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Development of Small-Diameter Vascular Grafts Based on Silk Fibroin Fibers from *Bombyx mori* for Vascular Regeneration

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Abstract

In the field of surgical revascularization, the need for functional small-diameter (1.5–4.0 mm in diameter) vascular grafts is increasing. Several synthetic biomaterials have been tested for this purpose, but in many cases they cause thrombosis. In this study, we report the development of small-diameter vascular grafts made from silk fibroin fibers from the domestic silkworm *Bombyx mori* or recombinant silk fibroin fibers from a transgenic silkworm. The vascular grafts were prepared by braiding, flattening and winding the silk fibers twice onto a cylindrical polymer tube followed by coating with an aqueous silk fibroin solution. The grafts, which are 1.5 mm in inner diameter and 10 mm in length, were implanted into rat abdominal aorta. An excellent patency (*ca.* 85%, $n = 27$) at 12 months after grafting with wild-type silk fibers was obtained. Endothelial cells and smooth muscle cells migrated into the silk fibroin graft early after implantation, and became organized into an endothelium and a media-like smooth muscle layer.

Keywords

Silk fibroin fiber, small-diameter vascular graft, transgenic silkworm

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1. Introduction

Artificial vascular grafts manufactured from synthetic materials, for example, expanded polytetrafluoroethylene (ePTFE) and Dacron [1–3], have been routinely used to reconstruct blood flow in patients with various cardiovascular disorders. Large-diameter vascular grafts have sufficient patency for implantation into the human body. However, only 15–30% of small-diameter vascular grafts remain patent after 5 years *in vivo* [4]. Moreover, *in vivo* studies have shown only a 20–25% patency rate with 1-mm-diameter PTFE microvessels, while all vein grafts in similar settings remained patent [5, 6]. The reason is that the vascular grafts made using Dacron or PTFE did not affect the cellular proliferation on the lumen of the vascular graft.

Conventional grafts have clinically shown satisfactory durability; however, they still have several disadvantages, such as thrombogenicity, late stenosis and occlusion from intimal hyperplasia (especially in small-diameter grafts), susceptibility to infection and lack of growth potential [7]. To overcome these limitations in the search for an ideal artificial graft, various tissue-engineered vascular grafts have been developed. The utility and clinical experience of these new grafts has been reported [8–14].

In this study, we report the development of new vascular grafts with a small diameter made from silk fibroin from the domestic silkworm, *Bombyx mori*, on the basis of our accumulated information on the structures of the silk fibroin. *B. mori* produces a protein fiber with excellent mechanical properties such as high strength and high toughness, which has a long history of use as suture [15]. In addition, from aqueous or organic solutions of the silk fibroin, it is possible to prepare a form of film, sponge, powder, gel and regenerated fiber. Much information on its structures and structural changes including the excellent physical properties of silk fibroin in several forms has been accumulated by us and other investigators [16–28]. Recently, there has been a significant increase in the number of reports of applications of silk fibroin to biomaterials [17–20, 27–32]. Moreover, the recent development of biotechnology permits an improvement of the cell-adhesive character of *B. mori* silk fibroin by incorporation of cell-adhesive amino-acid sequences into the silk protein [33–41]. In particular, the incorporation of cell-adhesive sequences into silk fibroin fiber and the production of such a recombinant silk fiber directly from the cocoon was possible using a transgenic silkworm. This recombinant silk fiber was also used for preparation of vascular grafts with a small diameter, and in preliminary studies showed improved performance compared to the wild-type silk.

2. Materials and Methods

2.1. Preparation of the Silk Fibroin Fiber

Cocoons produced by *B. mori* were placed in water at 95°C after which the threads were reeled. The dried silk threads were placed in a mixture of sodium carbonate

(0.08%, w/v) and Marseille soap (0.12%, w/v) at 95°C for 120 min [16–18]. This process was repeated in order to remove silk sericin from the raw silk fibers completely. The removal of silk sericin was checked by a scanning electron microscope (SEM VE-7800, Keyence, Japan). Pure silk fibroin fibers prepared in this way were used for the braiding and winding process, and also for preparing aqueous fibroin solutions during the production of the small-diameter grafts.

