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Design, Expression and Characterization of Collagen-Like Proteins Based on the Cell Adhesive and Crosslinking Sequences Derived from Native Collagens

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Two recombinant collagen-like proteins consisting of cell adhesion domains derived from native type I collagen were designed and synthesized by a genetic engineering method. The cross-linking sequence, GPPGPCCGGG, derived from collagen III was used to promote triple helix formation through the disulfide bonds formed among three chains by flanking the peptide at the C-terminal of the collagen-like proteins. SDS-PAGE and western-blotting data suggested possibility of the formation of a triple helix structure for both recombinant proteins. CD spectra and thermal stability analyses indicated that the triple-helix structure in the collagen-like proteins was pH-dependent and stabilized under acidic environmental condition. Moreover, the collagen-like protein flanked with the cross-linking sequence at the C-terminal showed the most stable triple-helical conformation under acidic conditions.

Key words: cell adhesion, collagen, cross-linking, genetic engineering, recombinant protein.

Collagen is the most abundant protein found in animals and is mainly associated with the extracellular matrix. As much as 25% of the protein in mammals is collagen. Collagen has been widely used in many medical applications, including tissue engineering and drug delivery materials, because of its strength and stability as well as its general compatibility with living tissues (*1*, *2*). Currently, the main source of collagen is cow skin. This source carries a high risk of contamination and can also cause allergic reactions (*3*). Thus, there is a need for alternative sources of collagen that can produce large quantities.

Fibrillar collagen comprises the largest and best characterized group, consisting of five collagen types: I, II, III, V and XI. The tensile strength of collagen fibrils depends on intermolecular cross-links among collagen molecules and on the fibril diameter (*4*–*6*). Collagen molecules have a central triple-helical domain consisting of an uninterrupted series of GXY triplets, and N- and C-terminal peptide domains. Type I collagen is well known as the major fibrillar collagen and was the first to be characterized. It is widely distributed in most organs, including skin, bone and muscle, in higher vertebrates. In type I collagen, X and Y can be any residues, but are frequently Pro and Hyp (hydroxyproline), respectively, which accounts totally for about 44% of the total tripeptide units, while GXY tripeptide units with no imino acid constitute the remaining 44% (*7*). In addition to providing the structure of connective tissue, type I collagen can mediate intracellular communication. Cell-collagen interactions play a role in a number of processes including cell migration (*8*), collagen catabolism (*9*), and the aggregation of platelets (*10*).

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Several distinct sequences derived from collagens have been identified as cell adhesion sites. The activity of some sequences may require the triple-helix, while others might be nonfunctional in the native, triple helical conformation but revealed in the denatured state (*11*). The unit GER is the fifth most frequently occurring tripeptide sequence in collagen I (*12*), which was demonstrated that the collagenous sequences containing the GER triplet support both cell attachment and platelet aggregation, and that GER is essential for angiogenesis (*13*, *14*). On the other hand, Grab et al. examined the potential cellular recognition sites within type I collagen and suggested that the peptide GPQGIAGQRGVVGLP* (P*, Hyp) in the triple-helix structure has a highly active fibroblast binding property in addition to promoting cell signaling and the production of matrix metalloproteinase (*15*). It had been postulated that Hyp imino acids transmit significant stability to the triple-helix structure via inter- and intrachain hydrogen bonds involving water molecules(*16*, *17*). However, the use of Hyp-deficient variants has offered the possibility of stabilizing the triple-helix formed in collagen-like peptides and proteins. For example, Mechling and Bächinger (*7*), and Boudko et al. (*18*) demonstrated that the crosslinking domain of collagen III, GPPGPCCGGG, has the ability to promote triple helix formation through disulfide bonds formed among the three chains.

In this study, we designed two collagen-like proteins with the following amino acid sequences.

