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BRIEF COMMUNICATION

Long-term results of tissue-engineered small-caliber vascular grafts in a rat carotid arterial replacement model

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Abstract The concept of tissue engineered small-caliber vascular grafts (TE-SCVGs) is theoretically ideal. In this study, we evaluated the long-term (more than 1 year) course of TE-SCVGs using a rat carotid arterial replacement model. We fabricated a TE-SCVG scaffold (0.7 mm in diameter) with electrospun nano-scale fibers. Poly-ε-caprolactone was used as a biodegradable polymer. These artificial vessels were then used in carotid arterial replacement performed on Sprague–Dawley rats. The implanted grafts were removed at an early phase (1, 2, 6 weeks), middle phase (12, 24 weeks), and late phase (48, 72 weeks) after implantation. Twenty-nine patent grafts from among the 40 implanted grafts (patency 72.5 %) could be evaluated. No aneurysm formation was observed during the follow-up period. Endothelial cells positive for immunostaining with von Willebrand factor were found to be already attached to the inner surface of the TE-SCVGs in the early phase. The percentage of smooth muscle cell specific marker (α-smooth muscle actin and calponin with fluorescent immunostaining) positive cells, which seemed to be mesenchymal cells in the graft wall, increased with time, while, in contrast, the scaffold material decreased. Even after 72 weeks, however, although the scaffold material had degraded, it had not disappeared completely. These results show that the novel TE-SCVGs we developed were still functioning in the rat carotid arterial circulation after more than 1 year. However, further investigations will be required with regard to regeneration of the SMC layer and the complete degradation of graft materials.

Keywords Tissue engineering · Small-caliber vascular graft · Long-term result

Introduction

Current artificial small caliber vascular grafts (SCVGs), especially those less than 6 mm in diameter, are clinically unsatisfactory because of their low patency [1]. Causes of this low patency are thought to be:

1. thrombosis because of reaction with foreign bodies or lack of endothelial cells; and
2. intimal hyperplasia caused by inflammatory reaction and compliance mismatch of the native vessel and the prosthetic graft at the anastomosis site [2, 3].

To overcome the shortcomings of artificial SCVGs, current focus is on development of tissue-engineered SCVGs (TE-SCVGs) that can ultimately change into viable autologous cells, a concept regarded as ideal. Such TE-SCVGs are being investigated actively worldwide [4, 5]. In these tissue-engineering studies, however, much remains unclear about the cells that will constitute such vessels, selection of scaffold materials, and growth factors to promote regeneration. Moreover, no strategy has yet been established as to whether it is better to seed autologous vascular cells on to the tubular scaffold before surgical

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implantation (in-vitro tissue engineering) or to directly implant a tubular scaffold (non-cell seeded graft) only (in-vivo tissue engineering). The disadvantage of the former is that development will be costly, time-consuming and technically difficult. We therefore focused on TE-SCVGs using a biodegradable polymer scaffold without cell seeding [6]. It has been hypothesized that if cells are not seeded on to TE-SCVG before implantation, the cells that will constitute the vessel might be supplied mainly from circulating stem cells [7]. It is possible that these stem cells could be induced to differentiate into the different types of cell that make up a vessel, such as endothelial cells (ECs) and smooth muscle cells (SMCs). In addition, the biodegradable scaffold material is degraded by hydrolysis gradually over time. However, if the speed of material degradation is faster than regeneration of the tissue in the vascular graft, the graft may rupture. It is, therefore, of utmost importance to achieve a balance between the rate of material degradation and the speed of tissue regeneration in order to obtain appropriate vascular regeneration. So, long-term observation of implanted grafts is needed to elucidate these phenomena.

There are few reported long-term observations of the course of implanted TE-SCVGs. For the development of this promising procedure, however, studies on the process of regeneration of implanted TE-SCVGs, including the transition of component cells making up blood vessels, must be investigated. In this study, we examined time-dependent histological changes of implanted TE-SCVGs over a long period, using a rat carotid arterial replacement model.

