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Histological maturation of vascular smooth muscle cells in in situ tissue-engineered vasculature

Noriko Isayama [a], Goki Matsumura [a,*], Hideki Sato [b], Shojiro Matsuda [b], Kenji Yamazaki [a]

[a] Department of Cardiovascular Surgery, The Heart Institute of Japan, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
[b] Gunze Ltd., Research and Development Department, 1 Ishiburo, Inokurashinmachi, Ayabe, Kyoto 623-8512, Japan

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ABSTRACT

The goal of regenerative medicine is to achieve histological and functional recovery to the level of the original tissue. For this purpose, we have developed a biodegradable scaffold to create cell-free in-situ tissue-engineered vasculature (iTEV) with good long-term results. However, the regeneration process of vascular smooth muscle cells (VSMCs) over time has yet to be examined. To evaluate the regeneration ability of VSMCs, the inferior vena cava of experimental animals was replaced with iTEV, and tested at 1, 3, 6, 12, and 24 months ($n = 6$ each) after implantation. Six animals were enrolled to compare 24-month iTEV and native vasculature in single individual samples. There were no complications throughout the study. Immunohistology, protein expression analysis, and biochemical findings indicate that iTEV can gradually regenerate and develop into a mature vessel within 24 months using our biodegradable scaffold. These results provide a time course for the regeneration of VSMCs within the tissue-engineered vascular autograft constructed using a biodegradable scaffold.

1. Introduction

There is a great need for a substitute material suitable for pediatric cardiovascular implants that can develop into native-like tissue, with the potential to adapt or remodel into the shape required by the living body. Furthermore, scientists have been pursuing a method for vascular tissue engineering that is simple and versatile. Recently, we have developed a biodegradable scaffold to create a cell-free in-situ tissue-engineered vasculature (iTEV) with good long-term results in both the inferior vena cava and pulmonary artery in vivo in canine studies [1,2]. In these studies, we showed that the histological, biomechanical, morphological, and biochemical properties of iTEV revealed similarities to the native vessels, which remodeled into the native-like vasculature within approximately 2 years. Furthermore, a previous study revealed that the regenerated endothelial cells proliferated rapidly and covered the entire inner surface of the iTEV within approximately 1 month. In contrast, vascular smooth muscle cells (VSMCs) appeared to develop gradually with time. In an inferior vena cava reconstruction study [3], VSMC elastin and collagen content was achieved at approximately 2 years post-implantation. However, the process of regeneration and the changes that occur over time in protein expressions after implantation are still not clear. As our protocol for iTEV can be accomplished by a self-renewal process, individual differences in regeneration are avoidable. The present study investigated the regeneration process of VSMCs in iTEV after implantation of the biodegradable scaffold over time. Furthermore, it should be noted that VSMCs in the healthy vena cava distribute randomly and locate in a non-uniform fashion [4]. We speculated that differentiated VSMCs in iTEV also distribute sporadically within the tissue, so we first examined the proliferation levels and maturity of VSMCs in iTEV to confirm the duration of histological integrity. We then compared the VSMC characteristics of iTEV to original native tissue at individual time points to show the degree of remodeling by VSMCs in each sample case to exclude individual variations. Additionally, other vascular components such as collagen, elastin, and calcium contents of iTEV were examined.

2. Materials and methods

2.1. Characteristics of iTEV

The material used in this study was the same used in our previous studies [1,2]. This scaffold was composed of three layers with different non-enzymatic hydrolysis periods in vitro as follows: polyglycolide (PGA) knitted fibers, 1 month; L-lactide and ε-caprolactone copolymer (P(LA/CL)) sponge, 6–12 months; and a glycolide ε-caprolactone copolymer (P(GA/CL)) monofilament reinforcement, 2 months. These different biodegradable periods produce strength for stenosis and external pressure,

* Corresponding author. Tel.: +81 3 3353 8111; fax: +81 3 3356 0441.
E-mail addresses: smatumur@hij.twmu.ac.jp, n4mb4a@gmail.com (G. Matsumura).