Coll: $[GERGDLGPQGIAGQRGVV(GER)_3GAS]_8$
Coll-Fusion: $[GERGDLGPQGIAGQRGVV(GER)_3GAS]_8$ GPPGPCCGGG

Using a genetic engineering technique, we synthesized these two recombinant proteins, abbreviated as Coll and Coll-Fusion, in *Escherichi coli* system. The CD results suggest that the Hyp-deficient variants offer the possibil-

(a) Oligonucleotide sequences of synthetic adapter inserted into pUC118 to create pUC118-linker

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                 Xba I          Spe I      Apa I      Nhe I          Xba I
Adapter-1  5'  CTAGA ATG ACT AGT GGG CCC GCT AGC ATG T    3'
Adapter-2      3'   T TAC TGA TCA CCC GGG CGA TCG TAC AGATC   5'
                    Met Thr Ser Gly Pro Ala Ser Met
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(b) Oligonucleotide sequences of collagen-like block

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                 Spe I
Coll-1     5'  CTAGT GGC GAA CGT GGT GAT CTG GGC CCG CAG GGT ATC GCG GGC CAG
Coll-2         3'   A CCG CTT GCA CCA CTA GAC CCG GGC GTC CCA TAG CGC CCG GTC
                    Gly Glu Arg Gly Asp Leu Gly Pro Gln Gly Ile Ala Gly Gln
                                                                  Nhe I
                    CGT GGT GTG GTT GGC GAG CGT GGT GAA CGC GGC GAG CGT GGT G  3'
                    GCA CCA CAC CAA CCG CTC GCA CCA CTT GCG CCG CTC GCA CCA CGA TC  5'
                    Arg Gly Val Val Gly Glu Arg Gly Glu Arg Gly Glu Arg Gly Ala Ser
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(c) Oligonucleotide sequences of fusion block

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                 Spe I                                                  Nhe I
Fusion-1   5'  CTAGT GGC CCG CCA GGT CCG TGC TGT GGC GGT GGC G    3'
Fusion-2       3'   A CCG GGC GGT CCA GGC ACG ACA CCG CCA CCG CGATC   5'
                    Gly Pro Pro Gly Pro Cys Cys Gly Gly Gly Ala Ser
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Fig. 1. **The designed oligonucleotide sequences of the collagen-like proteins.** (a) Oligonucleotide sequences of synthetic adapters inserted into pUC118 to create the pUC118-linker; (b) Oligonucleotide sequences of the collagen-like blocks and (c) oligonucleotide sequences of fusion blocks. All of the oligonucleotides were purified by HPLC and phosphorylated at the 5′ end.

ity of stabilizing the triple-helix formed in these recombinant proteins. Finally, cell adhesion assays were performed.

EXPERIMENTAL PROCEDURES

Materials—Plasmids pUC118 and pET30a(+) were obtained from Takara Bio. Inc. and Novagen, respectively. *E. coli* DH5α was used for the propagation and construction of plasmids, and BL21(DE3)pLysS was used for the expression of proteins. Mouse fibroblast BALB3T3 cells were obtained from Dainippon Pharmaceutical Co., Ltd. The oligonucleotides were purchased from Asahi Techno Glass Co., Japan. Restriction and ligase enzymes were purchased from Takara Bio. Inc.

Production of Collagen-Like Proteins—The method for the production of recombinant proteins has been described in detail previously (*19–22*). The duplex DNA obtained by oligonucleotide annealing, shown in Figure 1a, was ligated into pUC118 to construct the pUC118-linker vector. For the construction of genes for the Coll and Coll-Fusion proteins, the duplex DNAs of the collagen-like block (Fig. 1b) and fusion block (Fig. 1c) were inserted into the *Nhe*I–*Spe*I site of the pUC118-linker, respectively. Linearized vectors and insert DNA fragments were extracted by digestion at *Nhe*I and/or *Spe*I. After polymerization of the DNA sequences, the inserts of the target DNAs were excised from the above two vectors by *Bam*HI and *Hin*dIII digestion, subcloned directionally into expression vector pET30a(+), and transformed into BL21(DE3)pLysS, respectively. Larger scale cell cultivation was performed using a fermenter (Marubishi BioEng. Japan), with protein expression was induced by IPTG (isopropyl-thio-β-D-galactopyranoside). Both recombinant proteins were purified with Nickel-chelate chromatography, and identified by SDS-PAGE and western-blotting analyses.

