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Bombyx mori silk fibroin (SF) was successfully used for vascular grafts implanted in rats or dogs. Current transgenic technology can be developed to produce SF with improved properties. In this study, vascular endothelial growth factor (VEGF) or the repeated fibronectin-derived sequence, TGRGDSPAS, arginylglycylaspartic acid (RGD), were introduced into the SF heavy chain to improve its properties. A blood compatibility assay was performed for lactose dehydrogenase (LDH) activity for both transgenic and wild type SF. Human umbilical endothelial cells (HUVECs) growth showed greater enhancement of cellularization behaviour for the transgenic SF samples (VEGF and RGD) than for the wild type (WT) SF. VEGF SF also showed lower platelet adhesion than the RGD SF and WT SF. An *in vivo* implantation study supported these *in vitro* results. In particular, early endothelialisation was observed for VEGF transgenic SF, including the occurrence of native tissue organization at three months after implantation in rat abdominal aorta.

Introduction

Cardiovascular disease is the leading cause of death in the developed world. Currently, synthetic vascular grafts, with large diameters (>6 mm), from Dacron (polyester) or expanded polytetrafluoroethylene (ePTFE) are being successfully used for revascularization.[1, 2] However, graft implantation in the small-diameter vessels (<6 mm), including coronary arteries and arteries in the lower extremities, has not yet been achieved; mostly due to rapid occurrence of thrombosis or formation of intimal hyperplasia.[3, 4] Graft thrombosis due to material surface incompatibility, and altered flow dynamics at the site of anastomosis or distal outflow are recognized as primary reasons for blood-contacting device failure.[5-7] Reduction of platelet adhesion is crucial for improving graft patency, and several methods have been developed by modification of the graft surface. Most studies of graft surface involve the adhesion of antithrombogenic factors,[7-9] designing of surface roughness,[10, 11] coating with cell adhesive materials to promote adhesion to endothelial cells,[12,13] and seeding of vascular cells or their precursors.[14, 15] However, these methods have some problems: less mechanical properties for their use in arterial systems and they require long periods for cell seeding. Therefore, at present, none of these materials can be successfully used to produce small-diameter grafts.

Bombyx mori (*B. mori*) silk fiber has been used as surgical suture for centuries due to its high strength and toughness.[16] Moreover, silk fibroin (SF) has been reported to have many superior, inherent properties as a biomaterial including advantageous mechanical properties, environmental stability, biocompatibility, low immunogenicity and biodegradability.[17-20] In addition, silk protein is known to increase cell adhesion activity, which is necessary for its use as a biomaterial.[21-24] In the last decade, many studies have concentrated on SF vascular graft application, produced by aqueous gel spinning techniques,[25] electrospinning methods,[26, 27] braiding and winding [28], or coating SF to silk fabric or other materials.[29, 30] These SF grafts have shown promising results in terms of their mechanical properties, early cell adhesion, and patency compared with other materials. However, some *in vitro* studies have shown that the biocompatibility of the *B. mori* silk remains insufficient for usage for grafts, and therefore, an improvement of the properties of this silk is required.[21, 22] The introduction of functional peptides is useful for improving silk character. In the last decade, the germ line transformation method for the silkworm was developed using the transposon

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piggyBac. [31, 32] This method can produce larger numbers of transgenic silkworms than methods using *Escherichia coli*.[22] It is known that proteins fused with fibroin light chain (L-chain) and GFP, or fibroin heavy chain (H-chain) and GFP, can be produced in the posterior silk gland and expelled in the cocoon silk. [32-34] These findings suggest that another fusion protein could be expressed using this method and that we could modify the character of silk by the introduction of a novel gene fused with the fibroin L-chain or H-chain gene. In our previous study, we fused some functional peptides into fibroin L-chain or H-chains. The SF with RGD showed enhanced cell adhesion property. [21] Also, the SF with YIGSR fused to the H-chain showed enhanced cell adhesion activity and greater cell migration compared to wild type SF. [23] These results showed that fused fibroin has the potential to improve silk character and graft patency. We then focused on using two kinds of functional sequences, Arg-Gly-Asp (RGD) and vascular endothelial growth factor (VEGF), for improving adhesion to endothelial cells and anti-thrombogenic properties. The tripeptide, RGD, found in fibronectin, had been shown to possess cell adhesion activities,[21, 22, 24] suggesting that the addition of these peptides could increase the cell adhesion activity of the *B. mori* silk. On the other hand, VEGF is a specific endothelial cell mitogen that enhances adhesion and proliferation of endothelial cells, and it is believed to prevent thrombosis. [12, 13, 35]

In this study, SF grafts were improved by the action of active sites of VEGF or RGD peptides that were fused with H-fibroin, which facilitated the obtainment of excellent anti-thrombogenic properties in the silk. Usability of the antithrombotic, small-diameter vascular graft was evaluated by an *in vitro* study using endothelial cells and platelet adhesion tests and an *in vivo* study through implantation into rat abdominal aorta models.

Materials and methods

Production of transgenic (TG) silk

The DNA of the open reading frame sequence of the VEGF (Human VEGF–165 A) gene (AB021221), excluding the signal-peptide coding sequence, was synthesized (GenScript, USA) and then cloned into the BamHI and SalI sites of a H-fibroin fusion vector, pHC-EGFP. [32] To introduce the RGD tripeptide into H-fibroin, the DNA fragment coding $[TGRGDSPAS]_8$ peptide[21] was cloned into the H-fibroin fusion vector. The DNA of the vectors was injected into the eggs of silkworm in order to produce the TG silkworms, as reported previously. [36] The TG silkworms were established using a non-diapausing strain, w1-pnd, and were mated with the diapausing strain, w1, to maintain the TG silkworms as diapausing strains. The silkworms were reared on an artificial diet (Nosan, Japan) or on fresh mulberry leaves at 25 °C. Due to the poor yield and robustness of the silk of the TG silkworm strains, these were repeatedly backcrossed with the commercial strains, Gunma and 200, respectively, which have been shown to have high silk productivity. The resulting hybrid strains were then used for mass rearing of the silkworms and silk reeling from the cocoons. [36] The wild type *B. mori* SF without improving silk character (WT SF) was used for a comparison of TG SF.

