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Recombinant Silk Fibroins incorporated Cell-adhesive Sequences produced by Transgenic Silkworm as a Possible Candidate for Use in Vascular Graft

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Abstract

Interest in vascular grafts has recently grown because more patients are undergoing procedures that involve these grafts. However, smaller grafts with diameters < 6 mm made from conventional biomaterials are associated with a high incidence of thrombosis, and therefore the development of improved materials suitable for small vascular grafts is highly desirable. In this paper, four kinds of recombinant *Bombyx mori* silk fibroins were prepared using transgenic techniques for use as silk vascular graft with a diameter of <6 mm. The peptide sequence TS(CDPGYIGSRAS)$_8$ derived from the laminin B1 chain or the combination of two kinds of sequences, TS(CDPGYIGSRAS)$_8$ and (TGRGDSPAS)$_8$ derived from fibronectin, was incorporated into the light (L)-chain or heavy (H)-chain of the silk fibroin. The fractions of the incorporated peptide sequences range from 0.8% to 7.2% by weight in the recombinant silk fibroins. This incorporation causes a very small increase in random coil fraction of silk fibroin and a decrease in the tensile strength. Compared with native silk fibroin, the adhesive activities of mouse endothelial and smooth muscle cells increase significantly with the recombinant silk fibroin films incorporating only TS(CDPGYIGSRAS)$_8$ sequence independent of the L- or H-chains . A similar tendency was observed for the high migration activities of the endothelial cells *in vitro* and also the longer migration distance of the endothelial cell from the anastomotic part of rat abdominal aorta *in vivo* when this recombinant silk fibroin was used as a coating material for the silk graft. In view of the results, the recombinant silk fibroin incorporating the laminin peptide sequence can be potentially used as a vascular graft material.

Introduction

For many years, vascular grafts prepared from Dacron or expanded polytetrafluoroethylene have been used for the revascularization of blood vessels with inner diameters of ≥6 mm. The interest in reengineering these materials has recently grown as the number of patients requiring such grafts has increased. However, replacement of small-diameter vessels (<6 mm) including coronary arteries and arteries in lower extremities with these synthetic materials has failed, mostly due to rapid thrombosis.[1-3] In attempts to address this problem, various tissue-engineered vascular grafts have been developed; poly-L-lactic acid, polyglycolic acid, polyurethane, poly-ε-caprolactone, cellulose, chitosan, and polyvinyl alcohol together with respective composites have been attempted for such grafts. Nevertheless, at present none of these materials can be successfully used to produce small-diameter grafts.

Bombyx mori (*B. mori*) silk fiber has a long history of use in textiles. Moreover, silk fiber has also been used as surgical suture for centuries due to its high strength and toughness.[4] Recently, there have been many reports of using silk fibroin as biomaterials.[5] In order to develop silk fibroin with appropriate properties for biomaterials,[6] a transformation method to produce recombinant silk protein has been developed using a transposon, *piggyback* as the transgenic (TG) technique.[7] Thus, an improvement of the properties of silk fibroin for biomaterials has been attained by incorporating suitable DNA sequences. This method has many advantages: 1) The TG silkworms are easy to handle and the larvae well adapted for artificial rearing. 2) The adult moths cannot fly away or live in the wild environment. 3) The expression of the recombinant proteins can be confirmed visually by fusion of Green Fluorescent Protein (GFP) as a marker protein.[8,9] *B. mori* silk fibroin contains two chains: fibroin heavy chain (H-chain; MW: 350 kDa) and light chain (L-chain; MW: 25 kDa).[10] Fibroin H-chain is united with L-chain and P25 in the silk gland cell and is secreted in the silk gland**.** In general, the mixing ratio of H- and L- chains in the cocoon is 1:1. The new gene that follows the fibroin gene is expressed as a new protein in the silk gland.

The cell-adhesive tripeptide sequence, RGD, which has been found in major protein components of extra-cellular matrix (ECM) such as fibronectin was the most intensively investigated cell-binding sequence.[11,12] By introducing the RGD sequence into the silk fibroin through covalent coupling or bioengineering techniques, better biomaterials have been developed with the modified silk fibroin because of the ability of RGD to promote cell attachment.[13] In fact, recombinant silk fibroins incorporating RGD in the sequence after cloning and expression from *E. coli* are now available as commercial products, e.g., Pronectin® F.[14] Kaplan's group has emphasized the potential of the RGD-coupled silk fibroin in tissue engineering. For example, silk fibroins coupled with RGD have been reported to increase osteoblast-like cell adhesion and expression of both alkaline phosphatase and osteocalcin.[15] The RGD-coupled silk fibroins can also support human bone marrow stromal cell attachment and are suitable for autologous bone tissue engineering.[16-18] In our previous studies,[19-23] we designed and produced several recombinant silk proteins incorporating the repeated sequences of RGD within the longer sequences ASTGRGDSPA by expression in *E. coli*. Recombinant silk fibroin films prepared on a plastic plate by cell culture or recombinant silk fibers after electrospinning were used for cell-adhesive experiments with Normal Human Dermal Fibroblasts (NHDF) or VERO cells. In addition, we constructed TG silkworms through the insertion of modified fibroin L-chain genes and prepared recombinant silks incorporating RGD.[7,24] In all cases, higher cell adhesion activities were obtained for the recombinant silk fibroin compared with wild type (WT) silk fibroin without incorporating RGD.