Circular Dichroism (CD) Measurements—The solution state CD spectra (205–260 nm) of the recombinant proteins were recorded on a JASCO J-820 spectropolarimeter with a thermostatted 0.2 cm path-length quartz cell. Thermal stability was determined by monitoring the change in the ellipticity at a fixed wavelength of 220 nm, θ_{220}, as a function of temperature. A heating rate of 5°C/min was used. Data analysis was performed with the software packages attached to the spectropolarimeter by JASCO.

Cell Adhesion Assay—Mouse fibroblast BALB3T3 cells were used for the cell adhesion assay (*23*). The recombinant proteins were dissolved in PBS at a concentration of 1 mg/ml. The solutions were added to 48-well microplates at 500 μl/well. The plates were desiccated at room temperature for 30–60 min, and 500 μl BALB3T3-containing Eagle's MEM (FBS) medium was added to each well. The plates were kept at 37°C incubator for 2 hours. Cultured cells were rinsed with PBS, and detached with

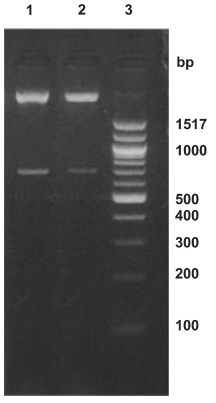

Fig. 2. ***Spe*I and *Nhe*I digestion analyses of multimerized cloning vectors.** Lane 1, pUC118-linker-Coll(8); Lane 2, pUC118-linker-Coll(8)-Fusion, and lane 3, 100 bp DNA ladder.

a. N-terminal sequence:

MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSSRM

b. C-terminal sequence:

MSRVDLKLAAALEHHHHHH

Fig. 3. **Amino acid sequences at (a) the N-terminal and (b) the C-terminal of collagen-like proteins.** His-tags are underlined.

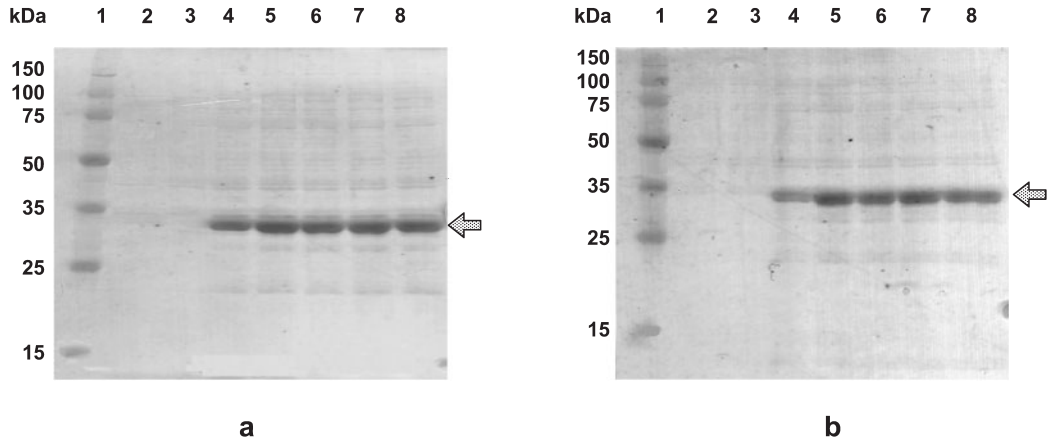

Fig. 4. **SDS-PAGE analyses of the expression level of (a) Coll(8) and (b) Coll(8)-Fusion after different induction times.** Lane 1, Perfect Protein™ Markers; Lanes from 2 to 8 are the cultured solutions 0, 0.5, 1, 2, 3, 4 and 5 h after the induction of expression by IPTG addition. Arrows indicate the target proteins with molecular mass of 32 kDa for Coll(8) and 33 kDa for Coll(8)-Fusion.

