [2,3]. Anastomotic intimal hyperplasia, aneurysm formation, infarction, and atherosclerotic disease progression are major health problems worldwide and may be addressed by using small-caliber vascular grafts [2–6]. During the last half century, researchers have investigated the potential of clinical applications of different types of grafts, such as endothelial cell-seeded synthetic materials [7–9], biodegradable polymers [10–15], cell sheets [16,17], and biopolymers [18–20]. The majority of these studies used 1–2-mm ID very short grafts transplanted into the abdominal aorta in rats or 3-mm ID grafts measuring approximately 5 cm in length transplanted for carotid artery replacement in dogs [10,12,14,21–23]. Although these studies provided a large amount of fundamental information regarding tissue response, antithrombogenicity, and patency of small-diameter artificial blood vessels, clinically usable small-diameter vascular grafts have yet to be developed. One explanation for this may stem from the discrepancies in required diameters and lengths between animal models and humans. For a coronary artery or limb distal bypass graft to be clinically relevant, small-diameter vascular grafts with IDs of less than 2 mm and lengths greater than 10 cm are required. Indeed, the majority of previous efforts have focused on obtaining a much more effective antithrombogenic surface, and the development of appropriate grafts yielding good patency has not been achieved.

Rapid endothelialization on the luminal surface of the artificial graft must inhibit the initial thrombosis and lead to long-term patency. In this study, we focused on four important features for designing small-caliber vascular grafts: (i) compliance matching and high tensile strength to facilitate easy suturing and to prohibit rupture; (ii) achieving high endothelial cell affinity of the modified luminal surfaces [24]; (iii) ability of the engineered graft to be replaced with host tissue in order to achieve life-time usage; and (iv) creating a graft with adequate size for clinical use, such as for a coronary artery bypass or distal limb bypass. Our final design was a 2-mm ID decellularized vascular graft with a length of 20–30 cm.

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To this end, decellularized ostrich carotid arteries were employed as the graft material due to their straight and branchless structure. We have been studying larger sized decellularized cardiovascular tissue using non-detergent technology [25,26] and reported their good patency and performance. However we did not achieve high patency in this thin and long vascular graft. The purpose of this study was to achieve the patency of the small-diameter long-bypass decellularized graft by modifying the luminal surface with the integrin α4β1 ligand peptide, REDV. Increasing of the endothelial cell binding affinity through peptide-modification was indicated in vitro, and the mechanical property of the graft was also evaluated. The patency and neointima-inducing activity were assessed by transplanting to pig in the femoral–femoral artery crossover bypass method.

2. Materials and methods

2.1. Decellularization of the graft

Carotid arteries were isolated from African black ostriches weighing 90–130 kg (Shimizu-Laboratory Supplies Co., Ltd, Kyoto, Japan). Fat tissue was removed from the arteries, and trimmed arteries were washed and packed with saline. They were then decellularized using a modified the ultrahigh hydrostatic pressure (UHP) method 20. Arteries were treated with the high-hydrostatic pressure with a cold isostatic pressurization machine (Dr. Chef; Kobelco, Kobe, Japan) containing pressure-transmission fluid which consists of ethylene-glycol and water [25,26]. The pressure was increased up to 980 MPa at a rate of 65.3 MPa/min and then maintained within the chamber for an additional 10 min. After decreasing the pressure until atmospheric pressure was reached, the specimen was washed with saline. The sample was then immersed with saline containing 40 U/mL of DNase I (Roche Applied Science, Indianapolis, IN, USA), 20 mM $MgCl_2$, and antibiotics for 3 days under 37 °C. After washing with saline, the specimen was immersed in saline containing 20 mM $MgCl_2$ and antibiotics for 3 days at 37 °C to remove the remaining DNase I. Finally, the sample was washed with saline and preserved in the same medium until experimental use. The quantification of the remaining DNA was carried out using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA), and fluorescence DNA quantification kit (Bio-Rad, Richmond, CA).

The physical and morphological features of the graft were evaluated. To evaluate histological staining, tissues were fixed with 10% formalin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and samples were embedded in paraffin. Paraffin-embedded tissues were then sectioned and stained with hematoxylin-eosin, Elastica van Gieson, or von Willebrand factor. Staining was carried out by the Applied Medical Research Laboratory (Osaka, Japan). The mechanical properties were evaluated by stress–strain curve measurements. The specimen was fastened to the stage of a tensile strength tester (AGS-H autograph, Shimadzu Co., Kyoto, Japan). The specimen was stretched with the speed of 2 N/min, and the stress and strain curves were analyzed.

