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Functional peptide of dermatopontin produces fibrinogen fibrils and modifies its biological activity

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ABSTRACT

Background: Dermatopontin (DP), a small extracellular matrix protein, interacts with both fibrinogen and fibrin. DP accelerates fibrin fibril formation and enhances cell adhesion to fibrin fibrils but DP does not influence fibrinogen fibril formation. We have previously demonstrated that DP-4 (PHGQVVVAVRS) is a functional dermatopontin peptide (Wu et al., 2014).
Objective: Identification of biological functions of DP-4.
Methods: Protein–protein interactions were examined by solid-phase assay. The kinetics of fibrinogen/fibrin polymer formation was monitored by turbidity change, SDS-PAGE, and electron microscopy. A cell adhesion assay was performed using human umbilical vein endothelial cells.
Results: Although DP promoted fibrin formation, the DP-4 peptide promoted fibrinogen polymerization but did not apparently affect fibrin formation. The polymerized fibrinogen formed straight solid fibrils comparable to the normally formed fibrin fibrils. A minimum functional sequence of the DP-4 peptide was determined to be VVVAVRS. An αC domain in fibrinogen was involved in the fibril formation. Fibrinogen fibrils made by DP-4 enhanced endothelial cell adhesion and spreading in a dose-dependent manner. This cell adhesion was inhibited by heparin and by anti-αvβ3 and β1 integrin antibodies.
Conclusion: DP-4 did not reproduce the full functional biological activities of DP with fibrin but DP-4 did promote fibrinogen fibril formation. The fibrinogen fibrils produced by DP-4 are useful as a novel synthetic biomaterial for therapeutic applications.

1. Introduction

Dermatopontin (DP) is a small non-proteoglycan extracellular matrix (ECM) protein [1–3]. The dermis and the heart are the richest DP sources [4,5], and the content of DP in the dermis is approximately 50 μg/g wet weight [2]. Several structural functions of DP have been identified [6–9], and involve interactions with other extracellular matrix proteins or with a growth factor [6,10]. However, in spite of the abundance in the ECM, knowledge about DP is still limited. We have hypothesized that DP has additional biological functions and have investigated its potential functions.

Recently, we found that DP is a potent cell adhesion protein for epidermal cells, HaCaT [11], and that it is present in the serum and in a provisional matrix that is formed at the time of wounding [12]. The provisional matrix is a temporary structure mainly made of fibrin, fibronectin, and other serum-derived proteins [13]. The components in the provisional matrix function as scaffolds for migrating endothelial cells and fibroblasts to develop a granulation tissue, and subsequently, wound healing is achieved [14,15]. We also found that DP interacts with both fibrin/fibrinogen and fibronectin, and activates fibronectin [12]. DP is suggested to have

Abbreviations: ECM, extracellular matrix; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; BSA, bovine serum albumin; FITC, fluorescent isothiocyanate; HRP, horseradish peroxidase; β-ME, β-mercaptoethanol.

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multiple roles during the process of wound healing and to have the potential as a therapeutic tool.

Fibrinogen is a serum protein that primarily functions in the process of blood coagulation by conversion to fibrin *via* thrombin action [16]. Fibrinogen is a dimeric protein composed of three chains, namely, Aα, Bβ, and γ chains [16,17]. These chains form a central E domain and two outer D domains [16,17]. When small fibrinopeptides A and B in the Aα and Bβ chains are cleaved from the rest of the chains by thrombin, fibrinogen molecules are converted to fibrin monomers, and crosslinking with active sites in the D domains of the adjacent fibrin molecules proceeds, and linear fibrin protofibrils are formed [18,19]. The fibrin protofibrils bind to each other, and finally fibrin fibrils form and become visible as a fibrin clot [18,19].