200 μl of 0.5% Triton-X100/PBS solution. The solution was treated with a lactate dehydrogenase (LDH) detection kit (Roche Diagnostics Co.) according to the manufacturer's protocol. The activity was determined using a plate-reader spectrophotometer (ThermoMax Microplate Reader, Molecular Devices) by measuring the rate of increase of absorbance at 340 nm due to the conversion of NADP+ to NADPH. The data are the averages of 6 samples selected from the 48 wells at random.

RESULTS

Gene Construction—DNA sequences of the collagen-like and fusion blocks shown in Figure 1, b and c, were designed with 5′-*Spe*I and 3′-*Nhe*I terminals. After insertion into vectors, the inserts can be liberated from the recombinant plasmids by digestion with *Spe*I and *Nhe*I. These inserts are able to self-ligates, and also to ligate with the vector. Optimal codons for each amino acid were selected based on codon usage in *E. coli* as reported previously (*24*). Since the base composition and sequence of the DNA can influence the polypeptide expression level and the stability of the DNA itself, the codons were selected to maximize the A-T contents as possible. Figure 2 shows 2.0% agarose electrophoresis results of the *Nhe*I and *Spe*I digested recombinant cloning vectors, pUC118-linker-Coll(8) and Coll(8)-Fusion (lanes 1 and 2), corresponding to the construction of 8 repeats of the monomer DNA of the collagen-like block and the DNA ligated with the fusion block, comprising 720 bp and 750 bp, respectively, and verified by the 100 bp DNA ladders (BioLabs) (lane 3).

Construction and Expression of Collagen-Like Proteins—In this research, the purified DNA fragments were inserted into the commercial available expression plasmid pET30a(+) between the *Bam*HI and *Hin*dIII restriction sites. Therefore, the resulting recombinant expression vectors encode the Coll(8) and Coll(8)-Fusion of interest, both of which are flanked by N- and C-terminal extensions of 53 and 19 amino acids as shown in Fig. 3, a and b, respectively. The host used for protein expression was *E. coli* strain BL21(DE3)pLysS. In this strain, a gene encoding T7 RNA polymerase is incorporated into the bacterial chromosome under *lacUV5* control, and protein production is induced by the addition of IPTG. (*25*) The pLysS plasmid provides low levels of T7 lysozyme, which inhibits T7 RNA polymerase and suppresses the basal level of protein expression.

The protein expression level at 37°C in rich medium (TB) was checked at different induction times. Aliquots of cultures representing approximately equal numbers of cells (as determined by optical density at 600 nm) were removed periodically after the induction of protein synthesis (t = 0, 0.5, 1, 2, 3, 4, 5 and 6 h), and loaded onto a 12% polyacrylamide gel for SDS-PAGE by the method of Laemmli (*26*). SDS-PAGE analyses (Fig. 4, a and b) suggest that the expression level of both recombinant proteins, shown as a bands at about 32 kDa for Coll(8) and 33 kDa for Coll(8)-Fusion, increases with induction time up to 4 h, and that further induction is not likely to provide any additional increase in the level of expression. Large scale protein production was performed by batch culture. For this, the proteins were synthesized in a soluble state in the host and none appeared to accumulate in inclusion bodies at growth and induction temperatures of

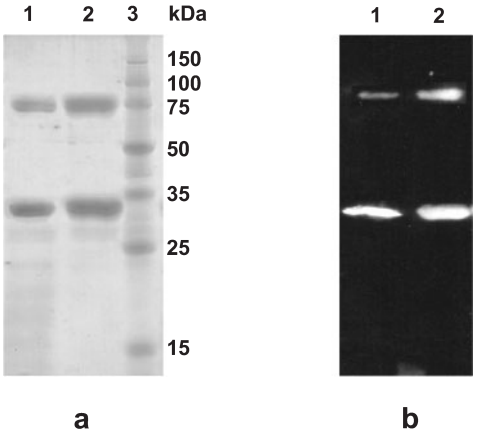

Fig. 5. **(a) SDS-PAGE and (b) western-blotting analyses of the purified proteins.** Lane 1, Coll(8), lane 2, Coll(8)-Fusion, and lane 3, Perfect Protein™ Markers.