2.2. Peptide-modification

Two peptides, having the sequences (Pro-Hyp-Gly)$_7$-Gly-Gly-Gly-Arg-Glu-Asp-Val (POG7G3REDV) and (Hyp-Pro-Gly)$_7$-Gly-Gly-Gly-Arg-Glu-Asp-Val (OPG7G3REDV) (Hyp = hydroxyproline), were purchased from Sigma–Aldrich Japan (Tokyo, Japan) as custom-made synthesized peptides. Decellularized carotid arteries were immersed in 10 μM peptide solution in saline, and samples were then incubated at 60 °C for 1 h. The solution containing the specimen was then cooled to room temperature. Before experimental use, the decellularized tissue was washed with saline.

Peptide modification on the luminal surface was verified using Alexa Fluor 633-labeled peptides under a confocal laser scanning microscope (CLSM). Alexa Fluor 633 NHS esters (Life Technologies, Gaithersburg, MD, USA) were added to the Gly-Gly-Gly-(Pro-Hyp-Gly)$_7$ peptide solution and incubated overnight at room temperature. After the incubation, labeled peptide was purified with a PD-10 column. Peptide modification was carried out using the same procedure. Cross sections of the modified graft were observed using a FV1000-D CLSM system (Olympus, Tokyo, Japan).

2.3. Cell binding assay

Human umbilical vein endothelial cells (HUVECs, Kurabo, Osaka Japan) were cultured on a tissue culture polystyrene surface (Iwaki, Tokyo, Japan) using endothelial basal medium (EBM-2; Lonza, Switzerland) supplemented with EGM Single Quots supplements and a growth factor kit (Lonza, Switzerland). Human endothelial progenitor cells (EPC; Biocat GmbH, Heidelberg, Germany) were also cultured on a collagen-coated polystyrene surface using endothelial basal medium (EBM-2; Lonza, Switzerland) supplemented with EGM-2-MV supplements and a growth factor kit (Lonza, Switzerland). The cells were grown to confluence. The cultures were placed in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C. The culture medium was changed every two days, and cells typically reached confluence in 6–8 days. After the cells reached confluence, they were washed with room-temperature HEPES buffer and then immersed in 0.025% trypsin/HEPES solution containing 0.01% EDTA (Lonza, Switzerland). After 2–5 min, the cells became round, indicating detachment from the surface. After neutralization of the trypsin, 5×10^5 cells were seeded on 10-cm culture dishes and cultured until reaching confluence. NIH/3T3 cells were cultured using the same procedure used on HUVECs, except that Dulbecco's modified Eagle's medium with low glucose was used instead. All disposable materials and subculture media were the same as those used in HUVEC cultures.

To evaluate the cell-binding efficiency for peptide-modified decellularized tissue, porcine aortic tissue was selected. The tissue was decellularized using UHP treatment, and cells were labeled with a Q-dot 625 cell labeling kit (Life Technologies, Grand Island, NY, USA). The labeling procedure was performed according to the manufacturer's instructions. Peptide-modified and unmodified 8 × 8 mm sections of decellularized tissue were placed into cell culture multiplates (Iwaki, Tokyo, Japan), and the cell suspension containing 2×10^5 cells was added to the chamber. The plates were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C for 24 h. The tissues were then washed three times with PBS, and adherent cells were eluted using cell lysis buffer (Promega, Madison, WI, USA). The fluorescence intensities of the lysed solutions were measured, and adherent cell numbers were calculated from a standard curve. Additionally, 2×10^5 EPCs were seeded on peptide-modified and unmodified 8 × 8 mm sections of decellularized tissue. After 24-h incubation, the tissue was washed with PBS. The tissues were fixed with 3.7% formaldehyde solution for 10 min at room temperature, and then the cells were immersed in 0.1% Triton X in PBS for 5 min. The fixed cells were stained with rhodamine phalloidin (Life technologies, Grand Island, NY, USA) and DAPI solution (Dojin chemical Co., Kumamoto, Japan). After staining, specimens were observed using the FV1000-D CLSM system (Olympus, Tokyo, Japan).

2.4. Transplantation of grafts

All animal experiments were conducted in accordance with the Guidelines for Animal Experiments established by the Ministry of