Removal of silk sericin from cocoons

The SF fibers obtained from TG silkworms were degummed in a mixture of sodium carbonate (0.08 % (w/v)) and Marseille soap (0.12 % (w/v)) at 70°C for 45 min, in order to remove the silk sericin completely. For WT yarns, the degumming process was carried out as described previously, [29] followed by rinsing several times with distilled water to remove the sericin. The SF was then dissolved in 9 M LiBr (adjusted to 10% (w/v)) by incubating for 1 h at 37°C. The solution was dialysed against distilled water for 3 days.

SDS-PAGE and Western blotting of silks.

To validate the presence of the recombinant silk protein, SDS-PAGE and western blotting were performed. TG SF was dissolved at 9M LiBr/0.1M Tris- HCl (pH9), and dialyzed against PD-10 gel (5M Urea/20mM Tris- HCl (pH 8.0). SDS-PAGE was performed with using a 4- 12 % gradient acrylamide gel (NuPAGE, Life Technologies, USA), and the separated proteins were stained with Coomassie Brilliant Blue. When western blotting experiment was performed, the protein were separated by 10 % acrylamide gel (prepared by self) and the separated protein was performed using TBST containing 5% (w/v) skimmed milk and reacted with primary anti body (Anti-VEGF) and secondary antibody (Anti-Rabbit HRP).

Tensile Strength

Tensile strength test of the TG silkworm was evaluated by the method reported previously. [23] The breaking strengths of WT and TG SF fibers prepared here were measured by using a tensile testing machine (Auto-Graph AG-Xplus, SHIMADZU Co. Ltd. Japan). The diameter of each single fiber was determined by scanning electron microscopy (SEM : VE-7800, Keyence, Japan). The breaking strength was measured as the highest stress value attained during the test. Each value was obtained as average of 5 measurements. The standard deviations were calculated.

Cell adhesion and cell growth MTS assay

Human umbilical vein endothelial cells (HUVECs, Lonza, Inc., Switzerland) were used for experiments using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell adhesion assay. WT, RGD, and VEGF SF films were used for comparison. Each SF solution was diluted with water to a concentration of 0.2 % (w/v), and these solutions were used for cell adhesion and cell growth MTS assay experiment using 24-well microplates at 0.25 mg/cm^3. After drying at room temperature for 12 h, the plates were desiccated under vacuum conditions for 2 h. The silk-coated plates were treated with 70% ethanol for 15 min in order to insolubilize the silk proteins. These surfaces were then washed twice with physiological saline. HUVECs cultured in endothelial basal medium-2 (EBM-2, Lonza, Inc.) were kept in a 37°C-incubator for 1 day. Cultured HUVECs were

washed with PBS, detached with 0.25% trypsin, washed, and resuspended in medium. For cell adhesion experiments, this HUVEC suspension was added to silk-coated wells at 2.0×10^5 cells/well, and incubated for 3 h at 37°C, 5% CO_2. After removal of supernatant, live cells were stained with CalceinAM (Takara Bio Inc., Japan). The number of cells was counted using a fluorescence microscope BIOREVO BZ-9000 withBZ-H1C software (Keyence Co., Ltd., Japan).Cell growth experiments were performed with WT and TG SF film, using 96-well cell-culture plates. HUVEC suspension was added to silk coated wells at 2×10^3 cells/well, and incubated at 37°C, for 1, 3, and 5 days, at 5% CO_2. After incubation, and the medium was removed using an aspirator. For measurement of the number of live cells (HUVECs), the CellTiter 96 AQueous Non-Radioactive Cell Assay (Promega, Inc.) was used. A phenazine methosulphate (PMS) and MTS mixed solution was added to silk coated wells, and incubated at 37°C for 2 h at 5% CO_2. The number of live HUVECs was evaluated by measuring the absorbance at 490 nm, using a 96-well plate photometer (BIO-RAD, Inc., CA, USA).

Platelet adhesion and LDH activity

Canine platelet-rich plasma (PRP) was used in the platelet adhesion assay, analysed by SEM and the lactate dehydrogenase (LDH) activity method. Fresh blood samples were obtained from healthy and medication-free beagles, Canis lupus familiaris. Blood samples were drawn from jugular veins and collected directly into 50 mL citrated syringes (0.025 M= 9:1). Citrated blood was stored at room temperature and centrifuged at 2000 rpm for 15 to 30 min, collecting PRP within 1 h. Platelet adhesion experiments were performed with WT and TG SF films using 24-well cell-culture plates. Firstly, WT and TG SF solutions were air-dried at room temperature and insolubilized with ethanol and ethanol residue, then washed with PBS until complete removal of the alcohol. PRP was carefully added, drop wise, onto the film and incubated at 37°C for 3 h. Incubated samples were fixed with 1% glutaraldehyde and gradually dehydrated by ethanol. Gold vapour samples were evaluated by SEM. The number of adhered platelets were counted in 5 random points for each sample and calculated as the average adhered platelet number. The LDH activity assay was used to quantitatively evaluate platelet adhesion, performed according to a previously published protocol. [8, 21] Briefly, the amount of adherent platelets on the sample surface was determined by measuring the LDH activity released from adherent cells upon lysis with Triton X-100 (Wako Co. Ltd., Japan) and compared with the collagen samples. Platelet-rich plasma (PRP, 50 µL) was placed onto the samples and incubated at 37°C for 45 min. Samples were subsequently rinsed twice in PBS. After rinsing, 50 µl of (w/v) Triton X-100 (0.1%) was added onto the surface of the sample. Five minutes later, 25 µL of the lysates was taken from the surface of the samples and LDH activity was determined photometrically using a microplate reader at 340 nm through a kinetic assay with a substrate solution of 200 mL NADH and sodium pyruvate. The gradient of the curve was evaluated and calibrated with lysates of all platelets in the PRP volume.