37°C as judged from SDS-PAGE analysis (data not shown). Consequently, the proteins were purified under native conditions by immobilized nickel chelate affinity chromatography through the $[His]_6$ sequences positioned at both the N- and C-terminals (*20*). The yields of purified proteins were 60 ± 10 mg/liter of TB medium.

Figure 5 shows the SDS-PAGE and Western-blotting analyses of the recombinant proteins dissolved in PBS buffer (pH 5.8). As expected, bands corresponding to the collagen-like monomer molecules at about 32 kDa for Coll(8) (Fig. 5a, lane 1) and 33 kDa for Coll(8)-Fusion (Fig. 5a, lane 2) were observed. Moreover, bands were obtained at about 75 kDa were observed for both proteins. Western-blotting analysis (Fig. 5b) suggested that both bands originated from the His-tagged recombinant proteins. Since the molecular mass of the upper bands is 2–3 times larger than those of the lower bands, both bands might be attributable to collagen-like proteins formed through the trimerization of molecular chains.

Circular Dichroism (CD) Spectra of Collagen-Like Proteins—CD spectroscopy was used to determine the secondary structure of the collagen-like proteins. The far-UV spectra of both recombinant proteins at 4°C at a concentration of 15 mM are characteristic of collagen-like peptides in a triple helical state (Fig. 6, a and b) (*18*). With increasing temperature, both underwent thermal unfolding profile in PBS (pH 5.8) resulting in the partial degradation when heated to 60°C as judged from the peak intensity at 220 nm.

The structural roles of the fusion block and the pH value were investigated in PBS buffer. Figure 7 shows the thermal stability of both recombinant proteins at different pH values in PBS buffer determined by measuring θ_{220} as a function of temperature. The thermal transition temperature, T_m, did not change significantly, and was, about 21°C in all cases. The rate of decline lines with increasing temperature tended to be flatter at pH 5.8 (Fig. 7, a and b) than at pH 8.0 (Fig. 7, c and d). Of the four, the plot for Coll(8)-Fusion at pH 5.8 showed the most stable thermal character, and the molar ellipticity at 220 nm, θ_{220}, did not change significantly below the T_m. This suggests the appearance of a stable triple helical structure in the Coll(8)-Fusion recombinant protein at

