Improving Cell-Adhesive Properties of Recombinant *Bombyx mori* Silk by Incorporation of Collagen or Fibronectin Derived Peptides Produced by Transgenic Silkworms

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Silk of *Bombyx mori* can be used as various biomaterials. Especially, it is useful as a protein for coating the surface of cell culture plates since the silk possesses a biocompatibility to the cultured cells. However, the cell-adhesive ability is weaker than collagen or fibronectin, which are used for coating the plate more frequently (Yao et al. *J. Biochem.*, **2004**, *136*, 643−649). To increase the biocompatibility of the silk, we constructed transgenic silkworms, inserting the modified fibroin light-chain genes for making recombinant silks that possessed partial collagen or fibronectin sequences, that is, $[GERGDLGPQGIAGQRGVV(GER)_3GAS]_8GPPGPCCGGG$ or $[TGRGDSPAS]_8$, respectively. Films were made from the recombinant silks, and the cell-adhesive activity for cultured mammalian cells was observed. The results showed that the two types of recombinant silk films possessed a much higher cell-adhesive activity as compared to the original unmodified silk. Especially, the recombinant silk with the sequence $[TGRGDSPAS]_8$, produced by a transgenic *Nd-s*^{D} mutant, gave a 6 times higher activity than the original unmodified silk.

Introduction

Silk is a natural protein fiber produced by the silkworm, *Bombyx mori*, and contains mainly two components, fibroin and sericin, which are synthesized in the posterior and middles silk glands, respectively.[1] Silk fibroin consists of a fibroin heavy (H)-chain with a molecular weight of 350 kDa, a 25 kDa light (L)-chain, and 25 kDa P25/fibrohexamarin (FHX). Usually, the molar ratio between fibroin H-chain and fibroin L-chain is 1:1, and the two chains bind with a S−S bond.[2]

Silk fibroin has been used not only for textiles but also for biomaterials.[3,4] Since the biocompatibility of silk for mammalian cells is high, it has been used for many purposes. For example, the suture made from the silk has been used for a long time.[4] Sponge made from silk has been used as a scaffold for in vitro cartilage regeneration.[5] Similarly, silk can also be applied as a ligament to regenerate human bone tissues.[3,6] It is also used as an organic polymer for drug delivery.[7,8] In addition, silk protein is known to increase cell-adhesive activity when it coats the surface of plates for mammalian cell cultures. However, the biocompatibility of the silk is not enough for these purposes, and therefore, an improvement of the silk character is required.[9,10] Until now, peptides with amino acid sequences that consist of a crystalline domain of silk proteins and active sites in some proteins have been produced by *Escherichia coli*.[9−14] However, the cost for the production of such a silk-like peptide is high, and it is difficult to produce it in a large scale.

In 2000, the germ line transformation method for the silkworm was developed using the transposon *piggyBac*.[15] There are many advantages of using transgenic silkworms for the production of recombinant proteins. It is easy to handle large numbers of transgenic silkworms because the rearing system was improved by using mulberry leaves and an artificial diet, the adult moths are unable to fly, and the silkworms are unable to live in nature. In our previous experiments,[16,17] we reported that proteins fused with fibroin L-chain and GFP or procollagen can be produced in posterior silk gland and expelled in the cocoon silk. This suggest that another fusion protein can be expressed using this method and that it would be possible to improve the properties of *B. mori* silk. Since the fibroin H-chain and fibroin L-chain bind with a S−S bond, we can modify the character of silk by introducing the new gene fused with the fibroin L-chain gene. In this case, the fibroin complex of the H-chain, L-chain, and FHX is produced in the posterior silk gland and moves to cocoons. On the other hand, several distinct sequences derived from collagen and fibronectin already have been reported as cell-adhesion sites.[3,9,10] Particularly, the portion of peptide sequences in collagen and the tripeptide RGD found in fibronectin have been shown as a sequence with cell-adhesive activity, suggesting that adding these peptides increases the cell-adhesion activity of the *B. mori* silk.

In this study, we performed an experiment to change the *B. mori* silk character by introducing amino acid sequences that consist of active sites of collagen or fibronectin proteins. One of these peptides is abbreviated as **Coll-F**, which is the recombinant sequence $[GERGDLGPQGIAGQRGVV(GER)_3GAS]_8$ from several types of collagen, and the cross-linking domain of collagen III, GPPGPCCGGG, as described in detail in our previous paper.[9] Another peptide $[TGRGDSPAS]_8$ was prepared from cell-adhesive sequences from

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fibronectin and is abbreviated as **RGD** in this paper. The sequence, TGRGDSPAS, instead of the tripeptide sequence, RGD, was selected here because the former sequence has been reported to maintain a high activity in human lysozymes.[10] The results indicate that it is possible to change the cell-adhesive character of silk fibroin by introducing these peptide sequences. The *Nd-s* mutant especially was useful to increase the amount of introduced peptide and cell-adhesive activity of recombinant silk.