Methods

Fabrication of TE-SCVGs

Poly-ε-caprolactone (PCL), a biodegradable polymer, was used as the scaffold for our novel TE-SCVG. Nanofibers were produced by electrospinning. Briefly, a solution of PCL in dichloromethane was placed in a syringe with a needle that was given a positive charge (potential 10–15 kV). The polymer was charged electrically by electrospinning and collected on a spinning counter electrode where it formed a tube-like structure. We fabricated SCVGs with a diameter of 0.7 mm in this way (Fig. 1a–c) [8]. The diameter of the fiber was approximately 500–5000 nm. The measured porosity of this graft was approximately 78 %.

Surgical procedure

In previous work, we established a surgical method for carotid artery replacement using rats, and published early endothelialization results [6]. Using the same method, we performed carotid arterial replacement using the grafts we developed, without any anticoagulant agents, on Sprague–Dawley rats (9.7 ± 0.8 weeks old, male), after anesthetizing them by inhalation of diethyl ether (Wako Pure Chemical Industries, Osaka, Japan) and intraperitoneal administration of 20–30 mg/kg sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan), and 0.15–0.20 mg/kg atropine sulfate (Mitsubishi Tanabe Pharma, Osaka, Japan). The anastomosis was performed with end-to-end, interrupted sutures using 10-0 nylon (Crownjun, Kono Seisakusyo, Chiba, Japan) (Fig. 1d).

The grafts were removed in the early phase (1 week, $n = 9$; 2 weeks, $n = 7$; and 6 weeks, $n = 5$), middle phase (12 weeks, $n = 7$ and 24 weeks, $n = 7$), and late phase (48 weeks, $n = 4$ and 72 weeks, $n = 1$) after graft implantation (Fig. 1e). The patency of the removed grafts was examined with a test of saline passage through the graft and direct observation of cut grafts.

The animals were cared for in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (NIH Publication 85-23, National Academy Press, Washington, DC, revised in 1996). All procedures involving animals were approved by the Animal Experiment Advisory Committee of the Nagoya University School of Medicine.

Microscopic observation

Removed grafts were fixed in 7.5 % buffered formaldehyde sodium. After overnight dehydration using 20 % sucrose, samples were embedded (Tissue-Tek^{TM} O.T.C. compound, Sakura Finetec Japan, Tokyo, Japan) and cut into 5-μm slices. Light microscopic observation and immunofluorescent staining were performed.

The slices were stained with hematoxylin and eosin stain (H&E), elastica van Gieson stain (EVG), and von Kossa stain for histological examination. Immunofluorescent staining was also performed with von Willebrand factor (vWF; Dako, Glostrup, Denmark) as a primary antibody to identify ECs, and with α-smooth muscle actin (ASMA; 1:500, Sigma, Saint Louis, MO, USA) and calponin (1:500; Epitomics, Burlingame, CA, USA) to identify mesenchymal cells, including SMCs. Details of the immunofluorescent staining were as follows. After blocking using 5 % bovine serum albumin (Sigma), the slices were immunostained for 30 min with the primary antibodies. After rinsing three times, the primary antibodies were detected by use of secondary antibodies (1:500; Alexa Fluor 488; Invitrogen, Carlsbad, CA, USA; and 1:500; Alexa Fluor 546; Invitrogen) for 30 min, mounted with 4′,6-diamidine-2-phenylindole (DAPI, Vectashield, Vector Laboratories, Burlingame, CA, USA), and examined under a fluorescent microscope.