Vascular tube preparation and characterization

We used 1.5 mm inner diameter SF braided vascular grafts prepared from wild type and two types of TG silk multi yarns. A braiding structure is typically used because it is lightweight, has high strength, and has high flexibility and conformability in its body. Vascular grafts were degummed to remove silk sericin as described previously in this section. After removal of sericin, a ePTFE rod was inserted into the SF tube. The SF tube was dipped in the SF solution to make the SF filaments stick together, dried, and fixed in ethanol; then the SF grafts was removed from the central core. Details of the preparation had been reported in detail elsewhere. [29]

***In vivo* revascularization in rat abdominal aorta model**

WT and two different types of TG SF grafts (10 mm long, 1.5 mm inner diameter) were implanted into the rat abdominal aorta. All procedures were carried out as described previously.[29] Briefly, 3 rats after 2 weeks and 4 or 5 rats after 4 and 8 weeks, respectively, were evaluation for each sample (n=43). After recovery from anaesthesia, animals were maintained on a 12 h light/dark schedule and had ad libitum access to standard pellet food and water. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology. The graft patency was monitored by colour Doppler imaging (e-flow imaging), and pulse waves were recorded with a 7.5-MHz linear probe and echo-imaging apparatus (Prosound α-10, Hitachi-Aloka, Japan) at 1 week and again just before explantation, under anaesthesia with pentobarbital. Graft diameter, together with any sign of thrombosis or aneurysm formation, were carefully checked by B-mode imaging, and blood flow velocity was measured using a pulsed Doppler flow meter. Before euthanasia, rats underwent a general physical examination to evaluate their condition. At death, the rats were perfused with 0.9% saline solution through the left ventricle. The grafts were carefully removed, together with the surrounding tissue. Methanol-fixed explanted SF grafts were dehydrated in alcohol solutions of increasing concentration, clarified in xylene and embedded in paraffin. Paraffin-embedded sections (4 µm thick) were processed for hematoxylin and eosin (HE) staining and anti-rat CD 31 (clone TLD-3A12; BD Bioscience, San Jose, CA, USA) immunohistochemical staining. Rate of local tolerance was analysed in terms of presence of endothelialisation and tissue growth of samples.

Statistics

Data are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with Bonferroni post hoc tests was performed. A P level <0.05 was considered statistically significant. All calculations were performed using Microsoft excel (version 2010).

Results and discussion

Characterization of TG silk fibroin

To produce silk that possess the characteristic of early endothelialisation, we constructed two types of TG silkworm that produce recombinant silk with the peptide fused to SF H-chain. To confirm the presence of VEGF and RGD in SF H-chain, we first performed SDS-PAGE and western-blotting by anti VEGF antibody. Based on the predicted amino acid sequence, the estimated molecular weight was 31 kDa for the RGD fused SF H-chain, 43 kDa for the VEGF fused SF H-chain. SDS-PAGE analysis (Fig. 1 A) showed RGD fused SF H-chain and VEGF fused SF H-chain have shape band at 41 kDa and 53 kDa, respectively. These values were about 10 kDa larger than estimated molecular weight. Then, to get more information, western blotting by anti VEGF antibody was performed VEGF fused SF H-chain, as shown Fig. 1 B. A broad band by anti VEGF antibody reactivity was evident at 53- 58 kDa for VEGF fused SF H-chain. Thus, the molecular weight of VEGF fused SF H-chain and RGD fused SF H-chain was higher than estimated values. This tendency was reported previously. [23]

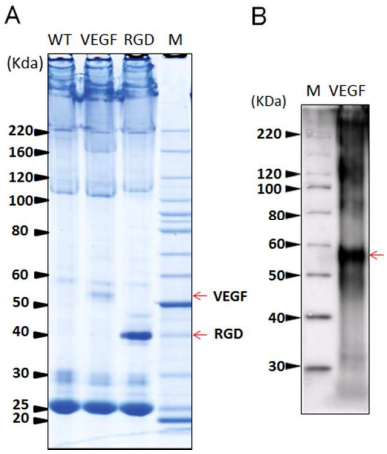

Fig. 1 SDS-PAGE and Western blot of WT and TG silk fibroins. (A) SDS-PAGE of silk fibroins with RGD and VEGF showing molecular weight, 41 kDa and 53 kDa, respectively (red arrows). (B) Western blot of silk fibroin with VEGF by anti-VEGF antibody. The band of anti-VEGF antibody reactivity was observed at 53- 58 kDa (red arrow) for VEGF fused silk fibroin H-chain. The standard molecular weights (M) were also shown.

Tensile Strength

The fiber diameters were 11.5 µm for WT SF, 12.6µm for VEGF SF and 10.6µm for RGD SF. Thus, fiber diameters were no significant difference among silk fibers. The tensile strength was 79 ± 24 MPa for WT SF [23], 65 ± 12 MPa for VEGF SF and 68 ± 13 MPa for RGD SF. Thus, each tensile strength of TG SF fiber was slightly smaller than that of WT SF fiber. This may be interpreted with small increase in the fraction of distorted turn and/or random coil conformation of TG silk fibroins.[23]

Vascular cell attachment and growth

After producing insolubilized SF film from each sample, the HUVEC adhesion experiment was performed by fluorescent microscopy. Attachment intensity of HUVECs for each SF was shown in the fluorescent microscope images as shown in Fig. 2 A-C. The average number of HUVECs is shown in Fig. 2 D and observations of HUVEC growth are shown in Fig. 2 E. The VEGF and RGD SF showed significantly higher attachment and growth activity of HUVECs than WT SF. Cell adhesion activity of RGD [21, 22, 24] and vascular endothelial growth activity of VEGF[12, 13, 35] worked effectively for adhesion and growth of endothelial cells.