Materials and Methods

Construction of Vectors. The DNA sequence of **Coll-F** reported elsewhere[9] was used. Single-chain DNA corresponding to the TGRGDSPAS sequence of fibronectin was used from the following synthesized single-chain DNAs: 5′-CTAGTACCGGCCGTGGT-GATTCTCCGG-3′ and 5′-CTAGCCGGAGAATCACCACGGCCGGTA-3′ (Operon Biotechnologies). Then, the DNA was annealed and inserted into the *Spe*I and *Nhe*I sites of modified pUC118. The construction of the repeat sequences of the **Coll-F** and **RGD** DNA fragments was performed using the method reported elsewhere.[10] The DNA fragments with repeated **Coll-F** or **RGD** were subcloned into the plasmid pET30a (Novagen Inc.) and transformed into the *E. coli* strain BL21(DE3)pLysS.

For the production of the transgenic silkworm, the plasmids pigFiLp**Coll-F**GFP/3xP3DsRed and pigFiLp**RGD**GFP/3xP3DsRed were constructed using the repeated **Coll-F** and **RGD** DNA sequences of the plasmids of pET30a. The two oligonucleotides, 5′-GATCCCAC-TAGT-3′ and 5′-CCGGACTAGTGG-3′, were used as linkers for constructing the *Spe*I restriction site, which was used to introduce the **Coll-F** or **RGD** sequences into the *Bam*HI and *Age*I sites of pBac-FiLpGFP/3xP3DsRed[16] and the *Spe*I−*Nhe*I fragment of pUC118 with repeated **Coll-F** or **RGD** sequences into the *Spe*I site of pigFiLpGFP/3xP3DsRed.

Production of Coll-F and RGD Peptides by *E. coli*. Larger scale cultivation by *E. coli* was performed in 2.4 L TB medium using a fermenter (Marubishi Bio Eng. Japan), and the expression of the recombinant proteins was induced with IPTG. The **Coll-F** or **RGD** peptides were purified with nickel-chelate chromatography, which was examined by SDS-PAGE and Western blotting analyses to identify the expression.[9]

Construction of Transgenic Silkworm. Construction of the transgenic silkworm was performed by the method reported elsewhere.[15,16] Transgenic insects were screened for the expression of DsRed in the stemmata of G_1 embryos. The fluorescence of DsRed and GFP of the silk gland was observed under a fluorescence microscope (Leica) equipped with a filter set for DsRed and GFP, respectively.

SDS-PAGE and Western Blotting of Silks. Recombinant silk of the transgenic silkworm was dissolved in 60% LiSCN for 30 min at 37 °C and dialyzed against the 20 mM Tris-HCl (pH 8.0) and 5 M urea for 3 days. The concentration of the silk protein was determined by a BCA Protein Assay Kit (PIERCE). SDS-PAGE was performed using a 12.5% polyacrylamide gel (DRC), and the separated proteins were stained with Coomassie Brilliant Blue. Blotting of the separated protein was performed using TBST (10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 0.1% (v/v) Tween 20) containing 5% (w/v) skimmed milk and reacted with the GFP antibody. Proteins that reacted with the antibody were detected by the ECL Plus Western Blotting Detection System (Amersham Biosciences). The intensity of each band was calculated with KODAC 1-D Image Analysis Software (KODAC) in comparison with the strength of the purchased GFP (Clontech Laboratories Inc.) as a control.

Removal of Silk Sericin from Cocoon. The cocoons obtained from transgenic silkworms were boiled for 20 min in 0.5% (w/v) Ivory soap solution and then rinsed several times with distilled water to remove the sericin. Boiling for 20 min and washing were repeated. The silk fibroin was then dissolved in 9 M LiBr by incubating for 1 h at 37 °C and adjusted to 5% (w/v) solution. The solution was dialyzed against distilled water for 3 days, and the protein concentration was determined by a BCA Protein Assay Kit (PIERCE).