In this study, we incorporated another peptide sequence, YIGSR, derived from the laminin B1 chain into the L-chain or H-chain of the silk fibroin using TG techniques. The laminins are important components of the basement membrane and are composed of α-, β-, and γ-chains.[25, 26] Laminin can bind collagen IV, perlecan, and entactin and is thought to play a role in initiating the synthesis of the basement membrane.[27] It also contains an RGD sequence on

the α-chain and YIGSR on the β-chain, which facilitate cellular attachment.[28, 29] Unlike RGD, YIGSR does not interact with the integrin family of cell receptors but with the 67 kDa laminin binding protein.[27, 30] The introduction of the YIGSR into silk fibroins was performed as $TS(CDPGYIGSRAS)_8$ because multimeric forms of YIGSR strongly enhanced the activity of YIGSR in inhibiting tumor growth and metastasis.[31] Next, both peptide sequences, RGD and YIGSR, were incorporated into the silk fibroin. Thus, the two longer peptide sequences containing RGD and/or YIGSR were designed as follows.

YIGSR(8) : $TS(CDPGYIGSRAS)_8$

YIGSR(8)+RGD(8): $TS(CDPGYIGSRAS)_8\ (TGRGDSPAS)_8$

These peptide sequences were expressed as fusion proteins with the fibroin L-chain or H-chain and Enhanced GFP (EGFP) fusion in the TG silkworm to produce recombinant silk fibroin.[8, 9, 24] The fibroin-peptide-EGFP fusion protein was secreted by the posterior silk gland and was spun into the cocoon.

The use of these recombinant silk fibroins for the development of vascular silk graft with small diameter is significant because *B. mori* silk fibroin itself is known as a suitable material for vascular graft. In our previous studies,[5, 32,33] silk fibroin vascular grafts with 1.5 mm in diameter and 10 mm in length were prepared by repeated braiding and winding of the silk fibroin fibers. The grafts proved to be efficient through evaluation of long-term implantation in rat abdominal aorta.[34, 35] An initial evaluation of some properties of these recombinant silk fibroins relevant to their potential use in vascular graft is reported herein. The following results are included: the adhesive activities of mouse endothelial and smooth muscle cells, the migration activities of the endothelial cells *in vitro,* and the migration distance of the endothelial cell from anastomotic part of the vascular grafts coated *in vivo* with recombinant silk fibroins to rat abdominal aorta after implantation.

Experimental section

Design of oligonucleotides for cell-adhesive sequences

The design of the oligonucleotide of the sequence, RGD(8) was reported previously.[24] For the oligonucleotide of the sequence YIGSR(8) the design is shown in Figure 1. The synthesis of the oligonucleotides was performed by Hokkaido System Science Corp. (Sapporo, Japan). Plasmid pUC118 and *E. coli* DH5α, used for propagation and construction of plasmids, were obtained from Takara Bio Inc. (Shiga, Japan). The YIGSR(8) oligonucleotide was cloned into pUC118 and named as pUC118-YIGSR(8). The YIGSR(8) and RGD(8) oligonucleotides were ligated and cloned in the same way and named as pUC118-YIGSR(8) RGD(8). Restriction enzymes and ligase were purchased from Takara Bio Inc. Chemical reagents were provided by Wako Pure Chemical (Osaka, Japan), unless otherwise indicated.

We designed four transformation vectors to produce recombinant silk fibroins that contained either L-chain or H-chain cDNA with YIGSR(8) or YIGSR(8)RGD(8), and EGFP sequences: LcY, LcYR, HcY and HcYR, respectively (Figure 2). The L-chain or H-chain recombinants were prepared according to the previous papers.[8,24]

Production

The construction of the TG silkworm was performed by the method reported previously.[7-9, 36] TG insects were screened for the expression of DsRed2 or EGFP in the stemmata of embryos under a fluorescence microscope MZ16FA (Leica, Germany) equipped with a filter set for DsRed or GFP3 (Leica, Germany). The cocoons of TG silkworm was dissolved in 60% LiSCN at room temperature and dialyzed against the 20 mM Tris-HCl (pH 8.0) and 5 M urea for 2 days.[24] The concentration of the recombinant silk protein was determined by BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, USA). To validate the presence of the recombinant silk protein, SDS-PAGE and Western blotting with an anti-GFP antibody (Invitrogen, Life Technologies, USA) was performed. The fraction of the fused protein in the recombinant silk fibroin was calculated from the relative intensity of each band with Image J

(Image Processing and Analysis in Java) by comparison with the intensity of the purchased recombinant GFP (Clontech, Takara, Japan) as a control in the Western blotting. The fraction was very low for Hc-YR, and therefore we did not use Hc-YR for further experiments.