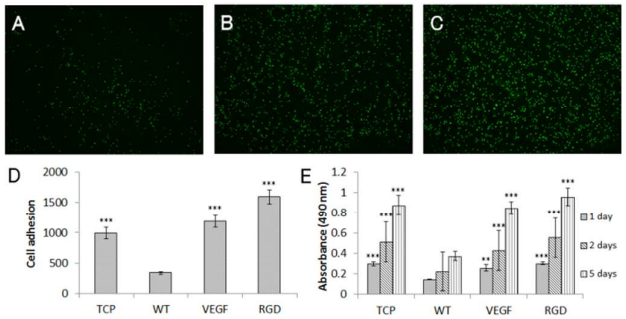

Fig. 2 HUVEC adhesion observation by fluorescent microscopy. (A) WT, (B) VEGF-fused silk fibroin and (C) RGD-fused silk fibroin. (D) number of adherent HUVECs at 2 h after incubation and (E) HUVEC growth at 1, 3 and 5 days after cultivation. Silk fibroins with VEGF and RGD showed higher numbers of the attached HUVECs than the WT silk fibroin.

Blood compatibility by platelet attachment and LDH assay

After producing insolubilized SF film from each sample, platelet adhesion was examined by SEM and LDH activity, as shown in Fig 3. Attachment intensity of canine platelets for each SF was shown by SEM images, represented in Fig. 3 A– C and the average number of platelets is shown in Fig. 3 D. The attachment intensity of platelets was observed to be higher for the RGD and WT SF using the SEM images. The VEGF SF showed significantly lower platelet attachment with a mean of 174.8 (± 40.9) whereas these values were 284.3 (± 33.5) and 319.2 (± 48.9) for the WT and RGD SF, respectively. Values for LDH activity were compared with the common material, collagen. In this case, lysis of platelet cells was clearly lower for SF than for collagen samples. (Fig. 3 E). RGD SF showed greater attachment of HUVECs (Fig. 2 C) and platelets (Fig. 3 C) than the WT SF. RGD SF promoted attachment of both endothelial cells and platelets by functioning as a cell adhesion factor. Endothelial cells and platelets were attached in the RGD SF through the functioning of integrin. [37]

On the other hand, VEGF SF showed greater attachment only of HUVECs (Fig. 2 B), and lesser attachment of platelets than WT SF (Fig. 3 B). The VEGF protein is known to promote growth of endothelial cells and to organise endothelial cell rolling inhibition of

platelet attachment.[12, 35] These results suggested that VEGF SF was a better material than RGD SF and WT SF to fulfil the role of an anti-thrombosis small-diameter graft.

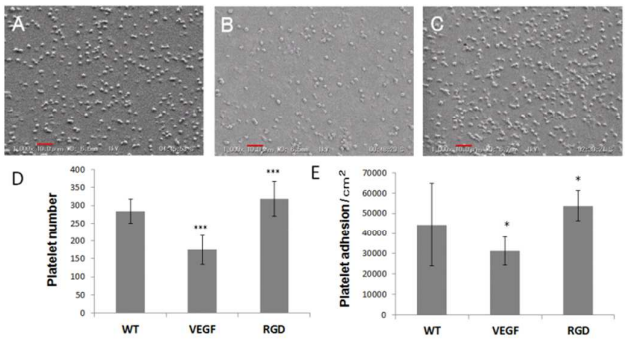

Fig. 3 Canine platelet-rich plasma (PRP) evaluation of SEM images of (A) WT silk fibroin, (B) VEGF-fused silk fibroin and (C) RGD-fused silk fibroin. (D) Platelet count by SEM evaluation and (E) Lactate dehydrogenase (LDH) lysis assay. The VEGF silk fibroin showed significantly lower attachment of platelets compared with WT silk fibroin and RGD silk fibroin. Scale bar=200 µm.

Applications of revascularisation in rats

Fig. 4 shows representative SEM images of the inner part of the fabricated braided SF vascular grafts before and after coating. The surface of the SF fibers was not observed after coating. However, large open spaces among the bundles of fibers observed before coating were not buried. These braided SF grafts had adequate stretch and elasticity, and were easy to handle for implantation. Implanted samples were evaluated at 1, 2, 4 and 8 weeks to observe blood contact, tissue ingrowth, and endothelialisation. One week after implantation, all grafts were patent and the inner surface of the samples was covered with thrombi. However, as shown in Fig. 5, 2 weeks after implantation, WT and VEGF SF grafts showed smooth white inner surfaces with no fibrinogen or platelet attachment (Fig. 5 A, B), but the RGD SF graft at the same time, this inner surface was covered with a reddish blood component (Fig. 5 C).

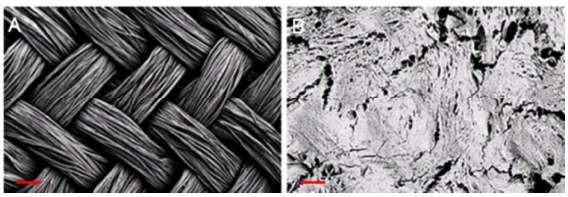

Fig. 4 SEM images of silk fibroin vascular graft at the inner surface, (A) before and (B) after coating treatment. The surface of the silk fibroin fibers was not observed after coating. Scale bar=200 µm.

