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Long-term results of cell-free biodegradable scaffolds for in situ tissue engineering of pulmonary artery in a canine model

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ABSTRACT

We previously developed a cell-free, biodegradable scaffold for in-situ tissue-engineering vasculature (iTEV) in a canine inferior vena cava (IVC) model. In this study, we investigated application of this scaffold for iTEV of the pulmonary artery (iTEV-PA) in a canine model. In vivo experiments were conducted to determine scaffold characteristics and long-term efficacy. Biodegradable scaffolds comprised polyglycolide knitted fibers and an L-lactide and ε-caprolactone copolymer sponge, with an outer glycolide and ε-caprolactone copolymer monofilament reinforcement. Tubular scaffolds (8 mm diameter) were implanted into the left pulmonary artery of experimental animals ($n = 7$) and evaluated up to 12 months postoperatively. Angiography of iTEV-PA after 12 months showed a well-formed vasculature without marked stenosis, aneurysmal change or thrombosis of iTEV-PA. Histological analysis revealed a vessel-like vasculature without calcification. However, vascular smooth muscle cells were not well-developed 12 months post-implantation. Biochemical analyses showed no significant difference in hydroxyproline and elastin content compared with native PA. Our long-term results of cell-free tissue-engineering of PAs have revealed the acceptable qualities and characteristics of iTEV-PAs. The strategy of using this cell-free biodegradable scaffold to create relatively small PAs could be applicable in pediatric cardiovascular surgery requiring materials.

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1. Introduction

Complex heart defects often require foreign substitutes to accomplish vascular continuity and achieve normal circulation. In terms of repairing an anomalous pulmonary artery (PA), autopericardium, homograft and artificial grafts are currently used. However, during long-term follow-up some patients require further surgical intervention because of material-related events [1–5]. Because operative survival has improved for infants and children with complex congenital heart defects, the number of adults with congenital heart disease (CHD) is drastically increasing [6]. Furthermore, the number of secondary operations to replace outgrown or degenerative prosthetic conduits has also increased. One reason for this is that commonly used for the substitutes are foreign materials. We have therefore focused on development of optimal materials, possessing suitable repair characteristics to address current material concerns for CHD treatment.

We previously reported long-term efficacy results of in-situ tissue-engineering vasculature (iTEV) in a canine model [7]. The objective of this study was to determine the feasibility of using any cell source and to identify the ideal biodegradable material to achieve a versatile cell-free protocol. iTEV using our biomaterial resulted in native tissue-like histological regeneration, with acceptable biomechanical characters [7]. To overcome graft stenosis of the tissue engineered vasculature, we explored the use of this biodegradable scaffold in achieving acceptable long-term results in a venous position.

To investigate the use of our biomaterial for PA regeneration, in vivo experimentation was conducted to evaluate the applicability, basic characteristics and long-term efficacy of our biodegradable scaffold for cell-free in-situ tissue-engineering vasculature of the pulmonary artery (iTEV-PA), in a canine model.

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2. Materials and methods

2.1. In vivo experimentation

All animals received care according to the Principles of Laboratory Animal Care (NIH, 1985). The ethical committee of Tokyo Women's Medical University reviewed and approved the study protocol (Permit Number: 11-75). Seven healthy adult female beagles (NARC, Tomisato, Japan) with a mean weight of 9.6 kg (8.6–10.2 kg) were used in this study. Two animal was euthanized at 6 months and 5 animals were euthanized at 12 months for angiographies, echocardiograms, and for histological and biochemical analyses. We assessed 5 dogs after 12 months because the histological results in 2 dogs after 6 months were acceptable. For surgeries, animals were anesthetized with pentobarbital (1 mg/kg body weight) and heparin (700 U/kg body weight) was administered intravenously for anticoagulation during anastomoses.