^{13}C solid state NMR observation

After removal of silk sericin from the cocoons by degumming treatment,[4] we obtained the silk fibroin fibers. ^{13}C solid state CP/MAS NMR experiments were performed on a Bruker Avance 400 spectrometer at a sample spinning rate of 8 kHz in a 4 mm diameter ZrO_2 rotor. The ^{1}H 90° pulse was 3μs and a 70 kHz rf field was used during CP and decoupling. A total of 10-20 K scans for the non-labeled sample were collected over a spectral width of 35 kHz with a recycle delay of 3 s. All spectra were obtained using a CP contact time of 2 ms and TPPM decoupling. The Ala Cβ region of the ^{13}C CP/MAS NMR spectra was expanded and decomposed by assuming Gaussian line shape.[19] The ^{13}C chemical shifts were calibrated indirectly using the adamantane methylene peak observed at 28.8 ppm relative to TMS (tetramethylsilane) at 0 ppm.[19,23]

Tensile strength

The breaking strengths (MPa) of wild type (WT) and recombinant silk fibers prepared here were measured by tensile testing machine (Auto-Graph AG-Xplus, SHIMADZU Co. Ltd. Japan) with 1kN lad cell at 23 ^{o}C and 50% relative humidity. Each single fiber diameter was determined by SEM and set to tensile testing machine on samples of 10 mm length. The breaking strength was measured as the highest stress value attained during the test. The rate of crosshead was 300 mm/min. Force and displacement measurements were acquired at 0.01 s intervals. Each value was the average of 5 measurements. The standard deviations were calculated with all data for each experiment.

In Vivo Long Term Safety Evaluation

We used the recombinant silk fibroins, LcY and HcY together with WT silk fibroin. Salt (NaCl) /water-derived silk fibroin sponges were prepared as previously. [37] Briefly, salt particles were sieved through 300 and 500 μm meshes and packed into a plastic syringe. After the syringe had been filled with aqueous solution of recombinant and native silk fibroin, the piston was depressed as quick as possible to avoid a conformational change from random coil to β-sheet. Air in the syringe was carefully eliminated. After the aqueous solution had been allowed to stand for 24 h, the silk/salt composites were formed. These were immersed in distilled water to remove the salt. The average pore size was 425 μm. All *in vivo* procedures were approved by the University of Tokyo Hospital (the cardiovascular medicine division). Rat subcutaneous implantation test was conducted as described by ISO 10993-6, Biological evaluation of medical devices – Part 6: Tests for local effects after implantation.[38] Four sponges with 5mm diameter ×2.5 mm thickness were implanted into four positions selected from the paravertebral muscle of one Japanese white SD male rat (body weight 200-280 g, 8-weeks-old). The animals were humanely sacrificed under ether anesthesia 7 months after implantation.

Cell adhesion activity and cell migration activity assays

The recombinant silk fibroin solutions were diluted by water to a concentration of 1% (w/v), and 400 μl solution was dispensed to each well of 24-well microplates.[20,24] The plates were incubated at room temperature for 1 h and the solution was removed afterwards. The micro plate was then dried at 50°C for 30 min. The silk-coated plates were treated with 100% methanol for 30 min in order to insolubilize the silk proteins. Mouse fibroblast strain Balb/c 3T3 cultured in DMEM medium and mouse endothelial cell strain TDK2 cultured in RITC80-7 medium were added to each well, respectively. The plates were kept at 37°C, 5%

CO_2 for 2 hours. Cultured cells were rinsed with PBS, and fixated with 3% paraformaldehyde. The fixated cells were stained with DAPI solution and counted with the BAIOREVO BZ-9000 microscope (Keyence, Japan).

The migration of mouse blood vessel endothelial cell strain TDK2 was studied according to the method reported by Fittkau et al. [39] The cell was grown confluently in well of 24-well microplates where the cover glass was set within the plates for cell migration activity assay experiments. The RITC80-7 medium was added to each well and then incubated. The cell confluent cover glass was put in the micro plate. Then the plates were kept at 33°C, 5% CO_2 for 60 hours. The cultured cells were rinsed with PBS, and fixated with 3% paraformaldehyde and then stained with DAPI. The pictures of the fixated cells were taken, and cells were counted with the BAIOREVO BZ-9000 microscope.

Implantation of silk fibroin vascular graft to rat abdominal aorta

Vascular graft bases comprising WT silk fibers were made by the double-russell knitting method (Fukui Warp Knitting Co., Ltd., Fukui, Japan) [34,35] The size of the graft base was 1.5 mm in diameter and 10 mm in length. To remove silk sericin from the graft bases completely, it was boiled at 95°C for 2 h in a soap solution containing 20% Na_2CO_3 and then rinsed several times with water. The boiling and washing procedure was repeated three times. In contrast, the aqueous solution of the recombinant silk fibroin prepared with a similar degumming method was used for coating the surface of the silk graft bases. The silk fibroin graft bases were put in a mixed aqueous solution of the silk fibroin and PGDE (poly(ethylene) glycol diglycidyl ether) and then freeze-dried. PGDE was removed by allowing it to diffuse into an aqueous solution, and the silk fibroin grafts with coating of the recombinant silk fibroin sponge were obtained. Details of the preparation had been reported in detail elsewhere. [35,40] The silk fibroin graft was implanted into the abdominal aorta of each rat. The recombinant silk fibroins including native silk fibroin were used for coating the graft bases by

silk sponges. The implantation experiments were performed with n=3 and therefore a total of 12 rats were used.