2.2. Production of Recombinant Silk Fibroin from Transgenic Silkworm

We previously designed recombinant silk fibroins with a high cell-adhesive sequences and produced them from transgenic silkworms [41]. In this paper, for preparation of the vascular graft, we used a recombinant silk fibroin fiber incorporating the sequences of active sites from collagen. The details have been reported previously [41]. Only limited amounts of the silk fiber samples were available and, therefore, the grafts prepared from wild-type and recombinant silk fibroin fibers were compared only at the initial stage of the growth of endothelial cells after grafting.

2.3. ^{13}C CP/MAS NMR Analysis

^{13}C CP/MAS NMR spectra of wild-type silk fibroin fiber from *B. mori* and recombinant silk fibroin fiber from transgenic silkworm were observed to characterize the structure in the dried and hydrated states [18, 21]. The information on the structure in the hydrated state is important in order to characterize the structure *in vivo*. For the NMR observation of hydrated samples, two kinds of silk fibroin fiber which are wild-type and recombinant samples were hydrated by immersing them in water for 1 week. A small amount of water attached to the surfaces of the fibers was removed just before the NMR observation. ^{13}C CP/MAS NMR experiments were performed on a Bruker Avance 400 MHz spectrometer with an operating frequency of 100.6 MHz for ^{13}C at a sample spinning rate of 8 kHz in a 4 mm diameter ZrO_2 rotor. A total of 4096 scans for the samples were collected over a spectral width of 35 kHz with a recycle delay of 5 s. A 150 kHz radio-frequency field strength was used for ^{1}H–^{13}C decoupling with an acquisition period of 15 ms. A 90° pulse width of 4.5 μs with a 1 ms cross-polarization contact time was employed. Phase cycling was used to minimize artifacts. The experimental conditions were the same for dried and hydrated silk fibroin fiber samples. ^{13}C chemical shifts were calibrated indirectly using adamantane and are presented in ppm relative to tetramethylsilane.

2.4. Preparation of the Aqueous Solution of Silk Fibroin

Pure silk fibroin fibers were dissolved in 9 M LiBr aqueous solution to a concentration of 10% (w/v) at 60°C for 4 h and then dialyzed against distilled water for 3 days at 4°C using a cellulose membrane (Viskase Sales, USA) [16]. The final concentration of the fibroin/water solution was 3–4% (w/v). Freshly prepared silk fibroin aqueous solution was used for coating the silk graft [22].

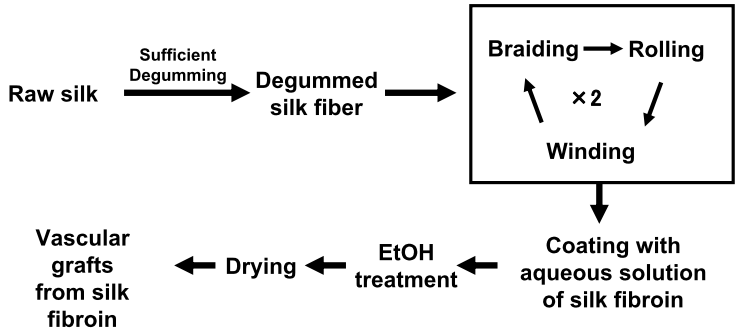

Figure 1. Preparation of the small-diameter vascular graft made of silk fibroin from *B. mori*.