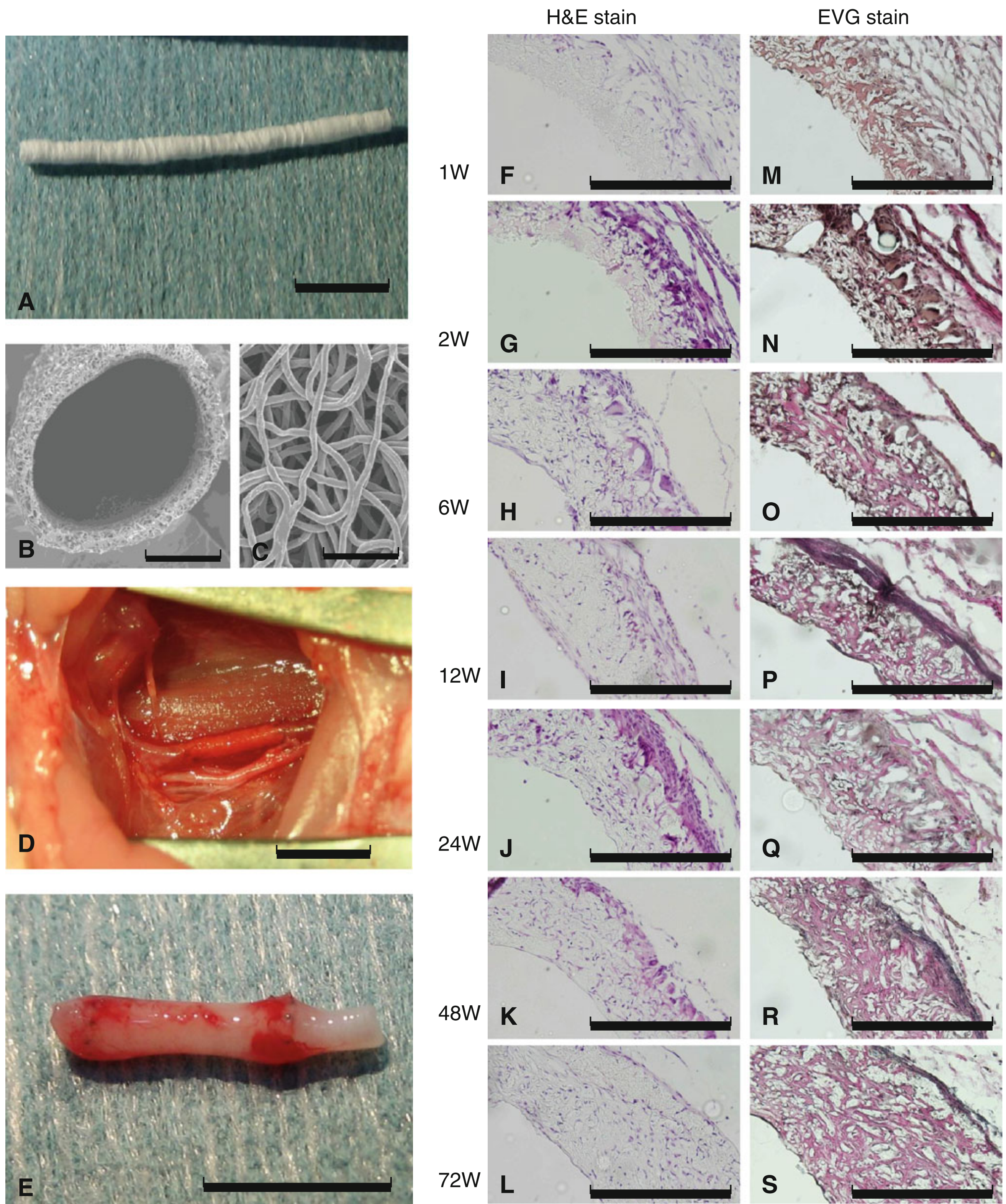

Fig. 1 Graft before operation (**a**), microscopic view of grafts obtained by use of scanning electron microscopy (**b**, **c**) view of surgical implantation (**d**), and removed grafts 2 weeks after implantation (**e**). Histological micrographs of the removed grafts: H&E staining of grafts removed 1 week (**f**), 2 weeks (**g**), 6 weeks (**h**), 12 weeks (**i**), 24 weeks (**j**), 48 weeks (**k**), and 72 weeks (**l**) after implantation. Elastica van Gieson staining of grafts removed 1 week (**m**), 2 weeks (**n**), 6 weeks (**o**), 12 weeks (**p**), 24 weeks (**q**), 48 weeks (**r**), and 72 weeks (**s**) after implantation. *Scale bar* in **a**, **d**, and **e** 5 mm, **b** 300 μm, **c** 60 μm, **f**–**r** 200 μm