After 2 weeks, the ultrasonic pulse-echo technique was used to assess patency.[35] The vascular grafts were then excised from rats, and embedded in paraffin. The paraffin-embedded specimen was cut; 300 μm and 5-μm thick sections were removed at 300-μm intervals on each paraffin-embedded specimen. To evaluate the migration of vascular endothelial cells, each cross section of the graft was immunohistochemically stained with anti-CD31 antibody (BD Biosciences Pharmingen (Franklin Lakes, NJ, USA) and secondary biotin-tagged anti-mouse IgG antibody (Dako, Tokyo, Japan). The sections were observed with the BAIOREVO BZ-9000 microscope, and distances of endothelial cell migration were measured.

Results and discussion

Confirmation of expression of recombinant silk fibroins

To construct the TG silkworm, the plasmid DNA was injected into eggs of *B. mori w1-pnd* strain at the preblastodermal stage in each vector. TG silkworms were verified by eye by screening for DsRed2 or EGFP fluorescence in the following generations. The appearance of the silk gland obtained from the TG silkworm at the 5^{th} day of the 5^{th} larval stage observed under normal condition and GFP3 filter was summarized in Figure 3. The green fluorescence was clearly evident in the middle and posterior silk glands, indicating that each fusion protein was produced in silk glands and secreted into the gland lumen. No fluorescence was detected in any other tissue of the TG animals at any stage or in the silk glands of non-TG silkworms which produced only WT silk fibroin (data not shown). The appearance of the cocoons of the TG silkworm under GFP filter indicates that the fusion protein produced in silk glands was expelled into the cocoon. Among four kinds of recombinant silk proteins, it is clear that the fraction expelled into cocoon was remarkably low for the recombinant silk fibroin, Hc-YR, which incorporated both the fibronectin and laminin peptide sequences into the H-chain. To

detect the EGFP fusion proteins in the fibroin-cell adhesion region, Western blotting was performed with anti-GFP antibody as summarized in Figure 4. Based on the predicted amino acid sequence, the estimated molecular weight was 62 kDa for the L-chain-YIGSR(8)-EGFP complex protein (Lc-Y), 69 kDa for the L-chain- YGSR(8)RGD(8)-EGFP complex protein (Lc-YR), 60 kDa for the H-chain-YIGSR(8)-EGFP complex protein (Hc-Y) and 67 kDa for the H-chain- YIGSR(8)RGD(8)-EGFP complex protein (Hc-YR). A single band of anti-GFP antibody reactivity was evident at 60 KDa for the protein Lc-Y, at 65 KDa for Lc-YR, at 70 KDa for Hc-Y and at 80 KDa for Hc-YR . Thus, the values of Western blots for the complex proteins, Hc-Y and Hc-YR were slightly higher than the value expected based on the predicted amino acid sequences. Such a tendency was reported previously;[4,24] for example, a silk fibroin H-chain-EGFP fusion protein with a theoretically calculated molecular mass of 50 kDa had an apparent molecular mass of 60 to 63 kDa based on SDS-PAGE and Western blot analysis possibly due to posttranslational modification[4]. When WT silk fibroin was used, no band of antibody reactivity was observed, as expected. Thus, we can conclude that the recombinant silk fibroins were produced by the TG silkworms. We calculated the fraction of the peptide sequences in recombinant silk fibroins according to the method described previously.[24] As listed in Table 1, the fraction of the incorporated peptide sequence (in % by weight) in the silk fibroin was calculated as 6.2% for Lc-Y, 7.2% for Lc-YR, 3.2% for Hc-Y and 0.8% for Hc-YR. Thus when the peptide sequence was incorporated in the H-chain, the fraction tended to be smaller than in the case of the L-chain. In particular, the fraction was very low for Hc-YR, possibly due to the construction of vector or the positional effect of the insertion site in the genome. Therefore, we did not use Hc-YR for further experiments.

^{13}C CP/MAS NMR spectra

Figure 5 shows the ^{13}C CP/MAS NMR spectra of WT, Lc-Y, Lc-YR and Hc-Y silk fibroins. There are apparently no significant differences among these spectra. Only the position of

spinning side band is different. Thus, it is difficult to discuss the differences among these spectra from Figure 5. In our previous papers,[40-43] we reported that the Ala Cβ peak in the ^{13}C CP/MAS NMR spectra was sensitive to the secondary and higher order structures of silk fibroin. Therefore, we analyzed the Ala Cβ peak carefully. Figure 6 shows the expanded Ala Cβ region of WT silk fibroin. The peak could be deconvoluted into three components reflecting the local conformation and inter-molecular arrangements of the chains. The highest field broad peak at around 16.7 ppm was assigned to a distorted β-turn and/or random coil, which ass characterized by a large distribution in torsion angles around an average conformation of a type II β-turn. The other two peaks were assigned to β-sheet structure where the inter-molecular silk fibroin chain arrangement was different although the back-bone conformation was the same. There were small differences among these spectra. In order to delineate the differences more quantitatively, the difference spectra were obtained by subtracting WT silk fibroin spectrum (black) from the spectra of three kinds of recombinant silk fibroins (red). An increase in the fraction of distorted β-turn and/or random coil and a decrease in that of β-sheet were observed although these differences were very small. The fractions of crystalline parts and distorted β-turn and/or random coil were determined by peak deconvolution to be 71/29 for WT, 69/31 for Lc-Y and Lc-YR and 68/32 for Hc-Y. Thus, the incorporation of the peptide sequences into the silk fibroin chain caused a very small increase in the distorted β-turn and/or random coil fraction in silk fibroin together with the destruction of β-sheet structure. This tendency was independent of L-chain or H-chain. The ^{13}C solution NMR spectra of these silk fibroins were also observed, but we could not observe new peaks from these peptide sequences, $TS(CDPGYIGSRAS)_8$ or $TS(CDPGYIGSRAS)_8$ $(TGRGD\text{- }SPAS)_8$ other than the usual peaks from WT silk fibroin (Data not shown). This might be due to peak broadening by restricted motion of the incorporated peptide sequences in the silk fibroin chain.