Under general anesthesia, the fifth intercostal was opened in a right lateral position. After heparinization, native left PA (LPA) was resected between the bifurcation of LPA and the superior lobar artery using a simple clamp technique. The distal end of the LPA was then anastomosed with the scaffold. The main PA (mPA) was then partially C-clamped and longitudinally incised followed by anastomosis of the scaffold. Finally, each remaining end on the scaffold was sutured together. The composite tubular scaffold used in this study was 8 mm in diameter and 5–6 cm long. The lengths of the scaffolds were trimmed according to the design of the anastomoses. The scaffold completely loses its strength within 6 months owing to non-enzymatic hydrolysis as previously described [7]. The scaffold was cut and trimmed to obtain natural curvature and anastomosis. All animals were assessed at the time of implantation and 3, 6 ($n = 2$), and 12 ($n = 4$) months post-implantation using a Terason t3000 ultrasound system (Terason, Burlington, MA) to evaluate blood flow in the iTEV-PA. Angiographies of iTEV-PA were carried out 6 and 12 months post-implantation. Subject animals were anesthetized using the protocol described above and placed in sterilized conditions for femoral vein dissection. An 8-Fr long catheter sheath (Terumo, Tokyo, Japan) was placed through the femoral vein adjacent to the inferior vena cava, close to the diaphragm. An X-ray fluoroscope was used for digital angiography. Approximately 10 ml of angiographic agent was injected to evaluate iTEV-PA features during the regeneration process. Animals were subsequently maintained without anticoagulant until euthanasia was performed as previously described [7–9]. After dissection, iTEV-PA length was measured directly between the two suture lines and the iTEV-PA and was then longitudinally dissected to perform histological and biochemical analyses. Samples of LPA and iTEV-PA were dissected, rinsed with phosphate-buffered saline (PBS) and stored at −20 °C until required for analysis.

2.2. Macroscopic and histological examination

After dissecting the iTEV-PA, macroscopic sample overviews were taken with a digital camera. Longitudinally incised iTEV-PA and control LPA samples were placed on hard cardboard and fixed at both ends using metal surgical clips to maintain the original length. The samples were then fixed in 4% paraformaldehyde in pH 7.0 PBS, embedded in paraffin and sectioned at 4–5 μm. Some sections were subjected to hematoxylin and eosin (H&E), Masson's trichrome, Victoria blue–van Gieson, or modified von Kossa staining. Immunostaining of remaining sections was performed using antibodies against factor VIII (1:1000; Abcam, Tokyo, Japan), alpha smooth muscle actin (ASMA, clone 1A4, 1:1000; Dako Japan, Tokyo, Japan), myosin heavy chain (MHC, 1:1000; Sigma), smooth muscle myosin heavy chain 1 (SM1, 1:1000; Yamasa, Tokyo, Japan and smooth muscle myosin heavy chain 2 (SM2, 1:1000, Abcam), as previously described [7–9]. LPA and iTEV-PA wall thicknesses were measured at 10 different sites in these sections.

To confirm endothelial cell distribution in iTEV-PAs at 6 months, specimens were co-incubated with Texas Red® Lycopersicon Esculentum (TOMATO) Lectin (1/15 mg/ml; Vector Laboratories, Burlingame, CA) for 1 h at 37 °C. To confirm ASMA distribution stereology, whole staining was performed on specimens as follows; iTEV-PA specimens were fixed in methanol/DMSO (4:1) at 4 °C overnight, washed with 100% methanol (3 × 10 min) at room temperature (RT) and stored in 100% methanol at −20 °C for 4 days. Dehydrated specimens were then bleached in methanol/DMSO/30% H_2O_2 (4:1:1) at RT overnight. Bleached specimens were then washed (1 × 10 min) in 75% methanol/0.1% Tween20/PBS (PBS-Tw) at RT, followed by washing in 50% methanol/PBS-Tw, 25% methanol/PBS-Tw (1 × 10 min) and PBS-Tw (2 × 10 min). Blocking was carried out by incubating specimens in 2% skimmed milk/PBS/1% Triton X-100 (PBSMT, 2 × 1 h) at RT, and then overnight at 4 °C. After blocking, specimens were incubated with Fluorescein isothiocyanate (FITC)-conjugated anti-ASMA (clone 1A4; Sigma, St. Louis, MO, USA) diluted 1:1000 in PBSMT for 3 nights at 4 °C. Specimens were then washed with PBSMT (5 × 1 h) at 4 °C with a further overnight PBSMT wash at 4 °C. iTEV-PA specimens were post-fixed for 1 h at 4 °C in 4% paraformaldehyde. Fixed specimens were washed in PBS (2 × 15 min) at RT. Finally, specimens were washed in PBS-Tw (2 × 15 min), prior to washes in each of 30% 50% and 80% Glycerol (1 × 15 min, diluted in 5% BADCO/PBS). After the analyses, the remaining sample was then embedded in paraffin and sectioned as described above to evaluate the sample in cross-section. The sectioned samples were labeled with rabbit anti-Von Willebrand Factor (1:1000; Abcam) and biotinylated anti-rabbit IgG antibodies (Vector), followed by Alexa Fluor 594 conjugated streptavidin (1:400; Molecular Probes) to detect the endothelial cells. The samples were also labeled with fluorescein isothiocyanate (FITC) conjugated anti-ASMA antibody (1:500; Sigma) to detect ASMA.