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and keep the shape of the scaffolds until regenerative tissue formation has occurred. The scaffold was sterilized with ethylene oxide gas prior to implantation.

2.2. Animal experiments

Thirty-six healthy adult female beagles (NARC, Tomisato, Japan) with a mean weight of 9.8 kg (range, 8.6–11.0 kg) were used in this study. At 1, 3, 6, 12, and 24 months, six animals were euthanized for histological and biochemical analyses. Dogs were observed for 24 months to compare the histological maturation of iTEV to original host tissue in each individual group. The Animal Care and Use Committee of Tokyo Women's Medical University approved the use of the animals in this study. The animals were anesthetized with pentobarbital (1 mg/kg body weight) and atropine sulfate (0.08 mg/kg body weight). Heparin (500 U/kg body weight) was administered intravenously for anticoagulation during anastomoses. The seventh intercostal was opened and each scaffold (8 mm in diameter and 2.5–3.0 cm in length) was implanted into the inferior vena cava using surgical techniques described previously. The length of the scaffold was decided according to the anatomical difference of the dog. The animals were maintained from 1 to 24 months after surgery without anticoagulants until euthanasia. At the time of euthanasia, the dogs were anesthetized as described above. The chest was then reopened with careful dissection to obtain the iTEV samples. The resected iTEV was then cut longitudinally and opened to observe the inner surface of the graft and obtain the sample for histological and biochemical analyses. Each sample of the native IVC and iTEV was rinsed with phosphate-buffered saline, and stored at −20 °C or embedded in 4% paraformaldehyde for analysis.

2.3. Macroscopic, histological, and immunohistochemical examination

Tissue samples were embedded in paraffin and sectioned at 4–5 μm thickness for histological examinations. All samples were stained with hematoxylin-eosin, Masson's trichrome, and Victoria blue-van Gieson. Immunostaining of the paraffin sections was performed with the following antibodies: ASMA (1:1000; Dako, Japan, Tokyo, Japan), calponin (1:5000; Sigma, St, Louis, MO, USA), MHC (1:500; Sigma), and SM2 (1:500; Abcam, Cambridge, MA, USA).

Twenty-four month iTEV samples and native vasculature were examined for vascular smooth muscle distribution by whole staining using fluorescein isothiocyanate-conjugated anti-alpha-smooth muscle actin (ASMA; FITC-1A4) antibody (1:1000; Sigma) and DAPI (Sigma) to identify cell nuclei.

All histological examinations and measurements were performed using a microscope (Biozero BZ-8000; Keyence, Osaka, Japan) and analysis software (BZ-Analyzer; Keyence). These procedures are identical to those described previously (Native, PA).

2.4. Biochemical analyses of protein, elastin, hydroxyproline, and calcium contents

After thawing, the iTEV and native IVC samples were weighed and homogenized at a 1:20 (w/v) ratio of tissue to Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA) to extract protein. The total protein content was determined using the Bradford assay. Equal amounts (15 μg) of the denatured proteins were added per lane for separation in 4–12% polyacrylamide gels (NuPAGER Novex® Bis–Tris [Bis (2-Hydroxyethyl) amino–Tris (Hydroxymethyl) methane-HCl] Midi Gels; Invitrogen, Carlsbad, CA, USA) and transferred to polyvinylidene difluoride (PVDF) membranes using an iBLOT dry blotting system (Invitrogen). The same antibodies were used for immunohistochemistry at the following concentrations: ASMA (1:1000; Dako), calponin (1:4000; Sigma), MHC (1:1000; Sigma), and SM2 (1:1000; Abcam). β-Actin (1:1000; Abcam) was used as an internal control. A WesternBreeze® Chemiluminescent Immunodetection Kit (Invitrogen) and BenchPro4100™ system (Invitrogen) were used for detection of antigen–antibody complexes immobilized on the PVDF membranes, according to the manufacturer's protocol. After enhancement of the membranes by treatment with a chemiluminescent reagent, images were acquired using a cooled CCD camera (LAS-