Tensile strengths

No difference was observed among the surfaces of the different fibers in the SEM pictures. The fiber diameters were 11.5 μm for WT, 11.4 μm for Lc-Y, 11.8 μm for Lc-YR and 14.1 μm for Hc-Y. Thus, the diameter of Hc-Y fiber was larger than others. The tensile strength (MPa) was different among these silk fibroin fibers: 79 ±24 for WT, 66 ± 15 for Lc-Y, 58 ± 9 for Lc-YR and 49 ± 11 for Hc-Y. Thus, all these tensile strengths tended to be smaller than that of WT silk fibroin fiber. This result seems to be related to the small increase of the fraction of distorted β-turn and/or random coil of recombinant silk fibroins.

***In Vivo* Long Term Safety Evaluation**

The four sponges with 5mm diameter ×2.5 mm thickness prepared with each recombinant silk fibroin, LcY and HcY together with WT silk fibroin were implanted into four positions of the paravertebral muscle of each rat. The toxicities together with the biocompatibilities of the silk fibroins were tested. During 210 days after implantation, the weights of the rats increased smoothly, and no significant difference was seen among rats implanted with the silk fibroin sponges. After 210 days, one small white piece of silk sponge (with the size less than 1mm cm^3) remained and other three sponges disappeared for WT. For Lc-Y silk fibroin, the sponges were decomposed completely and no sponge was obtained. For Hc-Y silk fibroin, all of the silk sponges remained, but the size of each sponge was less than 1mm cm^3. The color was white. Thus, it is concluded that there are no safety problems with these recombinant silk fibroins.

Cell adhesion activity and cell migration activity assays

Figure 7(a) shows the number of attached vascular endothelial TDK2 cells after 2h-culture on silk fibroins. The number of initially attached vascular endothelial cells on the recombinant silk fibroins Hc-Y tended to be high compared with those from the tissue cultured plates

(TCP) or WT silk fibroin ($p < 0.05$). The number for Lc-Y was also relatively high, but the number for Lc-YR was the lowest among the recombinant silk fibroins. The lower value of Lc-YR could not be explained on the basis of fractional amount of the incorporated sequence, since the fraction of the incorporated sequence (in % by weight) in silk fibroin was determined to be 6.2% for Lc-Y, 7.2% for Lc-YR, and 3.2% for Hc-Y. Thus the presence of R; RGD(8) in L-chain prevented the adhesion of vascular endothelial cells. On the other hand, in the case of vascular smooth muscle p53LMACO1 cells, the number of initially attached cells increased for all recombinant silk fibroins compared with TCP or WT silk fibroin as shown in Figure 7(b). In addition, the number was independent of the fraction of the incorporated sequence. These results indicated that both vascular endothelial cells and smooth muscle cells initially attached more on the recombinant silk fibroin that incorporated the YIGSR sequences. It is known that vascular endothelial cells play a key role in blood vessel formation through differentiation and migration. Thus, among the recombinant silk fibroins, Hc-Y is the most suitable materials for vascular graft because the number of the vascular endothelial cells is always high although the fraction of the incorporated sequence is not so high. Figure 8(a) shows the pictures of migration area of vascular endothelial TDK2 cells after 60 h of culture on TCP, WT silk fibroin, and recombinant silk proteins, Lc-Y, Lc-YR and Hc-Y. The migration area on either recombinant silk fibroin was significantly higher than that on WT silk fibroin ($p < 0.05$). The vascular endothelial cells migrated the most on the recombinant silk fibroin, especially on Lc-Y, and Hc-Y migrated the second fastest. This tendency (Figure 8(b)) seems to be related to the data in Figure 6(a) although the result is reversed between Lc-Y and Hc-Y. Thus, the findings from these cell assays indicate that the repeated YIGSR sequences, which were genetically incorporated into the silk fibroins, improved the properties of the silk protein; specifically, these sequences resulted in increased target-cell adhesion and migration.