Construction of Homozygous *Nd-s^D* Mutant Strain with the Fusion Gene of *L fibroin-GFP-RGD*. The homozygous *Nd-s^D* strain with the fusion gene of *L fibroin-GFP-RGD* was constructed by backcrossing the transgenic line with the homozygous *Nd-s^D* strain. The presence of *L fibroin-RGD-GFP* in F_1 and BF_1 was detected by observing the expression of DsRed in stemmata of embryos and larvae. The homozygous *Nd-s^D* silkworms in BF_1 were detected by PCR of the genomic DNA extracted from the hemeolymph of BF_1 larva without killing since *Nd-s^D* possessed the deletion in fibroin L-chain gene (R). For the identification of the normal gene, the primers 5′-TCGCTGC-CTACATAACCGCTCACTTACTTC-3′ and 5′-TTGCCAACGCCTCT-CAGG-AGCTGTGGATAA-3′ were used. For *Nd-s^D*, the primers 5′-AAATATTTATTA-CCCGAAACTAAGA-3′ and 5′-TCAATACGC-GACATTTATCTCTG-3′ were used.

Cell-Adhesion Activity Assay. A mouse fibroblast cell line, BALB/3T3, was provided from the Riken Cell Bank. The recombinant peptides, **Coll-F** and **RGD**, produced from *E. coli* were dissolved in PBS at a concentration of 1 mg/mL and then 500 μL of the solutions was added to each well of 48-well microplates. The peptides were left to stand for 30−60 min and were absorbed on the plates. Then, the plates were dried at room temperature for 12−24 h. Then, 500 μL of BALB/3T3 cells was trypsinized from the culture flasks (Nalge Nunc International) and then washed in Eagle's MEM medium (Nissui Pharmaceutical Co. Ltd.) with 10% fetal bovine serum (FBS; Gibco Invitrogen Co.) to prepare the cell suspension. Finally, the cell density was adjusted to 1.1×10^5 cells/mL in Eagle's MEM medium with 10% FBS. A total of 500 μL of the cell suspension was put in the each well, and the sample films were placed. The initial cell density was 5×10^4 cells/cm^2. Cell culturing was performed at 37 °C and 5% CO_2. After 2 h of incubation, the samples were washed twice with PBS to remove unattached cells, and then the samples were put in 0.5% Toriton X-100/PBS solution for cell counting by Lactate dehydrogenase (LDH) consumption.[18] The LDH activity of the dissolved solution was measured using the kinetics of NADH-consuming reactions at 340 nm absorbance.

Results

Cell-Adhesion Activity of Coll-F and RGD Peptides Produced by *E. coli*. For the production of the two peptides, **Coll-F** and **RGD**, we used the pET30a plasmids into which the DNA sequences corresponding to **Coll-F** and **RGD** (Figure 1A) were inserted. Therefore, the peptides produced possessed a His tag in both ends, and some additional peptides were derived from the vector plasmid. The two peptides purified using the nickel-chelate resin column were used to coat the surface of the plastic cell plate. The adhesion activity against the mouse fibroblast cells was observed and is shown in Figure 1B. The two peptides possessed cell-adhesive activity comparable to the corresponding pure collagen (type I) and fibronectin.

Construction of Transgenic Silkworm. To produce the silks that possess a high cell-adhesive activity, we constructed a transgenic silkworm that produces the recombinant silk with the peptide in the fibroin L-chain. In our previous reports,[16,17] we showed that the recombinant protein fused with the fibroin L-chain is produced in the posterior region of the silk gland, secreted into the lumen, and then expelled in the silk of cocoon. We designed the transformation vector pigFiLp**Coll-F**GFP/3xP3DsRed for the production of the recombinant fibroin that contained the fused gene with the fibroin L-chain cDNA, **Coll-F**, and GFP sequences (Figure 2A). We also constructed a similar vector containing **RGD** for the production of the silk-contained **RGD**. To construct the transgenic silkworm, we injected the plasmid DNA into about 900 eggs of w1-pnd strain

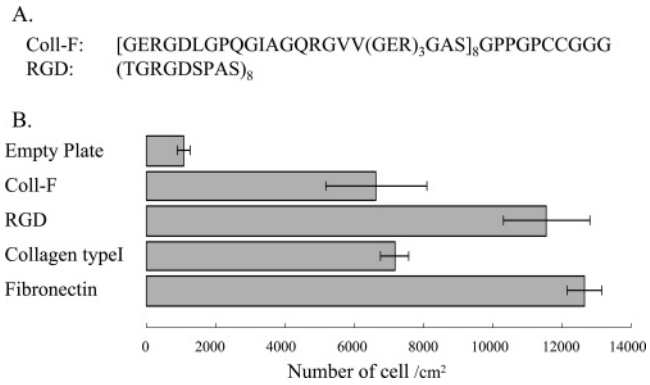

Figure 1. (A) Amino acid sequences of **Coll-F** and **RGD** peptides synthesized by *E. coli*. (B) Adhesive activity of peptides produced and commercially available proteins against the mouse BALB/3T3 fibroblast. The adhesive activity was determined by counting the number of cells cultured on plastic plates coated with the peptides produced. The bars are the average of five repeated experiments, and the standard error is shown by a line with vertical bar on both ends. Empty plate indicates the activity obtained using a non-coated plate. **Coll-F** and **RGD** show the activity obtained by using the coated plate with the **Coll-F** and **RGD** peptides, respectively, and collagen type I and fibronectin collagen type I indicate data obtained using the plate with purchased collagen type I and fibronectin proteins.