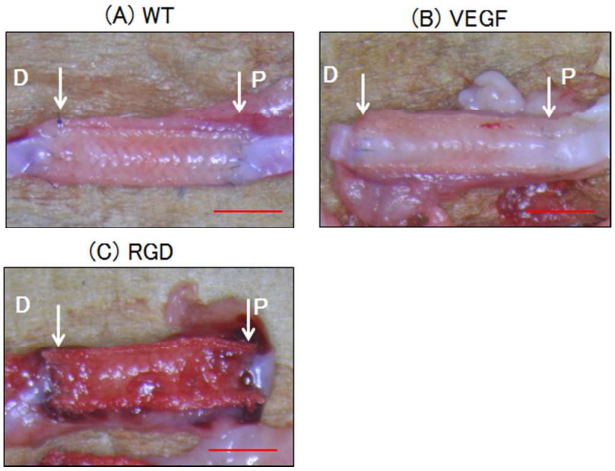

Fig. 5 Macroscopic gross observations of three types of vascular grafts at 2 weeks after implantation. Inner surfaces of (A) WT silk fibroin and (B) VEGF-fused silk fibroin grafts were covered with neointima. Thrombus was observed for the (C) RGD-fused silk fibroin grafts. The arrows indicate the suture site. D and P indicate distal and proximal part, respectively. Scale bar = 200 µm.

Table. 1 Patency results of each silk fibroin graft at 2, 4 and 8 weeks after implantation. (WT silk fibroin, VEGF-fused silk fibroin and RGD-fused silk fibroin)

sample	2weeks	4weeks	8weeks
WT Silk	3/3	3/4	3/4
VEGF Silk	3/3	5/5	5/5
RGD Silk	2/3	2/5	2/5

The patency rate of each SF is shown in Table 1. The patency rate of RGD SF graft was lower than that for the other two types of sample at 2, 4, and 8 weeks. VEGF SF graft showed a higher patency rate than the WT and RGD SF grafts at these same times. It was suggested that the cell adhesion activity of RGD promoted adhesion of platelets *in vivo*, and caused occlusion by thrombosis formation. These results were similar to our *in vitro* evaluation. Endothelialisation was evaluated by a CD31 immuno-staining method (Fig. 6). Endothelial cells covered the tissue ingrowth area of the VEGF SF graft edge at 2 weeks after implantation (Fig. 6 B). On the other hand, the WT SF graft did not show any endothelial cell appearance (Fig. 6 A), and the RGD SF graft showed some spots of endothelial cells at 2 weeks after implantation (Fig. 6 C). Early and uniformly organised endothelialisation occurred when using the VEGF SF graft compared with WT and RGD SF grafts. Early endothelialisation of the VEGF SF graft was also observed at the center of the graft at 4 weeks (Fig. 7 B). In contrast, the WT SF and RGD SF grafts did not show any endothelial cell presence (Fig. 7 A, 7 C). In addition, at 8 weeks after implantation, endothelial cells completely covered the center of the VEGF SF graft, while ndothelial cells partially covered that of RGD SF but very sparsely

covered that of WT SF graft. (Data not shown) Thus, VEGF SF showed greater attachment of endothelial cells. It was known that VEGF-165A protein worked specific signalling tyrosine-kinase receptor and activated endothelial cell growth [21]. In this study, the VEGF fused with SF H-chain seems to work this receptor. The VEGF remains permanently fused with the H- SF protein. The cDNA of an isoform of VEGF was fused with N- and C-terminal domains of H-SF gene, without the remaining domain of repetitive sequences. The cysteine residues at C-terminus of VEGF H-chain are thought to be bonded to SF L-chain by disulfide covalent bond and to SF P25 protein. The VEGF H-chain indicate a 53 kDa band as VEGF H-chain protein in the SDS-PAGE and Western blots under reducing conditions (Fig. 1)