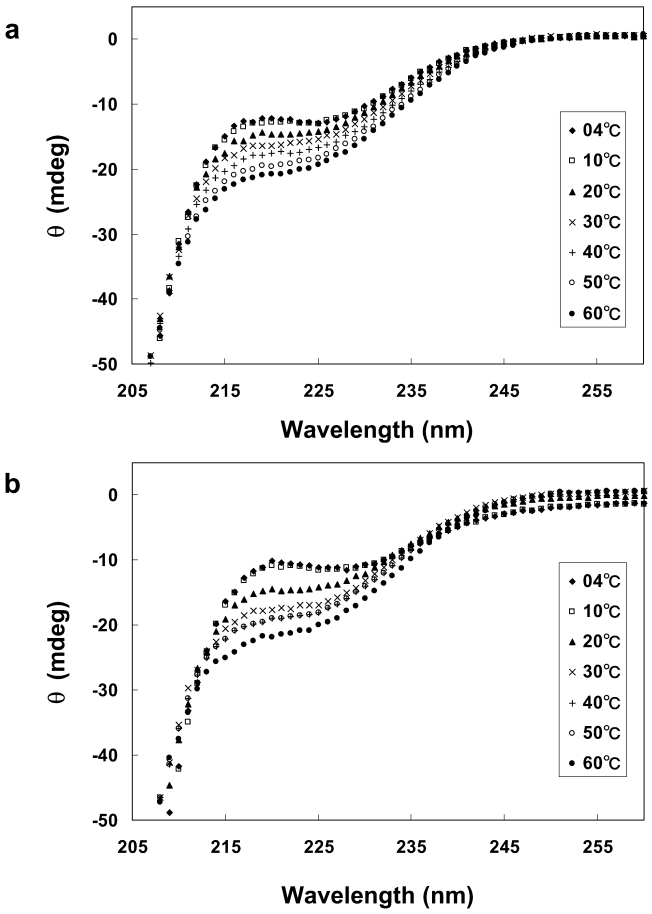

Fig. 6. **CD spectra of (a) Coll(8) and (b) Coll(8)-Fusion in PBS buffer (pH 5.8) at different temperatures.** Spectra were recorded at a protein concentrations of 15 μM.

pH 5.8 due to the possible formation of disulfide bonds among the molecular chains through the cysteine residues in the fusion block.

Cell Adhesion Assay of Collagen Proteins—Figure 8 shows the results of adhesion activity experiments of recombinant collagen-like proteins using mouse fibroblast BALB3T3 cells. The same cell adhesion assay was performed on an empty plate, and on native type I collagen and fibronectin for comparison. Fibronectin is the first well characterized adhesive protein, in which the core sequence is the triplet RGD (*27*). RGD has been found in many extracellular matrix proteins and usually forms the integrin-binding domain (*28*, *29*). As shown in Figure 8, although the substantial cell adhesion activity of both recombinant proteins produced in this work was somewhat lower than that of fibronectin, it was higher than that of native type I collagen. Thus, Coll(8)-Fusion and Coll(8) show high cell adhesive characters, which makes them suitable for use as biomaterials.

DISCUSSION

The collagen-like proteins produced in this research show very high cell adhesion to mouse BALB3T3 fibroblasts. Previous studies have shown that collagen has properties that promote cell adhesion (*8*), cell spreading (*30*), cell