All histological examinations and measurements were performed using light microscopy (Biozero BZ-8000; Keyence, Osaka, Japan and MVX10; Olympus, Tokyo, Japan) and accompanying analytical software. Specimens were labeled with DAPI (Sigma) to identify cell nuclei when necessary.

2.3. Biochemical analyses of protein, elastin, hydroxyproline and calcium content

LPA and iTEV-PA samples were prepared and biochemical analyses were performed as previously described [7]. For western blotting, ASMA (1:1000, clone 1A4; Dako) for vascular smooth muscle cell (VSMC) detection and CD146 (1:1000; Epitomics, Burlingame, CA, USA) for endothelial cell detection were used. An anti-GAPDH antibody (1:1000, clone I-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as an internal control.

Chemiluminescent images were acquired using a cooled CCD camera (LAS-3000 Mini; Fujifilm, Tokyo, Japan) and band intensities were analyzed using bundled software. Data were quantified as the ratio between the luminescence of the specified protein and the internal control. A commercially available elastin assay kit (Fastin Elastin Assay Kit; Biocolor, Belfast, Northern Ireland) was used to quantify elastin content and high-performance liquid chromatography (HPLC) was used to measure hydroxyproline content of the samples. The calcium concentrations of the

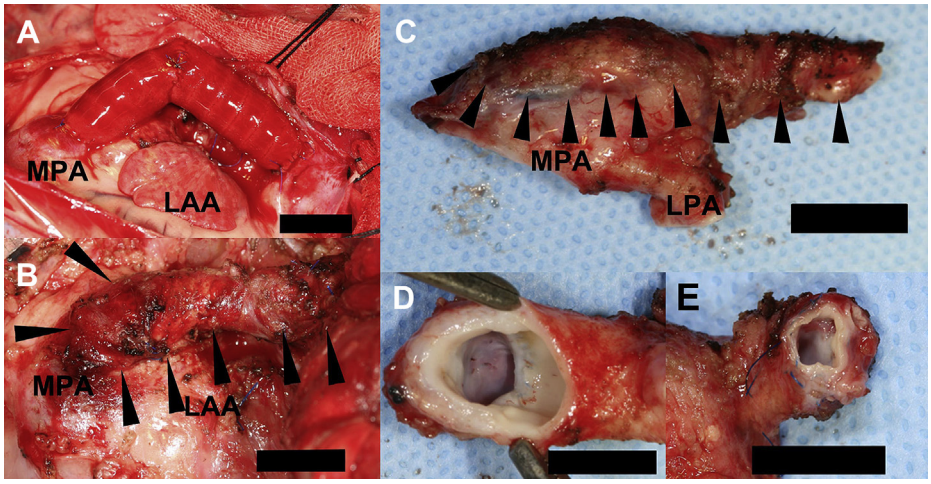

Fig. 1. (A) Macroscopic images of scaffold implantation for iTEV-PA (MPA, main pulmonary artery; LAA, left atrium appendage). (B) Macroscopic view of iTEV-PA 6 months post-implantation. Black arrowheads represent the iTEV-PA when dissected. (C) Macroscopic view of resected iTEV-PA (LPA, left pulmonary artery). (D) Proximal site and (E) distal site of resected iTEV-PA looking through the mPA and inferior lobar artery. Bars, 1 cm.

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