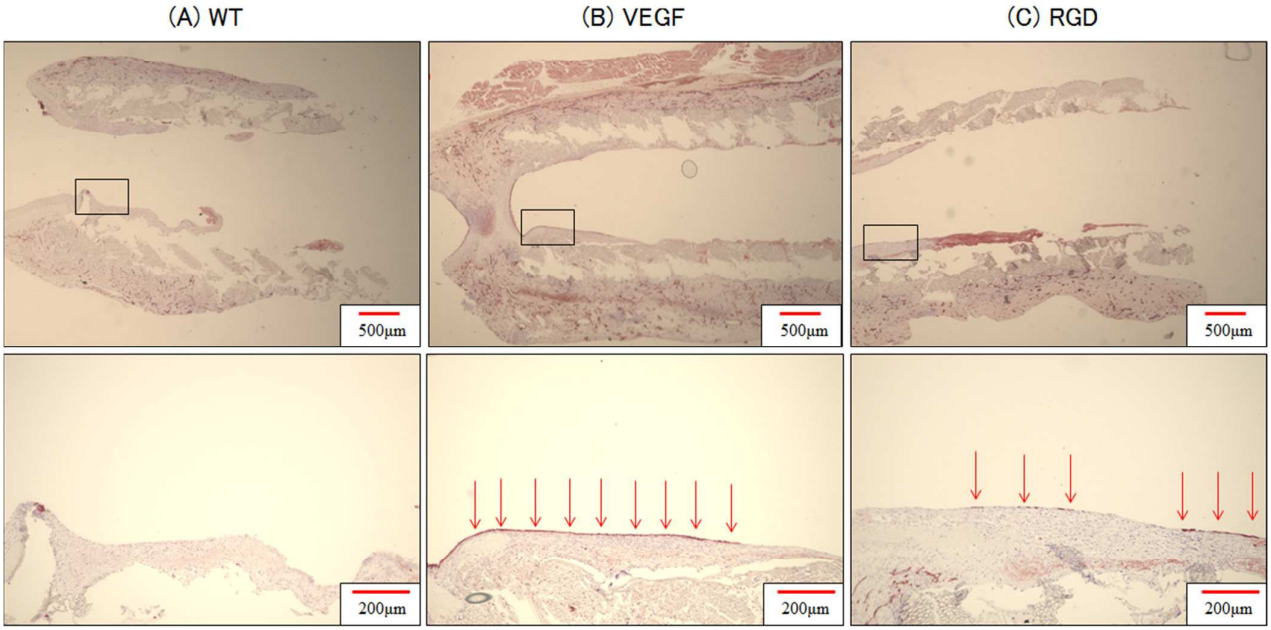

Fig 6 Pictures of longitudinal sections by CD31 immune-staining stain of three types of silk fibroin grafts at 2 weeks after implantation. (A) WT silk fibroin, (B) VEGF-fused silk fibroin and (C) RGD-fused silk fibroin. Lower images represent magnifications of areas encapsulated by boxes. Early endothelialisation was observed for the VEGF-fused SF graft (B- lower) at 2 weeks by endothelial cell specific CD31 method. However, WT silk fibroin graft (A- lower) did not show any endothelial cell appearance at the same time period. The RGD-fused silk fibroin graft (C- lower) shows few endothelial cells in an irregular confirmation. The arrows indicate the presence of endothelial cells.

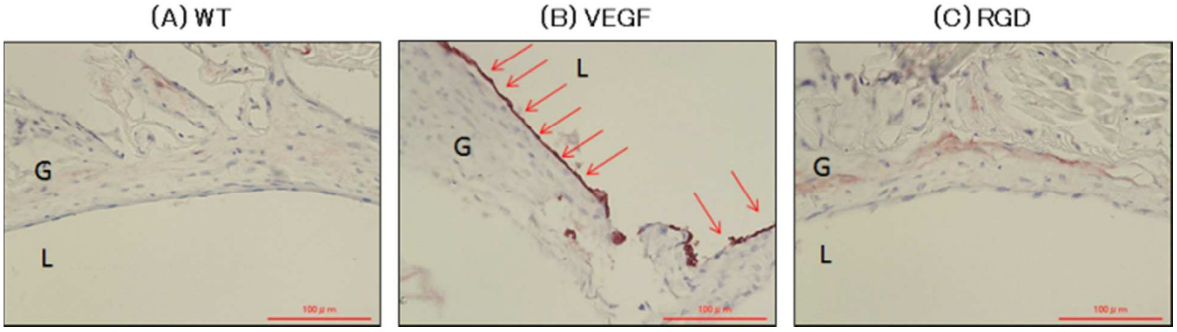

Fig. 7 Endothelialisation evaluations by CD31 specific immune-staining method at the center of three types of silk fibroin grafts at 4 weeks after implantation. (A) WT silk fibroin graft and (C) RGD-fused silk fibroin graft did not show any endothelial cell appearance, but the (B) VEGF silk fibroin graft showed a short line of endothelial cells in an irregular confirmation. The arrows indicate the presence of endothelial cells. **G** and **L** mean the graft and lumen.

When histological staining (by H&E) was performed at 8 weeks after implantation, tissue ingrowths were observed inside and outside of each graft (Fig. 8). The inner graft tissue was significantly thinner with VEGF SF graft (113.4 ± 10.3 μm) and RGD SF graft (92.2 ± 12.1 μm) compared with WT SF graft (210± 4.8 μm). In addition, these tissues of the inner graft surface were well organized for the VEGF and RGD SF grafts, while for the WT SF graft, the tissue was disorganized and neointimal hyperplasia occurred (Fig. 8 A). It was previously known that endothelial cells could prevent neointimal hyperplasia by inhibiting mitogen-induced proliferation of smooth muscle cells.[12, 35]