2.5. Preparation of the Small-Diameter Vascular Graft from Silk Fibroin Fibers

Figure 1 presents a schematic diagram of the preparation of the small-diameter vascular graft from silk fibroin. Pure silk fibroin fibers were braided on the surface of a 1.5-mm-diameter polyvinylchloride rod on a 16-bobbin braiding machine (Kokubun, Japan). By changing the number of bobbins, the pitch and spacing of the fibers can be controlled. Then, in order to smooth and flatten the surface of vascular grafts, the cylindrical silk fibroin tube covering the rod was rolled over flat plates ('rolling'). The surface of the cylindrical silk fibroin tube was then covered by a continuous winding by silk fibroin fibers. The entire process from braiding to winding was then repeated. The vascular grafts were then immersed in the fresh silk fibroin aqueous solution under partial vacuum at 700 mmHg. This coating step was repeated at least five times. Then the coated grafts were immersed in 50% ethanol to produce an insoluble state of the coated silk fibroin. The polyvinylchloride rod was stretched and pulled off from the vascular grafts after heating at 110°C.

2.6. Animals

Male Sprague–Dawley (SD) rats weighing 400–500 g were purchased from SLC (Japan). All rats were kept in microisolator cages with a 12-h light/dark cycle. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the University of Tokyo and complied with the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 86-23, revised 1985).

2.7. Implantation of the Vascular Grafts into Rat Abdominal Aorta

Vascular grafts, 1.5 mm in inner diameter and 10 mm in length, were implanted in the abdominal aorta of rat [32]. General anesthesia was induced by means of an intraperitoneal injection of 50 mg/kg Nembutal (sodium pentobarbital). In the operation, the abdominal aorta was approached *via* median laparotomy. The abdominal aorta was exposed and the aortic branches in this segment were ligated. After an intravenous injection of heparin (100 IU/kg body weight), the proximal and distal portions of the infrarenal aorta were clamped. A 10-mm segment of the aorta was removed and replaced by a fibroin graft by end-to-end anastomosis using inter-

rupted 9-0 monofilament nylon sutures (Bear, Japan), starting with two stay sutures at 180° to each other, then suturing the front wall followed by the back wall. The number of stitches used for each anastomosis ranged from 10 to 12. The distal, then the proximal vascular clamps were slowly removed, and flow was restored through the fibroin graft. Graft patency was monitored by color Doppler imaging and pulse waves recorded with a 12-MHz sector probe and an echo-imaging apparatus (En-Visor M2540A, Philips, Japan) under anesthesia with pentobarbital. Graft diameter and blood flow velocity were measured. Signs of thrombosis and aneurysm formation were carefully checked.

2.8. Histological Examination

The rats were killed with an overdose of Nembutal. At death, the rats were perfused with 0.9% NaCl solution *via* the left ventricle. The grafts were carefully removed with surrounding tissue, cut transversely in the midline into two pieces, and fixed in methanol or snap-frozen in OCT compound (Tissue-Tek, Japan) for histological analyses. Methanol-fixed samples were embedded in paraffin. Paraffin-embedded sections (4 μm thick) were processed for hematoxylin and eosin staining. For immunohistochemistry [32], the sections were incubated with primary antibodies (alkaline phosphatase-conjugated anti-α-smooth muscle actin (clone 1A4, Sigma, USA), anti-rat CD31 (clone TLD-3A12, BD Biosciences, USA) or anti-CD68 (clone ED1, Serotec, UK)), followed by incubation with biotinylated anti-mouse IgG secondary antibody (Dako, Denmark) and subsequent use of the avidin–biotin complex technique and Vector Red substrate (Vector Laboratories, USA). Nuclei were counterstained with hematoxylin. Sirius red polarization microscopy was performed to visualize interstitial collagen and fibroin. Frozen sections (5 μm) were rinsed with distilled water and incubated with 0.1% sirius red (Sigma-Aldrich, USA) in saturated picric acid for 90 ms. Sections were rinsed twice with 0.03 M HCl for 1 min each time and then immersed in distilled water. After dehydration with 70% ethanol for 30 s, the sections were coverslipped.