Silk fibroin vascular grafts transplant

In our previous studies,[28-31] silk fibroin grafts have proved to be efficient as vascular grafts

when implanted in the rat model. Specifically, individual small-vessel (1.5 mm in diameter, 10 mm in length) grafts were woven from silk fibroin thread and implanted into the abdominal aorta of rats. Patency of these fibroin grafts 1 year after implantation was significantly higher than that of ePTFE grafts used as a control (85.1% vs. 30%, $p < 0.01$). Based on immunostaining with antibodies against CD31 (cluster of differentiation 31) or against α-smooth muscle actin, endothelial cells and smooth muscle cells migrated into the fibroin graft shortly after implantation and organized into endothelial and medial layers, respectively. Sirius-red staining revealed that the fibroin content in the grafts decreased as the collagen content increased. Thus, in this work, silk fibroin grafts, each 1.5 mm in diameter and 10 mm in length, were prepared; specifically, double-raschel knitted silk fiber grafts were dipped in an aqueous solution containing fibroin and PGDE, which acted as a porogen-leaching reagent.

The silk vascular grafts coated with WT and recombinant silk fibroin were implanted into rat abdominal aota.[30] The implanted vascular grafts were taken out 2 weeks after the transplantation. The results are summarized in Table 2. Eight rats had patency at 2 weeks. Only the data on the migration of the endothelial cell are listed because there are no significant difference in the histological studies among the silk fibroin grafts coated with WT and recombinant silk fibroins. The migrating distance of endothelial cell on the TG silk coated vascular grafts tended to be longer than that of the WT silk fibroin although the predominance was not high. Just as the *in vitro* cell adhesive and migration activity, an increase in the cell migration activity is expected when the amount of expression increases.

Conclusions

In this work, we prepared four kinds of recombinant *Bombyx mori* silk fibroins that incorporated the laminin peptide sequence $TS(CDPGYIGSRAS)_8$ or the combination of two sequences, $TS(CDPGYIGSRAS)_8$ and fibronectin peptide sequence, $(TGRGDSPAS)_8$ into the

light (L)-chain or heavy (H)-chain. The adhesive activities of mouse fibroblasts and endothelial cells tend to increase in the TG silk fibroins relative to WT silk fibroin. In particular, the TG silk fibroins that incorporated the laminin peptide sequence into the H-chain seem to be the most suitable material for vascular graft.

We already reported that WT silk fibroin itself was excellent material for vascular graft when we used it for rat implantation[32,34] and therefore it might be difficult to show the superiority of TG silk fibroins compared with WT silk fibroin in rat implantation experiment. However, it is important to point out that TG silk fibroin is non-toxic for rat when we use it as a part of silk vascular graft although *in vitro* experiment shows non-toxic for mouse endothelial cell and smooth muscle cell. And the TG silk fibroin might be more useful in dog implantation experiment compared with WT silk fibroin. Thus we believe that it is important to have many kinds of silk fibroins as a possible candidate for use in vascular graft. We are planning to prepare silk vascular graft base with these recombinant silk fibers and do animal implantation experiment.

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Table 1 Fraction of the peptide sequences, Y or YR, in recombinant silk fibroins evaluated according to the method reported previously.[20]

	Molecular Weight	Weight %
Lc-Y	62.23kDa	6.2
Lc-YR	69.29kDa	7.2
Hc-Y	60.05kDa	3.2
Hc-YR	66.89kDa	0.8

Lc: Light chain Hc: Heavy chain Y: $TS(CDPGYIGSRAS)_8$
YR: $TS(CDPGYIGSRAS)_8(TGRGDSPAS)_8$

Table 2 Patency and cell migration distances from anastomotic part after implantation to rat abdominal aorta. The silk vescular grafts were prepared by coating with several recombinant fibroins on the double-raschek knitted silk fiber grafts.

	WT	Lc-Y	Lc-YR	Hc-Y
Patency	3/3	3/3	2/3	3/3
Cell migration distance(μm)	650±295	750±251	600±245	800±245

Lc: Light chain Hc: Heavy chain Y: $TS(CDPGYIGSRAS)_8$
YR: $TS(CDPGYIGSRAS)_8(TGRGDSPAS)_8$

Figure Captions

Figure 1 The designed TS(CDPGYIGSRAS)$_8$ sequence, which was incorporated into the fibroin genes of transgenic silkworms. The repeated sequence YIGSR is underlined. Details are given in the text.

Figure 2 The simplified schematic structures of A :wild type silk fibroin with H- and L-chains, B and C : recombinant silk fibroins incorporating Y and YR with GFP tag in the L-chain, respectively and D and E : recombinant silk fibroins incorporating Y and YR with GFP tag in the H-chain, respectively. Y: TS(CDPGYIGSRAS)$_8$
YR: TS(CDPGYIGSRAS)$_8$(TGRGDSPAS)$_8$

Figure 3 The appearance of the cocoons and silkglands from Lc-Y , Lc-YR, Hc-Y and Hc-YR silkworms under normal condition (1 st and 3 rd columns) and GFP filter (2 nd and 4th columns), respectively. Lc: Light chain Hc: Heavy chain Y: TS(CDPGYIGSRAS)$_8$
YR: TS(CDPGYIGSRAS)$_8$(TGRGDSPAS)$_8$

Figure 4 Western blotting analyses of recombinant silk fibroins from Lc-Y , Lc-YR , Hc-Y and Hc-YR silkworms, using anti-GFP antibody and wild type silk fibroin together with GFP. The standard molecular weights are also shown at the left sides.