Results

Patency

Twenty-nine of the 40 implanted grafts (72.5 %) were found to be patent. The respective numbers of patent grafts/implanted grafts (and the patency) at each graft removal time were: 6/9 (67 %) 1 week after implantation, 6/7 (86 %) at 2 weeks, 4/5 (80 %) at 6 weeks, 4/7 (57 %) at 12 weeks, 4/7 (57 %) at 24 weeks, 4/4 (100 %) at 48 weeks, and 1/1 (100 %) at 72 weeks. There was no aneurysm formation during the follow-up period, even in occluded cases.

Endothelialization

H&E and EVG staining of the removed grafts at each time point are shown in Fig. 1f–s. The results showed that the cells coating the inner surface of the graft walls had started to grow during the early phase. No calcium deposition was observed in TE-SCVG walls with von Kossa staining (data not shown). These cells were positive in immunofluorescent staining for vWF (Fig. 2a–e), and were thus confirmed to be ECs. Growth of endothelium was nearly complete in the early phase and remained unchanged after the middle phase according to optical and immunological microscopic visualization results. (There are no data for immunofluorescent staining for vWF, ASMA, and calponin in late phase.)

Mesenchymal cells in the walls of TE-SCVGs

H&E and EVG staining showed there were few cells in the middle layer of the graft wall during the early phase. However, the cells penetrating to the middle layer of the graft wall increased with time during the middle and late phases. The percentage of these cells in the middle layer of the graft wall also increased (Fig. 1f–s).

In the immunostaining results, immunofluorescent staining for ASMA and calponin was negative in the early phase. However, the grafts were positive for ASMA and calponin in middle phase (Fig. 2f–o).

Degradation of scaffold material

The area of biodegradable scaffold decreased with time, and starting in the middle phase the percentage of mesenchymal cells in the graft wall increased simultaneously. In H&E and EVG staining, unstained parts in the graft wall are thought to be remaining nanofibers. The increase in mesenchymal cells and decrease in scaffold in the vessel walls showed that the biodegradable nanofibers degraded over time. Even after 72 weeks, however, although the area of biodegradable scaffold had decreased they had not disappeared completely. In addition, the vascular smooth muscle layer had not completely regenerated (Fig. 1f–s).

Discussion

Tissue engineered vascular grafts (TEVGs) are a promising alternative concept for prosthetic vascular grafts. TEVGs for venous circulation have already been applied clinically in cases of congenital heart defect (total cavo-pulmonary connection for single ventricle) by Shin'oka et al. [9], and part of the process of vascular regeneration has been elucidated. However, a tissue engineering approach for arterial circulation has not been established, even experimentally (especially for SCVGs, which are most inconvenient clinically). Moreover, the regeneration processes for TEVGs in arterial circulation remains unclear. It is well known that early-stage patency and long-term durability/stability must be improved before artificial SCVGs can be used clinically. It is believed that early endothelialization prevents early-stage thrombosis of the graft. In a previous study, we improved early endothelialization by using similar TE-SCVGs with a specific trimeric peptide [6]. However, late-stage regeneration of tissue that makes up vessels, especially SMCs, and the degradation of graft material composed of nano-scale fibers could not be observed in that study because of the short observation period (6 weeks or less). In this study, therefore, we observed changes in the vascular walls of implanted TE-SCVGs over time and confirmed their long-term durability and stability (at least 72 weeks) in rat carotid arterial (peripheral) circulation.

Walpoth and his colleagues reported long-term observation of small diameter vascular grafts made using an electrospun biodegradable polymer (PCL) [10]. They observed chondroid metaplasia followed by calcification in the graft 1 year after the operation. For our graft, however, no calcification was observed after 72 weeks. The difference between these studies was the artery that was replaced (carotid arterial replacement vs abdominal aortic replacement). The aorta, including the abdominal aorta, is always under greater stress, for example high pressure and high shear stress, than peripheral arteries. Mechanical stress is one of the stimulating factors for osteogenic differentiation of vascular mesenchymal cells [11]. These differences might affect the long-term results in each prosthetic graft.