3. Results and Discussion

3.1. Morphological Examination of the Silk Fibroin Vascular Graft During the Preparation Process

SEM pictures of the silk fibroin during the preparation of the graft are shown in Fig. 2. It is important to remove silk sericin from the cocoon ('degumming') in order to avoid the possibility of allergy from silk sericin. Insufficient degumming produces a fiber with a rough surface (Fig. 2B), whereas the completely degummed fiber is smooth (Fig. 2C). Degummed pure silk fibroin fibers were braided onto a 1.5-mm-diameter polyvinylchloride rod using a braiding machine (Fig. 2D). However, a single braiding does not produce a continuous surface and, therefore, the tube was rolled over a flat surface, to produce the flattened tube shown in Fig. 2E. The surface of the tube was then covered by winding with silk fibroin fiber (Fig. 2F).

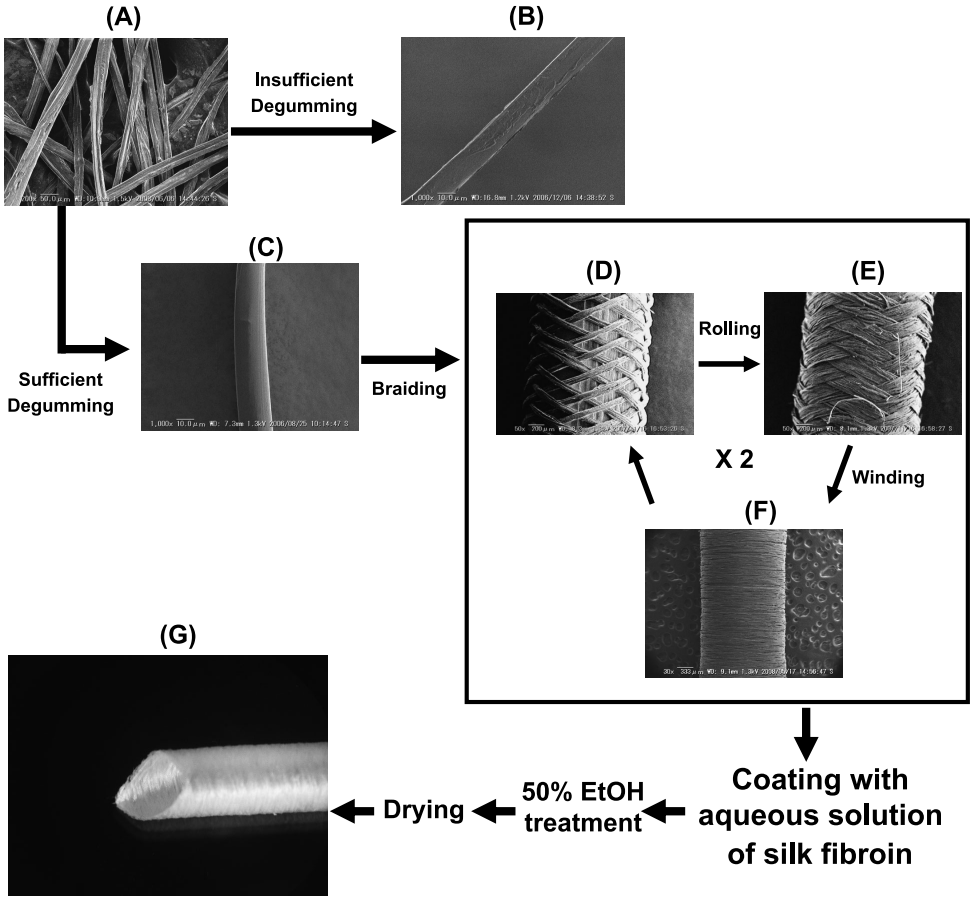

Figure 2. Scanning electron micrographs of silk fibers and silk grafts at several processing stages. (A) Raw silk, (B) a silk fibroin fiber after insufficient degumming, (C) a silk fibroin fiber after complete removal of silk sericin, (D) silk graft after braiding, (E) silk graft after rolling, (F) silk graft after winding and (G) the final silk graft.