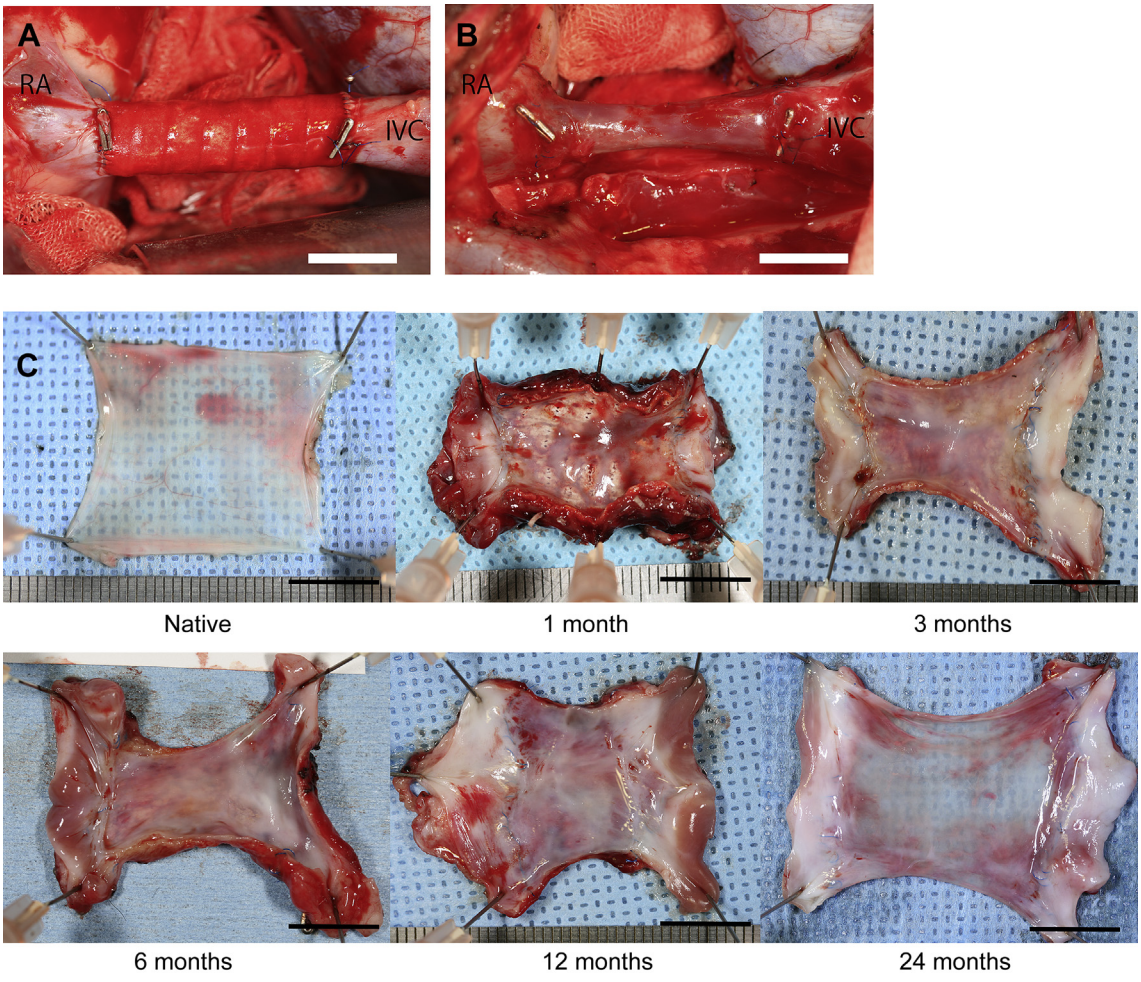

Fig. 1. (A) Macroscopic images immediately after implantation of iTEV and, (B) 24 months after implantation in the same individual. iTEV is fixed between two vascular clips (RA; Right atrium, IVC; inferior vena cava). (C) Macroscopic time course pictures of native, and 1-, 3-, 6-, 12-, and 24-month iTEV implants. iTEV is located between the blue sutured line. The thickness and structure of iTEV gradually decreased with time. Scale bars: 1 cm.