Figure 5 ^{13}C CP/MAS NMR spectra of A: wild type silk fibroin fibers together with recombinant silk fibroin fibers, B: Lc-Y , C:Lc-YR and D: Hc-Y. The asterisks denote the spinning side bands.

Figure 6 Expanded Ala Cβ region of ^{13}C CP/MAS NMR spectra of wild type silk fibroin fiber (black) together with recombinant silk fibroin fibers (red), Lc-Y , Lc-YR and Hc-Y.

The difference spectra are also shown. The fraction of crystalline and distorted β-sheet/random coil was determined to be 71/29 for WT, 69/31 for Lc-Y, 69/31 for Lc-YR and 68/32 for Hc-Y after peak deconvolution assuming Gaussian line shape.

Figure 7 Adhesive activities of (a): mouse endothelial cell and (b): smooth muscle cell on TCP (Tissue Culture Plate), WT silk fibroin, recombinant silk fibroins from Lc-Y, Lc-YR, and Hc-Y, respectively.

Figure 8 Migration activities of mouse endothelial cell on TCP (Tissue Culture Plate) , WT silk fibroin, recombinant silk fibroins from Lc-Y, Lc-YR and Hc-Y. The upper pictures (a) show selected pictures of the diffusion of the cells from the end of dish glass. The migration rates are summarized in histogram (b).

Table of Contents text

Transgenic silk fibroins that incorporated the laminin sequence were prepared. The adhesive activities tend to increase in the TG silk fibroins relative to WT silk fibroin.

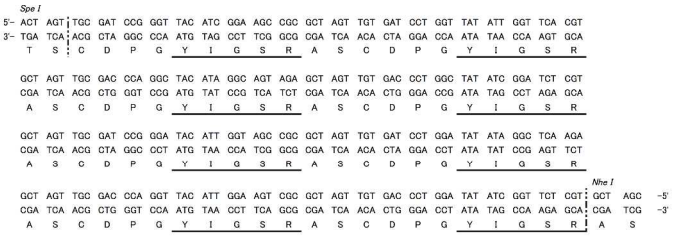

Figure 1

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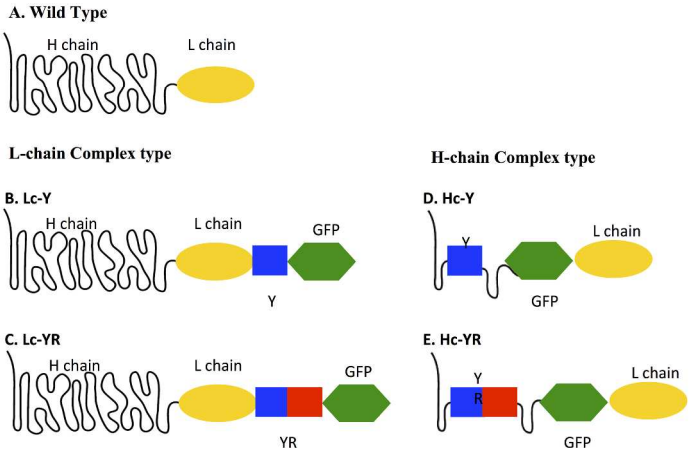

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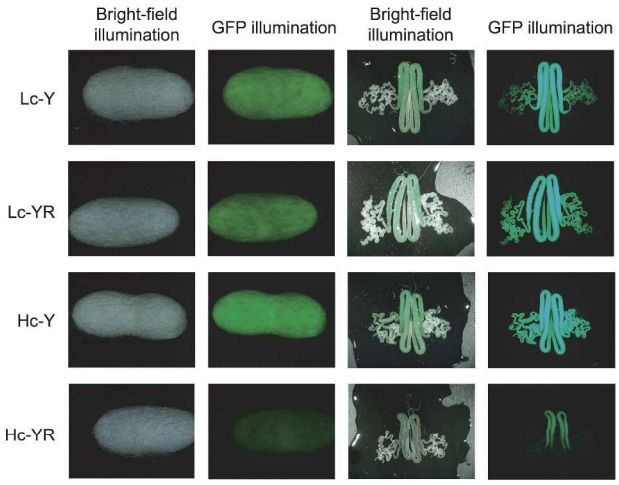

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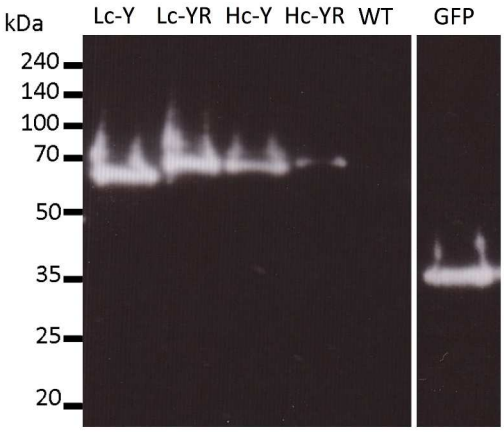