The entire braiding, rolling and winding process was then repeated to produce a graft of sufficient strength. In addition, in order to make the graft fully watertight, coating with silk fibroin is required. Vascular grafts were immersed in silk fibroin aqueous solution [22] under partial vacuum to help osmosis. The coating step was repeated at least five times. The graft was then immersed in 50% ethanol to make the coated silk fibroin insoluble by a structural change to β-sheet [18, 22]. Thus, vascular grafts with alternating braided and spiraled fiber layers, coated by silk fibroin, were prepared with a final appearance as shown in Fig. 2G.

3.2. Structural Analysis of Recombinant Silk Fibroin by ^{13}C CP/MAS NMR

We produced a recombinant silk fibroin fiber incorporating sequences of the active sites from collagen, i.e., $(GERGDLGPQGIAGQRGVV(GER)_3GAS)_8GPPGPCC$-

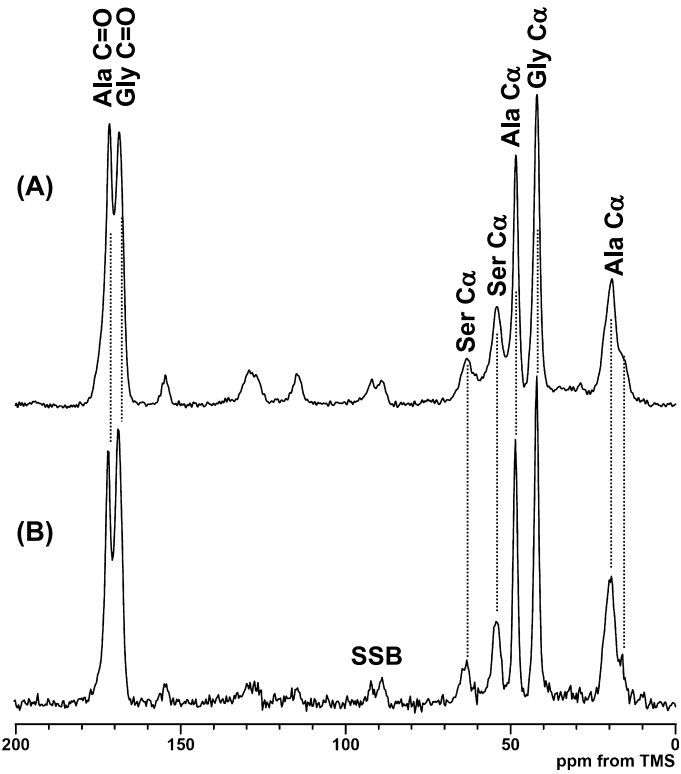

Figure 3. ^{13}C CP/MAS NMR spectra of recombinant silk fibroin fiber from a transgenic silkworm together with the peak assignment. (A) Dried state and (B) hydrated state. SSB, spinning sideband (an experimental artifact).

GGG, using a transgenic silkworm [41]. Before preparing a small-diameter vascular graft using the recombinant silk fibroin, the secondary structure of the fiber was examined by ^{13}C cross-polarization/magic angle spinning (CP/MAS) NMR [18–21]. Figure 3 shows ^{13}C CP/MAS NMR spectra of recombinant silk fibroin fibers from a transgenic silkworm in the dried (Fig. 3A) and hydrated state (Fig. 3B). The chemical shifts of the carbons from alanine residues indicate that the fiber mainly adopts a β-sheet structure in both the dried and hydrated states assigned by ^{13}C chemical shift contour plots (Table 1) [18, 42–44]. This result is important because a remarkable difference between the dried and hydrated states has been reported for the glycine-rich region of spider dragline silk [45]. Water molecules diffuse only into the glycine-rich region in the samples because this silk has crystalline polyalanine- and amorphous glycine-rich domains. However, in the case of *B. mori* silk fibroin fiber, the glycine residues are present mainly in the crystalline regions and, therefore, no change was observed between the dried and hydrated states [46]. The only difference is a slight narrowing of peaks from Ser C_α and C_β carbons in the hydrated state, although the conformation of Ser is still β-sheet. Thus, hydration