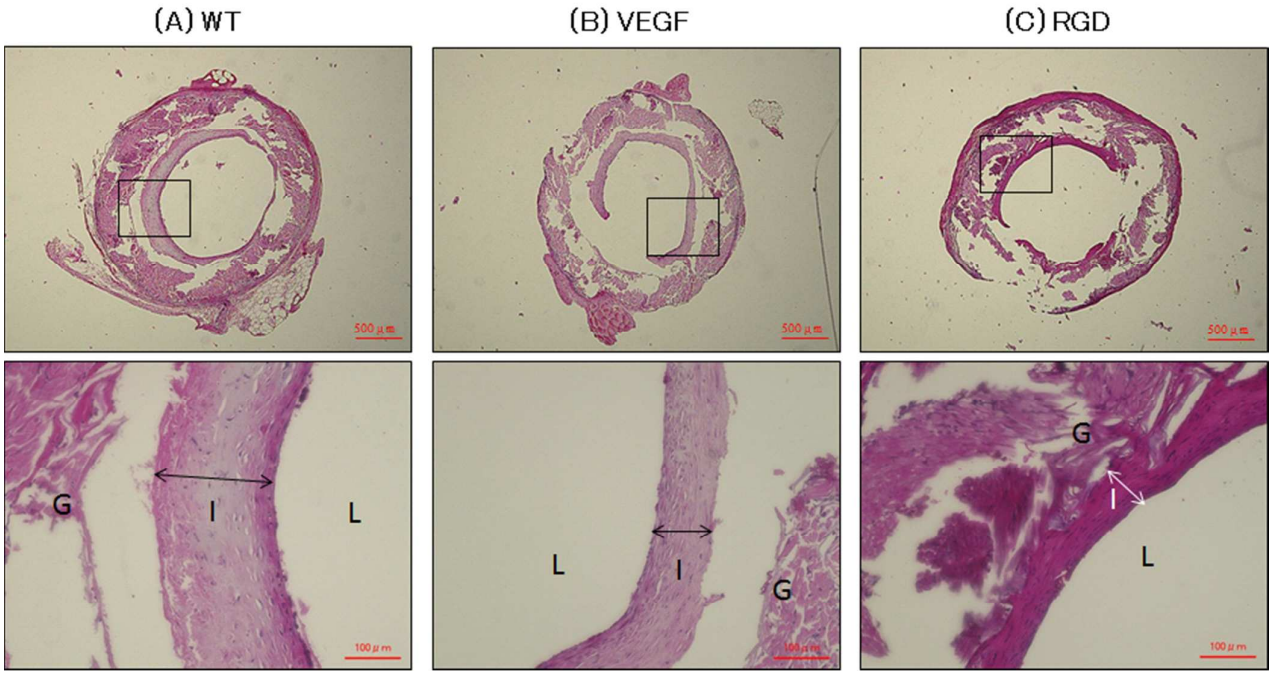

Fig. 8 Tissue organisation at the center of three types of silk fibroin grafts at 8 weeks after implantation. (B)VEGF-fused silk fibroin graft and (C) RGD-fused silk fibroin graft show lower tissue ingrowth than (A) WT silk fibroin grafts. **G** and **L** mean graft and lumen, and **I** thickness of intima.

Therefore, VEGF and RGD SF grafts could prevent neointimal hyperplasia by promoting growth or adhesion endothelial cells.

WT SF grafts had shown better patency and endothelialisation than ePTFE grafts when implanted into small animals such as rats.[25- 30] Also in our previous study,[28, 29] SF graft proved to be as efficient as vascular graft when implanted in rat models. Patency of fibroin grafts 1 year after implantation was significantly higher than that of ePTFE grafts (85.1% vs 30%). Endothelial cells and smooth muscle cells were organized in an inner layer of SF graft early. Sirius-red staining revealed that the collagen content of fibroin grafts significantly increased 1 year after implantation, with a decrease in SF content.[28] Thus the SF graft was adequate for using as a small-diameter graft for small animals. However, there were few successful studies of long term patency of SF small-diameter graft (inner diameter 3-4 mm) by implant to large animals because migration of endothelial cells at the center of the grafts became difficult, with increasing implantation length.[38] Absence of endothelial cells at the inner surface of the graft may cause formation of thrombosis and neointimal hyperplasia, as previously mentioned.

In this study, We focused on using two kinds of functional sequences, RGD and VEGF, for improving adhesion to endothelial cells. The *in vitro* experiment emphasize that RGD SF has the enhanced cell adhesion activity, but also higher platelet attachment simultaneously. Also, the *in vivo* experiment clearly showed that RGD SF was not suitable for the vascular graft because of formation of thrombosis. On the other hands, VEGF SF showed greater attachment only endothelial cells at *in vitro* experiments. In addition, VEGF SF graft showed early migration of endothelial cells and prevented both adhesion of platelets and hyperplasia at *in vivo* experiments. This result indicates that endothelial cells can completely cover the center of the graft in large animals by using VEGF SF and that the development of small-diameter grafts can be achieved.

Conclusions

In this work, we prepared two kinds of transgenic *Bombyx mori* SF that incorporated VEGF or the repeated fibronectin derived sequence, RGD. VEGF and RGD SF showed greater attachment of endothelial cell than that of WT SF. VEGF SF showed lower platelet

adhesion than the RGD and WT SF by an *in vitro* study. Moreover, VEGF SF graft showed early endothelialisation, good patency, and prevention of neointimal hyperplasia. Thus, VEGF SF has shown the highest potential for use as a silk vascular graft. This result is encouraging for the development of practical uses of this biomaterial for small-diameter vascular graft.