Figure 4

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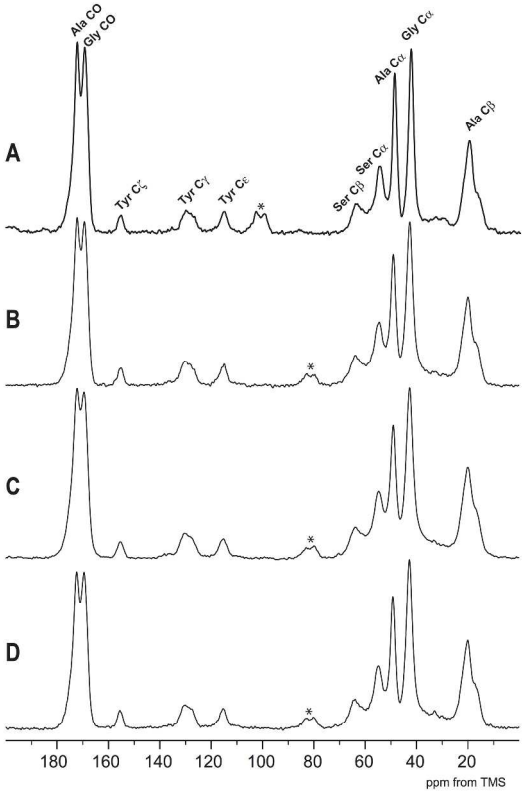

Figure 5

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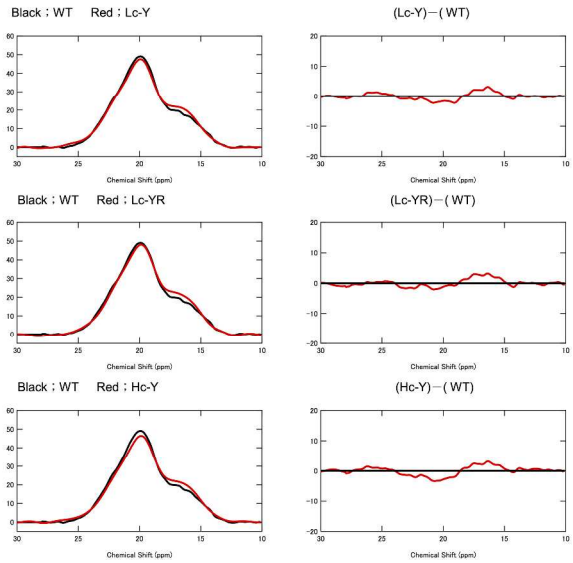

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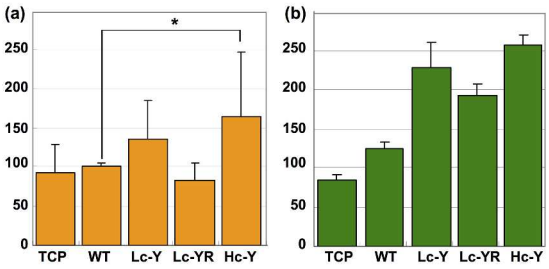

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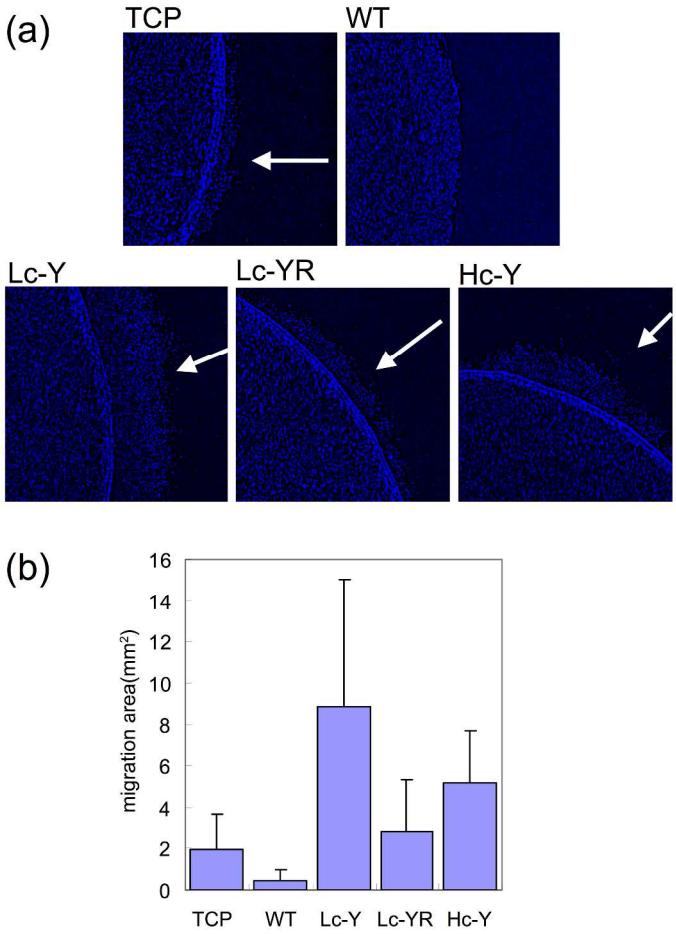

Figure 8

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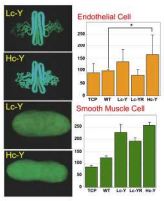

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