at the preblastodermal stage in each vector. We obtained one line that inserted the fusion construct of FiLpGFP**Coll-F** and three lines for FiLpGFP**RGD**. The green fluorescence in the posterior region of the silk gland and cocoon was observed in all transgenic silkworms (Figure 2B). The presence of the GFP fluorescence in the lumen of the silk gland and cocoon silk indicates that both fused proteins were produced in the silk gland, secreted into the lumen, and expelled into the cocoon silk. No fluorescence was detected in other tissues at any stages or the silk gland in the non-transgenic silkworm.

Characterization of Recombinant Silk Contained in Coll-F and RGD Peptides. To investigate the character of recombinant silk produced by the transgenic silkworms, we first performed SDS-PAGE and Western blotting. The silk proteins prepared from the cocoons by being dissolved in LiSCN were examined with SDS-PAGE after dialysis. The molecular weight of the fused fibroin L-chain was calculated from the fused genes; the fibroin L-chain is about 26 kDa, **Coll-F** is 24 kDa, **RGD** is 7 kDa, and GFP is 27 kDa. Therefore, the molecular weight of the fibroin L-chain-**Coll-F**-GFP fusion protein is 77 kDa, and the fibroin L-chain-**RGD**−GFP fusion protein is 60 kDa. As shown in Figure 3A, the protein corresponding to the previous molecular weights appeared as a band in the SDS-PAGE of the cocoon silk of the transgenic lines. The result of Western blotting against the anti-GFP antibody showed that the bands appearing in the SDS-PAGE reacted with the antibody (Figure 3B). The protein-contained GFP in the cocoon silk is only the fibroin L-chain fusion protein. Therefore, the protein shown by the arrows in the SDS-PAGE (Figure 3A) is the fusion protein expressed from the introduced gene of the transgenic silkworms. The silk prepared from the cocoon of the non-transgenic silkworm did not give the band observed in Figure 3 and possessed no reacted band with the antibody in Western blotting.

The cocoon silk contains two types of proteins: sericin and fibroin. The silk used for making textile mainly consists of fibroin, and fibroin is a complex of fibroin H-chain, L-chain, and P25/fibrohexamarine. To investigate whether the fusion protein produced by the transgenic silkworm is involved in fibroin or not, we performed Western blotting of the proteins extracted from the silk after removal of sericin. Although various