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Notes and references

1 H. Takagi, S. Goto, M. Matsui, H. Manabe and T. Umemoto, *J. Vas. Sur,* 2010, **52**, 232.
2 J. S. Budd, K. E. Allen, G. Hartley G and PR. Bell, *Eur J Vasc Surg,* 1991, **5**, 397.
3 S. Venkatraman, F. Boey and L. L. Lao, *Prog. Polym. Sci*, 2008, **33**, 853.
4 BL. Seal, TC. Otero and A. Panitch, *Mater Sci Eng R Rep*, 2001, **34**, 147.
5 W. M. Abbott, J. Megerrnan, J. E. Hasson, G. L'Italien and D. F. Warnock, *J. Vac. Sur*., 1983, **5**, 376
6 P. Qi, M. F.Maitz and N.Huang, *Surf. &Coat. Tech.*, 2013, **233**, 80.
7 C. Vepari, D. Matheson, L. Drummy, R. Naik and D. L. Kaplan, *J. Biomed. Mater. Res. A,* 2010, **93**, 595.
8 R. A. Hoshi, R. V. Lith, M. C. Jen, J. B. Allen and K. A. Lapidos, *Biomaterials*, 2013, **34**, 30.
9 C. K. Hashi, N. Derugin, R. R. R. Janairo, R. Lee, D. Schultz, J. Lotz and S. Li, *Arterioscler. Thomb. Vasc. Biol,* 2010,**30**, 1621.
10 V. Milleret, T. Hefti, H. Hall, V. Vogel, D. Eberli, *Acta. Biomat.,* 2012*,* **8**, 4349.
11 H. Bergmeister, C. Schreiber, C. Grasl, I. Walter, R. Plasenzotii, M. Stoiber, D. Bernhard and H. Scima, *Acta. Biomat.,*2013, **9**, 6032.
12 H. Zhang, X. Jia, F. Han, J. Zhao, Y. Zhao, Y. Fan and X. Yuan, *Biomaterials,* 2013, **34**, 2202.
13 B. Randone, G. Cavallaro, A. Polistenia, A. Cucina, P. Coluccia, P. Graziano and A. Cavallaro, *J. Surg. Res*, 2005, **127**, 70.
14 N. Hibino, T. Shinoka, G. Matsumura, Y. Ikada and H. Kurosawa, *The J. Thor. Card. Surg*, 2005, **129**, 1064.
15 T. L. Mirensky, G. N. Nelson, M. P. Brennan, J. D. Roh, N. Hibino, T. Yi, T. Shinoka and C. K. Breuer, *J. Pedi. Surg,* 2009, 44, 1127.
16 M. Demura, T. Asakura and T. Kuroo, *Biosensors,* 1989, 4, 361
17 T. Asakura, D. L. Kaplan, Silk Production and Processing in *Encyclopedia of Agricultural Science*, ed. C. J. Arutzen, Academic Press, NY, 1994, **4**, 1.
18 M. Demura and T. Asakura, *Biotechnol Bioeng*, 1989, **33**, 598.
19 M. Demura and T. Asakura, *J Membrane Sci,* 1991, **59**, 39.
20 C. Vepari and D. L. Kaplan, *Prog Polym Sci*, 2007, **32**, 991.
21 S. Yanagisawa, Z. Zhu, I. Kobayashi, K. Uchino, Y. Tamada, T. Tamura and T. Asakura, *Biomacromolecluess*, 2007, **8**, 3487.
22 T. Asakura, C. Tanaka, M. Yang, J. Yao and M. Kurokawa, *Biomaterials*, 2004, **25**, 617.
23 T. Asakura, M. Isozaki, T. Saotome, K. Tatematsu, H. Sezutsu, N. Kuwabara and Y. Nakazawa, *J. Mat. Chem. B*, 2014, **2**, 7375.
24 S. Sofia, M. B. McCarthy, G. Gronowicz and D. L. Kaplan, *J. Biomed. Mater. Res*, 2001, **54**, 139.
25 M. Lovett, G. Eng, J. A. Kluga, C. Cannizzaro, G. Vunjak-Novakovic and D. L. Kaplan, *Organogenesis*, 2010, **6**, 217.
26 B. Marelli, A. Alessandrino, S. Fare, G. Freddi, D. Mantovani and M. C. Tanzi, *Acta Biomat.*, 2010, **6**, 4019.
27 X. Zhang, X. Wang, V. Keshav, X. Wang, J. T. Johanas, G.. G. Leisk and D. L. Kaplan, *Biomaterials*, 2009, **30**, 3213.
28 S. Enomoto, M. Sumi, K. Kajimoto, Y. Nakazawa, R. Takahashi, C. Takabayashi, T. Asakura and M. Sata, *J Vasc. Surg.*, 2010*,* **51**,155.
29 T. Yagi, M. Sato, Y. Nakazawa, K. Tanaka, M. Sata, K. Itoh, Y. Takagi and T. Asakura, *J. Artif. Organs*, 2011, **14**, 89.
30 F. Huang, L. Sun and J. Zheng, *Artif. Organs*, 2008, **32**, 932.
31 T. Tamura, C. Thibert, C. Royer, T. Kanda, E. Abraham, M. Kamba, N. Komoto, J. L. Thomas, B. Mauchamp, G. Chavancy, P. Shirk, M. Fraser, J. C. Prudhomme and P. Couble, *Nat. Biotechnol.*, 2000, **18**, 81.
32 K. Kojima, Y. Kuwana, H. Sezutsu, I. Kobayashi, K. Uchino, T. Tamura and Y. Tamada, *Biosci. Biotechnol. Biochem*, 2007*,* **71**, 2943.
33 S. Inoue, T. Kanda, M. Imamura, G. X. Quan, K. Kojima, H. Tanaka, M. Tomita, R. Hino, K. Yoshizato, S. Mizuno and T. Tamura, *Insect Biochem. Mol. Biol.*, 2005, **35**, 51.
34 M. Tomita, H. Munetsuna, T. Sato, T. Adachi, R. Hino, M. Hayashi, K. Shimizu, N. Nakamura, T. Tamura and K. Yoshizato, *Nat.Biotechnol.*, 2003, **21**, 52.
35 A. H. Dorafshar, N. Angle, M. Bryer-Ash, D. Huang, M. F. Farooq, H. A. Gelabert and J. A. Freischlag, *J Surg. Res.*, 2002, **114**, 179.
36 T. Iizuka, H. Sezutsu, K. Tatematsu, I. Kobayashi, N. Yonemura, K. Uchino, K. Nakajima, K. Kojima, C. Takabayashi, H. Machii, K. Yamada, H. Kurihara, T. Asakura, Y. Nakazawa, A. Miyawaki, S. Karasawa, H. Kobayashi, J. Yamaguchi, N. Kuwabara, T. Nakamura, K. Yoshii and T. Tamura, *Advanced Functional Materials*, 2013*,* **23**, 5232.
37 U. Hersel, C. Dahmen and H. Kessler, *Biomaterials*, 2003, **24**, 4385.
38 G. Soldani, P. Losi M. Bernabei, S. Burchielli, D. Chiappino, S. Kull, E. Briganti and D. Spiller, *Biomaterials*, 2010, **31**. 2592.

Abstract;

Bombyx mori silk fibroin (SF) was successfully used for vascular grafts implanted in rats or dogs. Current transgenic technology can be developed to produce SF with improved properties. In this study, vascular endothelial growth factor (VEGF) or the repeated fibronectin-derived sequence, TGRGDSPAS (RGD), were introduced into the SF heavy chain to improve its properties. A blood compatibility assay was performed for lactose dehydrogenase (LDH) activity for both transgenic and wild type SF. Human umbilical endothelial cells (HUVECs) growth showed greater enhancement of cellularization behaviour for the transgenic SF samples (VEGF and RGD) than for the wild type SFs *in vitro*. An *in vivo* implantation study supported these *in vitro* cell seeding results. In particular, early endothelialisation was observed for VEGF transgenic SF, including the occurrence of native tissue organization at three months after implantation in rat abdominal aorta.