Regeneration of the tunica media is an important condition for TEVGs, because the SMC layer has important physiologic functions including regulation of vascular tonus and relaxation, synthesis of extracellular matrix, and

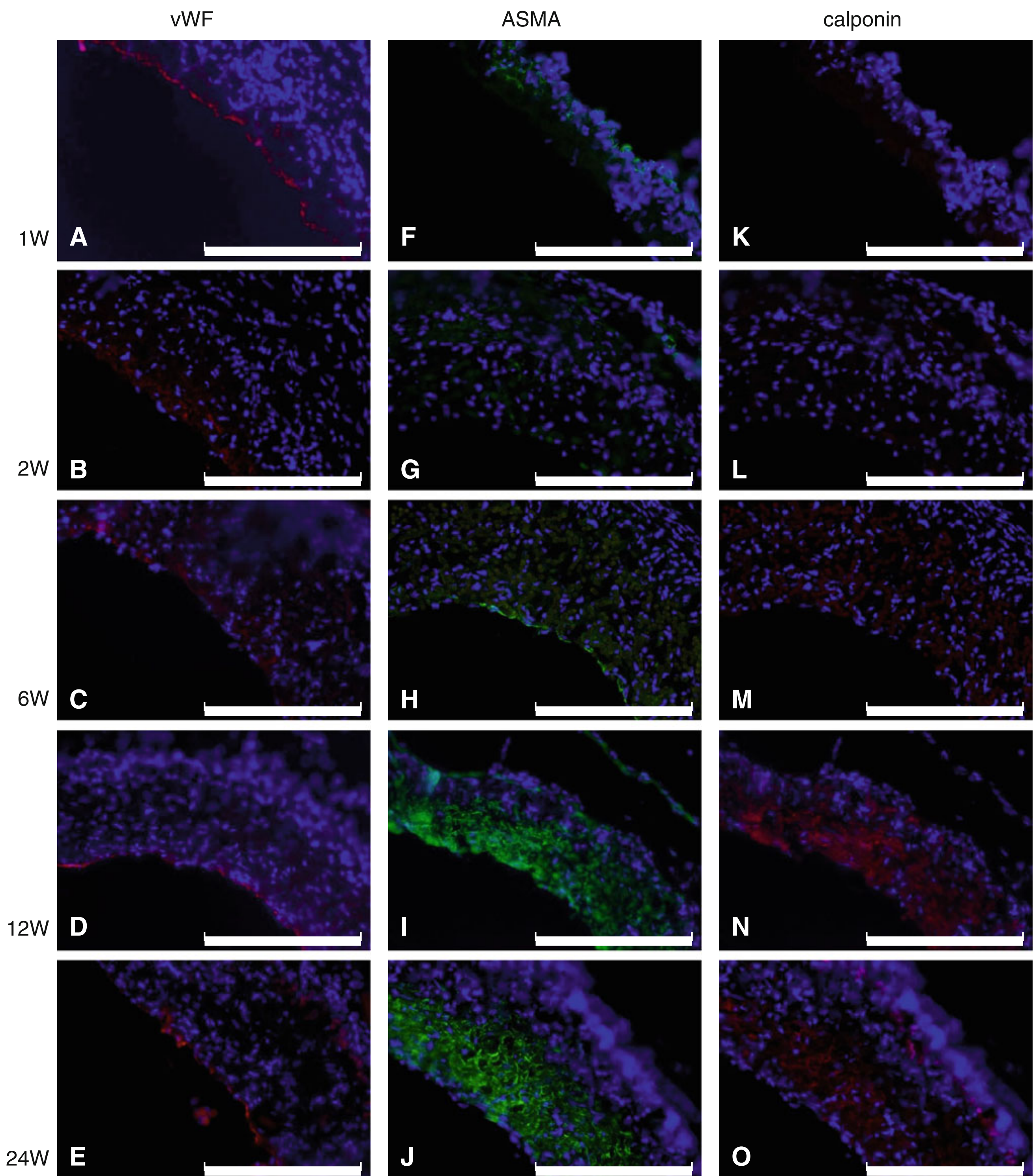

Fig. 2 Immunofluorescence staining for von Willebrand factor on grafts removed 1 week (**a**), 2 weeks (**b**), 6 weeks (**c**), 12 weeks (**d**), and 24 weeks after implantation (**e**). Immunostaining for α-smooth muscle actin on grafts removed after 1 week (**f**), 2 weeks (**g**), 6 weeks (**h**), 12 weeks (**i**), and 24 weeks (**j**). Calponin on grafts removed after 1 week (**k**), 2 weeks (**l**), 6 weeks (**m**), 12 weeks (**n**), and 24 weeks (**o**). *Scale bar* 200 μm

interaction with surrounding tissues. Inadequate regeneration of the tunica media may therefore adversely affect vessel patency [12]. For instance, SMCs and other vascular mesenchymal cells that proliferate (dedifferentiated) and migrate into the intima are known to produce extracellular matrix and cause intimal hyperplasia. In our study, we

observed cells stained with ASMA and calponin in the middle layer of the grafts. However, these cells did not form a layer like an SMC layer. In addition, these markers (ASMA and calponin) indicate not only well-differentiated SMCs but also dedifferentiated SMCs. Therefore, we need to devise some kind of new method to evaluate regeneration of proper tunica media that has a well-formulated SMC layer and that expresses well-differentiated markers, for example smooth muscle myosin heavy chains, which could not be detected in this study (data not shown).

An appropriate, well-designed scaffold is necessary to create good tissue-engineered products. Several biodegradable scaffold materials for TE-SCVGs have been reported [13], but opinions are divided with regard to the choice of a suitable scaffold material for TE-SCVGs. Our preliminary study showed that scaffolds of TE-SCVGs made with poly-L-lactic acid (PLLA) using an electrospinning procedure ruptured approximately 1 week after implantation, despite the same fabrication process and surgical procedure as in this study (data not shown). Nano-scale PLLA fiber prepared by electrospinning might be hydrolyzed earlier than