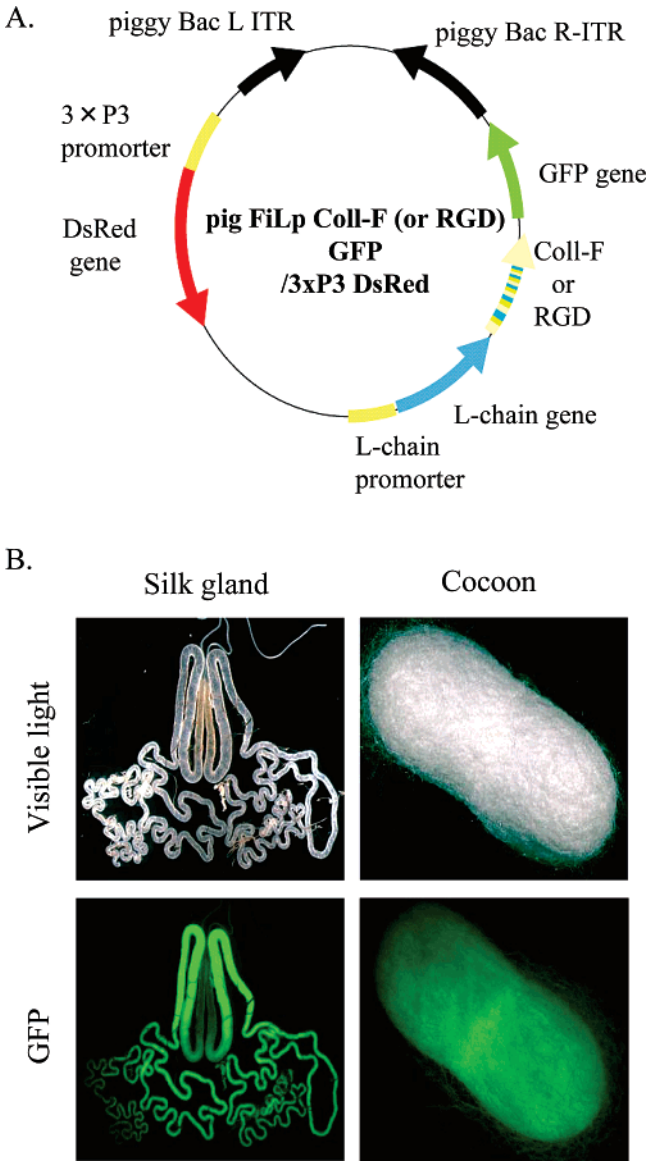

Figure 2. (A) Physical map of pBacFiLpGFPColl-F/3XP3DsRed and pBacFiLpGFPRGD-/3XP3DsRed. (B) Pair of silk glands at the fifth day of fifth instar larvae and cocoon of the transgenic silkworm with the FiLpGFPRGD gene inserted. The silk glands and cocoons are shown under bright field (upper), and the fluorescence of GFP is observed under the fluorescent stereo microscope equipped with a GFP filter (lower). Emission of the fluorescence shows the production and secretion of the recombinant protein in the silk gland and cocoon in the transgenic silkworm.

methods to remove sericin are reported, we used a method to remove sericin by boiling the cocoon in soap solution because no decomposition of the fibroin L-chain occurs by this treatment.[15] The recombinant silks after removing the sericin were dissolved in 9 M LiBr solution, dialyzed, and subjected to electrophoresis. The results of Western blotting of the silks produced by the transgenic silkworms with the fusion gene of **Coll-F** or **RGD** to the anti-GFP antibody indicate that specific bands with the molecular weight corresponding to the introduced fusion gene could be detected in both recombinant silks (data not shown).

To investigate the cell-adhesion activity of the recombinant silks, the silk-removed sericin was prepared and used to prepare the film to test the cell-adhesive activity. The cell-adhesive activity of recombinant silk is summarized in Figure 4. The average number of cells on the original unmodified silk was

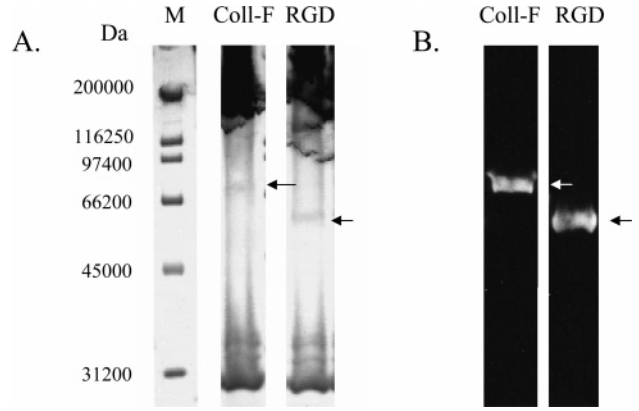

Figure 3. (A) SDS-PAGE and (B) Western blotting of recombinant silk proteins produced in the transgenic silkworm with the *FiLpColl-FGFP* (Coll) or *FiLpRGDGFP* (RGD) gene. The cocoon proteins were separated by SDS-PAGE after being dissolved with 60% LiSCN solution and subjected to Western blotting with anti-GFP antibody. Lane M is the molecular weight marker protein. The arrow shows the fibroin L-chain **Coll-F**-GFP and fibroin L-chain **RGD**-GFP fusion proteins.

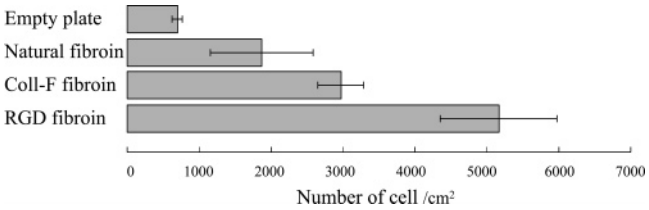

Figure 4. Comparison of cell-adhesive activities of the recombinant silks. The activity was measured using the films made by the silks produced by non-transgenic (natural fibroin), *FiLpColl-FGFP* (Coll-F fibroin), or *FiLpRGDGFP* (RGD fibroin) inserted transgenic silkworms to the mouse BALB/3T3 fibroblasts. Empty plate indicates the cell numbers growing on the surface of the non-coated plate.