Table 1.
^{13}C CP/MAS NMR chemical shifts of recombinant silk fibroin from transgenic silkworm and local conformation predicted from the chemical shifts

Carbon in amino acid	Observed shift (ppm)	Random coil shift (ppm)	β-Sheet shift (ppm)	α-Helix shift (ppm)	Conformation
Ala C_α	49.0	50.1	48.2	52.4	β-Sheet
Ala $C_\beta(1)$	20.0	16.6	19.9	14.9	β-Sheet
Ala $C_\beta(2)$	17.0	16.6	19.9	14.9	Random coil
Ala C=O	172.2	175.2	171.8	176.4	β-Sheet
Ser C_α	54.9	55.9	54.9	52.2	β-Sheet
Ser C_β	63.7	61.3	64.5	61.5	β-Sheet
Gly C_α	42.6	42.7	43.2	172.1	No prediction
Gly C=O	169.3	171.3	168.5	171.6	β-Sheet

through the side-chain OH group of the Ser residue might occur in the hydrated state. The spectrum (Fig. 3A) is exactly the same as that obtained for wild-type silk fibroin fiber in the dried state (data not shown). The small amounts of the sequences incorporated into the silk fibroin are less than 5% of the total sequence [41] and, therefore, the conformation of the silk fiber does not change.

3.3. Implantation of the Vascular Grafts into the Rat Abdominal Aorta and Histological Analysis

The grafts, which are 1.5 mm in inner diameter and 10 mm in length, were implanted into rat abdominal aorta by end-to-end anastomosis (Fig. 4A). Details of the implantation are described in the Materials and Methods section. The patency of the graft was examined by ultrasound Doppler flow studies every 2 weeks.

Figure 4B shows, as an example, a cross-section of the vascular graft made of silk fibroin fibers at 4 weeks after grafting. Vascularized tissues covering the silk fibroin graft were observed. We evaluated their utility up to 12 months after grafting. An excellent patency (*ca*. 85%) at 12 months after grafting was obtained (the number of rats for the experiment was 27, $P < 0.01$) [32]. The patency rate of the fibroin graft was significantly higher than that of PTFE graft, which caused occlusion within 2 months in almost all cases. This result suggests strongly that the silk fibroin fiber is a promising material to engineer vascular prostheses for small arteries.

An immunohistochemical study of the silk graft from wild-type silkworm after grafting was also performed (Fig. 5). In immunostaining of the vascular graft at 9 weeks after grafting, a uniform growth of endothelial cells was observed on the inner surface of vascular grafts (Fig. 5A). In a previous paper, we have reported that CD31-positive cells covered $92.2 \pm 2.4\%$ of the luminal surface in the fibroin graft at 12 weeks [32]. Thus, the coverage of endothelial cells at 9 weeks was not fully completed. However, it can be reasonably expected that the coverage of endothelial cell would be completed with each passing moment.