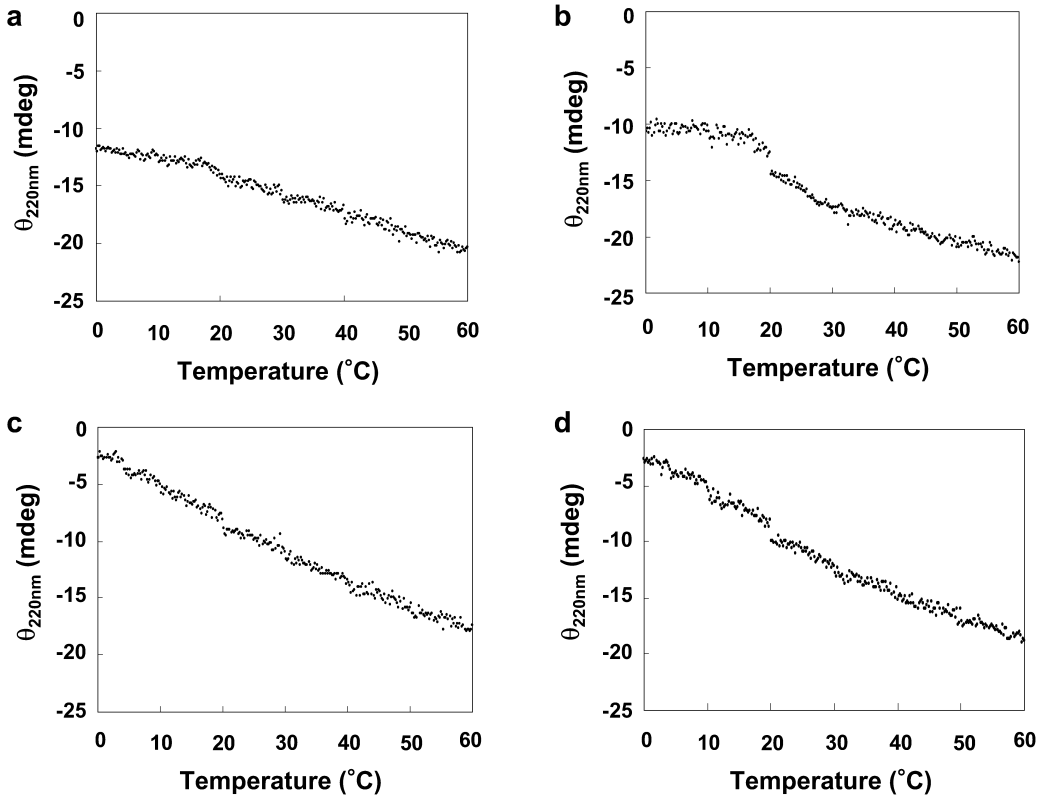

Fig. 7. **Thermal stability of the collagen-like proteins in PBS buffer.** (a) Coll(8), pH 5.8; (b) Coll(8)-Fusion, pH 5.8; (c) Coll(8), pH 8.0; and (d) Coll(8)-Fusion, pH 8.0.

migration (*31*), matrix metalloproteinase binding (*32*), and human platelet adhesion and aggregation (*33*). However, the recombinant expression of collagens and fragments of collagens is often difficult, as their biosynthesis requires specific post-translational enzymes, in particular prolyl 4-hydroxylase (*34*), the gene for which is not contained in most bacterial and yeast host expression systems (*35*). In this research, we recombined the repeating units of GER and GPQGIAGQRGVV, which originate from native type I collagen and show very high cell adhesion properties (*13–15*), and produced collagen-like proteins that can form a triple-helical structure under appropriate conditions. The cross-linking sequence, GPPGPCCGGG, derived from collagen III promotes triple helix formation through disulfide bonds formed among the three chains by attaching the peptide at the C-terminal of the collagen-like protein (*7*, *18*). It is noteworthy that the activity of Coll(8)-Fusion is almost the same as that of Coll(8), suggesting that the high activity of both recombinant proteins originates mostly from characteristic motifs, such as RGD, GER, etc. Namely, the complex formed between the RGD or GER motifs and integrin is an essential driving force behind the high cell adhesion activity.

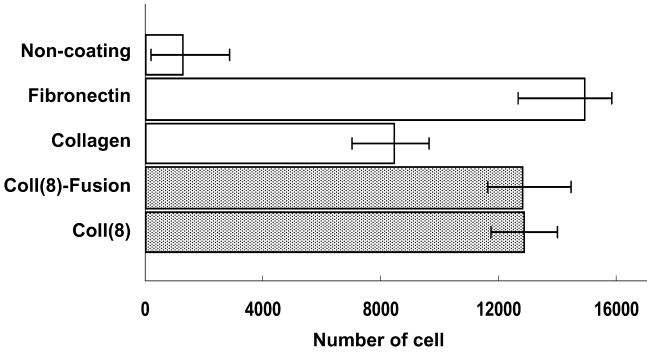

Fig. 8. **Histograms of the cell adhesive assay of collagen-like proteins to mouse BALB3T3 fibroblasts.** The assay results for empty plate, native type I collagen and fibronectin are shown for comparison.

Considering future applications, the combination of recombinant collagen-like proteins with other polymers or chemicals, such as hydroxyapatite (HAP), seems to be acceptable due to their high cell adhesion activities. The biomineralization process of the extracellular matrix in bone, dentin and cementum involves HAP-induced nucleation and crystal growth, where collagen acts as a structural matrix (*36*). For the collagen-like proteins produced in this work, the high contents of acidic amino acids (Glu and Asp) may play a key role in promoting nucleation activity in *de novo* mineral formation, which has been demonstrated for a non-collagenous protein, bone sialoprotein (*37*, *38*). Thus, a collagen-like protein-hydroxyapatite composite may promote the generation of new bone tissue with good functional and mechanical qualities, reducing the risks and expense of using autografts, allografts and metals.

In conclusion, we designed and produced two collagen-like proteins consisting of cell adhesion domains derived from native type I collagen using a genetic engineering method. The crosslinking sequence, GPPGPCCGGG, derived from collagen III was used to promote triple helix formation through disulfide bonds formed among the three chains by attaching the peptide at the C-terminal of the collagen-like protein.

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