about 2000, while the number on the empty plate hole was about 500. The result indicates that the original unmodified silk possessed a certain level of cell-adhesive activity. On the other hand, the recombinant silks introducing **Coll-F** or **RGD** gave a much higher activity as compared to the empty plate and original unmodifiedl silk, as shown in Figure 4. We concluded from the result that the introduction of **Coll-F** and **RGD** in the fibroin L-chain is effective in increasing the cell-adhesive activity of the silk. In comparison with the two recombinant silks, the silk-introduced **RGD** gave about twice as much activity as compared to the silk-introduced **Coll-F**. A similar result was already displayed in the two peptides produced by *E. coli* as described previously (Figure 1B). Thus, the introduction of **RGD** is more effective than **Coll-F**. However, the cell-adhesive activity in the **RGD** recombinant silk is lower than in the commercial fibronectin or **RGD** produced by *E. coli*. We estimated that the lower value might be due to the lower ratio of **RGD** fused L-chain in the recombinant silk.

Characterization of RGD Recombinant Silk Produced in the *Nd-s^D* Mutant. To increase the ratio of the **RGD** fused L-chain in recombinant silk, we backcrossed the transgenic silkworm to the *Nd-s^D* mutant. The presence of the FiLp**RGD**GFP construct was confirmed by the expression of GFP in the posterior region of the silkworm or in the cocoon. The genotype of *Nd-s^D* was determined by PCR using genomic DNA. After two repeated backcrosses, the fusion gene was introduced into the homozygous *Nd-s^D* mutant. The *Nd-s^D* mutant fibroin L-chain cannot form a S−S linkage with the fibroin H-chain because the mutant possesses a deletion in the

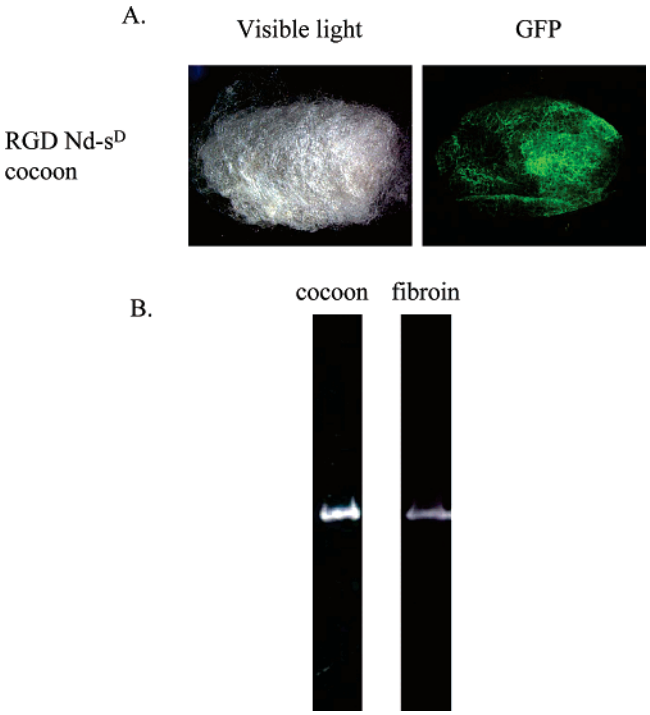

Figure 5. (A) Cocoon of *Nd-sD* homozygous transgenic silkworms with the *FiLpColl-FGFP* gene under visible light and a fluorescent stereo microscope. (B) Western blotting of the fibroin L-chain **RGD**-GFP fusion protein in *Nd-s^D* homozygous transgenic silk before (cocoon) and after (silk) removing sericin with the anti-GFP antibody.

Table 1. Amount of Recombinant Silks Contained in Cocoon Silk of Transgenic Silkworms[a]

	molecular weight (kDa)	wt % (in cocoon)	wt % (in fibroin)
Coll-F	77	0.2	0.2
RGD	60	0.6	0.9
RGD *Nd-s^D*	60	0.4	3.4

[a] Weight percentages of recombinant silks were calculated from the intensity of the band that appeared in Western blotting.