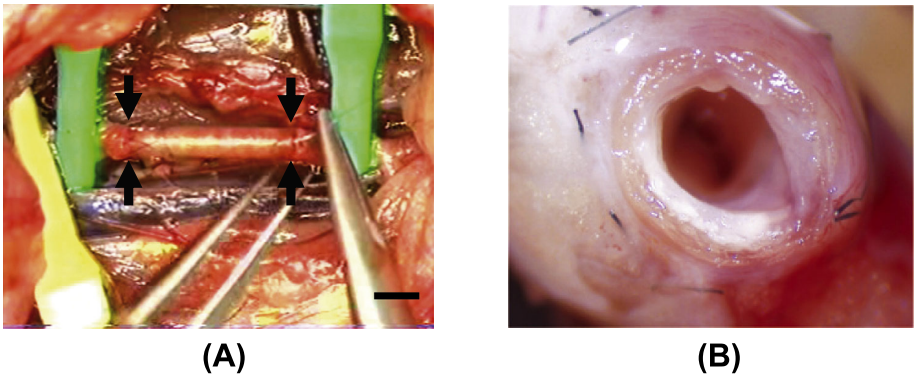

Figure 4. Implantation of the vascular grafts into the rat abdominal aorta. (A) The graft was implanted into the rat abdominal aorta by end-to-end anastomosis. The sites of the anastomosis are indicated by arrows. Scale bar = 2 mm. (B) Cross-section of the silk fibroin graft at 4 weeks after grafting. This figure is published in colour in the online edition of this journal, that can be accessed *via* http://www.brill.nl/jbs

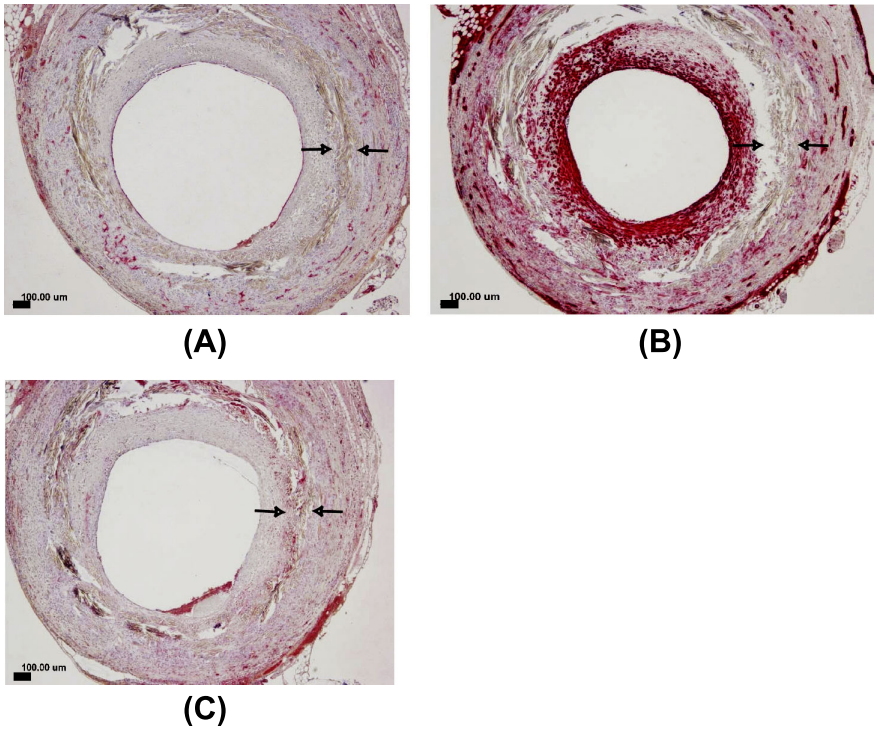

Figure 5. Immunostaining images of the vascular graft from silk fibroin based on wild-type silkworm at 9 weeks. (A) The confluent endothelial layer and (B) the SMC layer, as determined by immunostaining against CD31 and α-smooth muscle actin (α-SMA), respectively. (C) Substantial macrophage infiltration in the adventitia, as determined by immunostaining for CD68. The arrows mark the position of the silk fibroin. Scale bar = 100 μm. This figure is published in colour in the online edition of this journal, that can be accessed *via* http://www.brill.nl/jbs

The formation of a media-like smooth muscle layer was observed in the silk grafts (Fig. 5B). Moreover, anti-CD68 immunostaining revealed substantial infiltration of macrophages and phagocytic phenomena around the remnants of the fibroin.