we expected. The timing of the graft absorption and arterial regeneration are crucial aspects of the design of the TE-SCVG scaffold. In this study, the scaffold component remained at least 72 weeks after graft replacement. Therefore, our PCL graft needs more long-term follow-up observation (up to 72 weeks) until the scaffold component has completely degraded.

The different elasticity of the graft and the native vessel also causes stenosis at the anastomosis site [2, 3], a phenomenon called "compliance mismatch". The elasticity of the graft is thus another important aspect for the scaffold of TE-SCVGs. Unfortunately, our graft had no elasticity (data not shown). Improvement of this is an issue for creation of better TE-SCVGs. Further investigation to optimize the quality of material, thickness of nano-scale fiber, density of the grafts, and porosity of fabrication are needed for development of the best TE-SCVGs.

There are several major limitations to this study.

1. This study is a preliminary pilot study for TE-SCVG using biodegradable nano-scale fibers. Evaluations were, therefore, conducted by microscopic observation only, using H&E and immunological staining. We could not clarify the mechanisms of the long-term results of TE-SCVG. Further investigations (e.g., quantification of regeneration of the cells constituting the vessel, regeneration of ECM, and degradation of the polymer) will be required to elucidate the mechanisms of long-term patency of TE-SCVG.
2. Only one rat was observed 72 weeks after graft implantation, so we must verify reproducibility beyond that point.

Conclusion

We developed a graft that remained patent for over 1 year, and endured arterial pressure without aneurismal formation or calcification. In these TE-SCVGs, the intima (endothelial layer) had regenerated completely during the early phase, whereas regeneration of the media (SMC-layer) and scaffold degeneration progressed but did not reach completion in our observation period (72 weeks). These results suggest that our graft may be a useful material for TE-SCVGs, but further investigations will be required to assess regeneration of the SMC layer and complete degradation of nano-scale fibers in the graft wall.

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