fibroin L-chain gene and produces the mutant fibroin L-chain protein with no linkage site. Therefore, the mutant cannot secrete the fibroin in the lumen of the silk gland and cocoon. As shown in Figure 5, the cocoon produced by the transgenic silkworm is rather poor. However, strong fluorescence of GFP was observed in the cocoon, suggesting that the fused protein is contained in the cocoon silk. To confirm the presence of the fused protein in the silk, we studied the silk by Western blotting. As shown in Figure 5B, the fibroin L-chain fused with GFP and RGD was detected in the cocoon silk and the silk after sericin was removed. The amount of the fused protein was determined by comparing the strength of the bands observed in Western blotting. The fusion protein in cocoon silk was 0.4% (Table 1). This value was lower than that of the production of the FiLp**RGD**GFP construct in the normal strain. This probably is caused by a higher percentage of sericin in the *Nd-s^D* mutant cocoon. Actually, the percentage of the fusion protein in the silk after the removal of sericin was much higher as compared to the silk produced in the normal transgenic silkworm (Table 1). The cell-adhesion activity was measured using the silk made by the mutant transgenic silkworm (Figure 6). The value obtained was 6 times higher than that of natural original unmodified silk and 2 times higher than that of silk produced by the normal **RGD** transgenic silkworm.

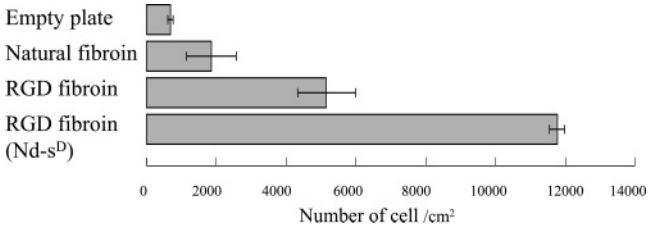

Figure 6. Comparison of cell-adhesive activities of the recombinant silks produced by non-transgenic (natural fibroin), ordinal transgenic silkworm with *FiLpRGDGFP* gene (**RGD** fibroin), and *Nd-s^D* homozygous transgenic (**RGD**) fibroin (Nd-sD) silkworms with the same gene. The activity was measured using the films made by the silks to the mouse BALB/3T3 fibroblasts. Empty plate indicates the results of non-coated plastic GE.

Discussion

The possibility and advantages of the transgenic silkworm in the production of recombinant protein have been reported previously.[16,17] In these cases, the fusion gene of fibroin L-chain cDNA and collagen or GFP were introduced, and the fibroin L-chain gene promoter was used for the expression of the introduced gene. In our experiment, a similar construct was used for making the transgenic silkworm. **Coll-F** and **RGD** sequences were connected to the position near the 3′ end of the fibroin L-chain cDNA and then followed by the GFP gene. The addition of GFP enables the confirmation of the production and secretion of the fused protein by stereo fluorescent microscopy equipped with a GFP filter. The constructed fusion genes of fibroin L-chain cDNA-**Coll-F**-GFP (*Fibp-Coll-F-GFP*) and cDNA-**RGD**-GFP (*FibLp-RGD-GFP*) were under the control of the fibroin L-chain gene promoter. We successfully constructed the transgenic silkworms that inserted the recombinant gene and the silkworms that produced the fusion protein in the posterior silk gland cells. The fusion protein produced was secreted into the lumen of the posterior silk gland. Then, the protein was moved to and accumulated into the lumen of the middle silk gland. The amounts of the fused proteins, fibroin L-chain-**Coll-F**-GFP and L-chain-**RGD**-GFP, of the cocoon filament were 0.2 and 0.6, respectively. The cocoon filament generally contained about 25% sericin and 75% fibroin. The fibroin molecule consisted of fibroin H-chain, L-chain, and FHX in a molar ratio of 6:6:1. The molecular weights of the fibroin H-chain, L-chain, and FHX were about 400, 30, and 25 kDa, respectively. From these values, the fibroin H-chain and fibroin L-chain occupied about 5% of the cocoon filament proteins. The amounts of **Coll-F** or **RGD** fused proteins in the silk were about 0.2 and 0.6%, respectively. Since the molecular weights of the fusion proteins, fibroin L-chain-**Coll-F**-GFP and fibroin L-chain-**RGD**-GFP, are 75 and 60 kDa, the molar ratio of the former corresponds to only about 2% fibroin L-chain in the modified silk, and the latter represents only 4%. Apparently, the level of expression of the introduced modified fibroin L-chain was much lower than the endogenous fibroin L-chain gene.

The measurement of cell-adhesive activity was performed using films made by the silks produced by the transgenic silkworm. Although the proportion of **Coll-F**-fibroin or **RGD**-fibroin in the silk produced by the transgenic silkworm was low, a large increase was observed in the recombinant silk as compared to the original unmodified silk produced by non-transgenic silkworms. Namely, the cell-adhesive activity of the film made from the silk-contained **Coll-F** fibroin is much higher than that of the natural silk but lower than that of **RGD** silk. The difference in cell-adhesive activity between films made by silk-contained **Coll-F** and films made by silk-contained **RGD** fibroin is probably dependent on the difference in the amounts of **Coll-F** and **RGD** proteins in the recombinant silks.