We have demonstrated a conspicuous increase of the collagen around the silk graft from 4 to 12 weeks [32].

These results and previous studies suggest that the organization of a vessel-like structure starts to occur from 4 to 12 weeks after grafting. In previous study, we have reported that the patency of the vascular graft of silk fibroin from wild-type silkworm and of PTFE was compared. In the case of PTFE graft, there was no cell attachment and thrombus formation was observed within the occluded. These results suggested that the availability of vascular graft from wild-type silk fibroin was significantly higher than that of PTFE graft. Additionally, it is important to the time-dependent change of the luminal diameter due to intimal hypertrophy. In a previous paper, the diameter of the silk fibroin graft was measured at 2, 4, 12, 24 and 72 weeks, giving values of around 1200–1400 μm [32]. The diameter remained unchanged in this system.

3.4. Implantation of Vascular Grafts Prepared with Wild-Type and Recombinant Silk Fibroins

Collagen tissues play an important role to provide recoil and prevent aneurysm formation in the artificial vascular grafts [47]. Therefore, we tried to develop a novel small-diameter vascular graft prepared with recombinant silk fibroin incorporating the sequences of the active sites from collagen produced by a transgenic silkworm. As mentioned above, the ^{13}C CP/MAS NMR spectrum is exactly the same between the wild-type and recombinant silk fibroin fibers. The preparation of the graft with recombinant silk fiber is basically the same as that with wild-type silk fibroin fiber. Because of the limited amounts of recombinant silk fibroin fiber samples, we only compared the initial stage of the growth of endothelial cells after grafting between wild-type and recombinant silk fibroin fibers.

Figure 6 shows a comparison of the number of endothelial cells attached to wild-type and recombinant grafts with distance from the anastomosis site by CD31 immunostaining at 2 weeks after grafting. This experiment was performed only one time and, therefore, it is difficult to discuss the difference clearly. However, the latter looks slightly more like an extended attachment of endothelial cells toward the inner part of the graft.

4. Conclusion

In this paper, small-diameter vascular grafts made from silk fibroin showed a good scaffold for cells which is essential to repair vascular tissue with attachment and proliferation cells. In addition, we demonstrated the utility of the small-diameter vascular graft based on wild-type and transgenic silk fibroins in the healthy animal models. This result suggested that this system will yield good patency rates and vascular reconstruction in the atheroslcerosis animal model.

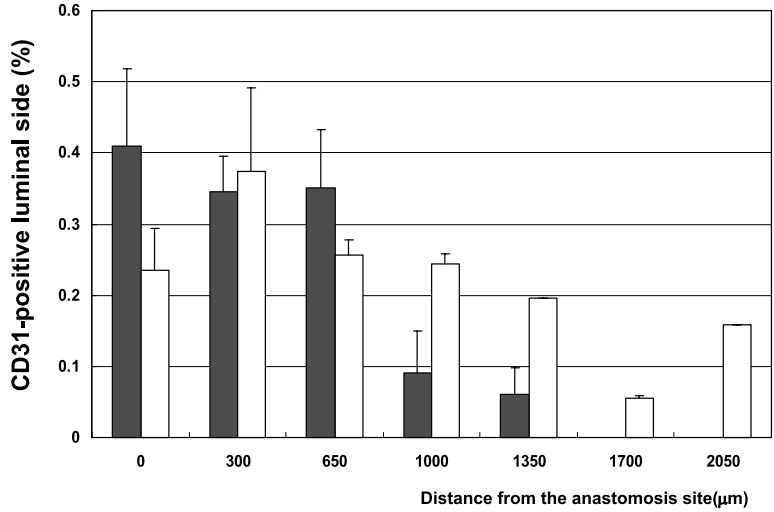

Figure 6. The frequency of CD31-positive cells on the luminal side. Black bar, the graft prepared from wild-type silk fibroin; white bar, the graft prepared from recombinant silk fibroin including the collagen sequence.

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