According to Inoue et al.,[16] the production of the modified fibroin L-chain protein can be increased by introducing the gene into the *Nd-sD* mutant, whose L-chain is deleted from the middle of the third exon. Therefore, the mutant L-chain cannot form the S−S linkage between the fibroin H-chains. Since the formation of the S−S linkage between the fibroin L-chain and the fibroin H-chain is required for the production of the fibroin complex and secretion into the lumen, the mutant cannot secrete the fibroin into the lumen. The modified **Coll-F**-fibroin and **RGD**-fibroin L-chain gene contained the deleted region of the fibroin L-chain gene in the mutant. Therefore, the fused protein was thought to be able to form a S−S linkage with the fibroin H-chain, suggesting that the increase of modified fibroin can be achieved by introducing the gene into the *Nd-sD* mutant. In our experiment, the introduction of the **RGD** fusion gene into the mutant was performed with repeated backcrosses with the mutant strain, and the genotype of the mutant was determined by PCR. The homozygous *Nd-s^D* mutant possessing the **RGD** fibroin L gene produced much smaller amounts of fibroin as compared to that of the transgenic silkworms inserted with the same gene into the normal strain because the expression of the introduced gene was generally more than 10 times lower than the endogenous gene, and the amount of silk produced by the transgenic silkworm inserted **RGD** fibroin gene was highly dependent on the amount of expression of the introduced gene. However, the proportion of **RGD** fibroin occupied in the silk was greatly increased, and the silk showed a very high cell affinity, almost comparable to pure fibronectine or the **RGD** peptide produced by *E. coli.* Thus, the introduction of the **RGD** fibroin gene into the mutant was very effective to produce silk with a higher cell affinity.

The biggest advantage in using the transgenic silkworm for the production of biomaterials is the possibility of obtaining large amounts of the materials at low cost. To increase production, it is possible to create the transgenic silkworm with multiple copies of the recombinant genes. In addition, it is easy to rear large numbers of silkworms, even more than 10 000, and produce more than 1 kg of product. The fibroin created this time can be applied to various biomaterials. Sofia et al.[19] combined the RGD peptide with natural fibroin and used a bone rework R. By adding the **RGD** peptide, the affinity to a bone cell increases, and good results are obtained. Moreover, it has been reported that silk-like peptides that have a special function can be created using *E. coli*. However, by these methods, the operation of purification and ornamentation results in a high cost for obtaining a sample in large quantities. If the method in this research is used, producing materials in a large scale is possible, and the same processing as natural silk is possible. In addition, excessive procedures, such as purification and chemical modification, are unnecessary. The modified silk can be used directly. Although the cell-adhesive activity was improved, it is possible to add improve other aspects as well. For example, the intensity of thread, elasticity, color, stainability, and size of cocoon can be altered. It is expected that silks that have even more beneficial functions will be created.

Conclusion

Genetically modified silk that contained partial sequences of collagen (**Coll-F**) or fibronectin (**RGD**) was produced by introducing a newly constructed fibroin L-chain gene into the

silkworm. We first showed that both peptides produced by *E. coli* possessed a higher cell-adhesion activity than that of the original unmodified silk. Then, the silk produced by the transgenic silkworm also possessed a higher cell affinity. Particularly, the silk produced by the *Nd-s*^{D} mutant inserted **RGD** sequence showed a 6 times higher affinity than the original unmodified silk.

One advantage of using the transgenic silkworm is the ability to produce large amounts of the modified silk at low cost. Therefore, the silk obtained might be used for commercial purposes. For example, the price of raw silk fiber has been $20 to ~25/kg in Japan in recent years. If the modified silk fibers are produced by transgenic silkworms according to the usual sericultural process system, the cost for production of the modified silk can be evaluated as being at the same level as that of raw silk, $20 to ~25/kg. On the other hand, the commercial prices of fibronectin and collagen are $70 000 000/kg and $650 000/kg, respectively. Therefore, if we assume that the amounts of these cell-adhesive proteins are 0.5% of the modified silk fibers, we can evaluate that the cost to obtain such proteins is about $4000 to ~$5000/kg. Although we should include the cost for extraction and purification for obtaining the proteins, it is possible to obtain cheaper materials as compared to collagen and fibronectin. If we use these modified silk fibers as biomaterials to improve the cell-adhesive character of the original unmodified silk fiber, the process of extraction and purification is not necessary. In this case, cost saving is greatly enhanced.

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