In our previous study, we found that DP interacted with the D domain of fibrin/fibrinogen [20]. Through interaction with DP, fibrin fibril formation was enhanced and the fibrils became thicker than that formed in the absence of DP. Furthermore, the structurally modified fibrin fibrils demonstrated enhanced endothelial cell adhesion and cell spreading [20]. We also found that a peptide, termed DP-4, was the interaction site of DP with fibrin and that the peptide was biologically active [20]. The DP-4 peptide is a part in the first loop structure of DP. In the present study, we further examined whether the DP-4 peptide can reproduce the effect of DP on the function of fibrin, or if it has distinct activity. We found that the DP-4 peptide produced fibrinogen fibrils without greatly enhancing the fibrin fibril formation, thus the function of DP-4 peptide was different from that of DP. We closely examined profiles of fibrinogen fibril formation, and discuss functional aspects and possibilities of therapeutic utilization of the fibrinogen fibrils.

2. Materials and methods

2.1. Materials

Overlapping and deletion peptides of DP were synthesized as described previously [11]. The designated terms and the amino acid sequences are the same as those described in our previous report [11] and they are shown in Table 1. Human umbilical vein endothelial cells (HUVECs) and culture medium KJB-210 were purchased from DS Pharma Biomedical (Osaka, Japan). Bovine fibrinogen and human D and E domains of fibrinogen were obtained from Merck-Calbiochem Japan (Tokyo, Japan). Thrombin was obtained from GE Healthcare (Buckinghamshire, UK). A sulpho-NHS-LC-biotin, HRP-conjugated streptavidin, and BCA reagent were obtained from Pierce (Rockford, IL).

Table 1
DP peptides and DP-4 related peptides.

DP-1	QYGDYGYSYHQY	DP-4	PHGQVVVAVRS
DP-2	YHQYHDYSDDGWV	DP-4S	VRVHVPVQGSA
DP-3	DGWVNLNRQGFSYQ		
DP-4	PHGQVVVAVRS	DP-4a	-HGQVVVAVRS
DP-5	AVRSIFNKKEGS	DP-4b	–GQVVVAVRS
DP-6	KEGSDRQWNYA	DP-4c	--VVVAVRS
DP-7	MPTPQSLGEPTE	DP-4d	---VVAVRS
DP-8	WWEEINRAGMEWYQT	DP-4e	PHGQVVVAVR-
DP-9	SNNGLVAGFQSRYFE	DP-4f	PHGQVVVAV–
DP-10	RYFESVLDREWQFY	DP-4g	PHGQVVVA-
DP-11	WLTTEYPGHYGEE	DP-4h	PHGQVVV--
DP-12	YGEEMDMISYNYD	DP-4i	PHGQVV---
DP-13	YNYDYYMRGATT		
DP-14	GATTTFSAVERD		
DP-15	VERDRQWKFIM		
DP-16	RMTDYD		

2.2. Fibrin and fibrinogen fibril formation assay

Fibrin formation was performed as described previously [20]. Briefly, fibrinogen dissolved in 50 mM HEPES buffer containing 0.14 M NaCl and 20 mM $CaCl_2$, pH 7.5, was added to wells of a 96-well plate, and a mixture of thrombin and DP-4 in the same buffer was added to the wells. The turbidity change of the solution was monitored at 405 nm at temperature between 25 and 29 °C. Final concentrations of fibrinogen and thrombin were 1.6 mg/ml and 1 U/ml, respectively. For fibrinogen polymerization assay, fibrinogen at the same concentration was mixed with various concentrations of DP-4 and the turbidity was measured as described above.

2.3. Fibrinogen polymerization assay

Fibrinogen was mixed with the peptides in PBS and was incubated at 4 °C overnight. The final concentration of fibrinogen was 300 μg/ml, that of the peptides was 200 μg/ml, and total volume of the mixture was 10 μl. After centrifugation, the supernate and pellet were separated and were analyzed using a 5% conventional SDS-PAGE gel under nonreducing conditions, or 10% gel under reducing conditions containing 1% β-ME in sample buffer [21]. In an experiment, to highlight the difference of MWs between Aα and A, Bβ and B chains of fibrinogen/fibrin, the electrophoresis was performed at low voltage (around 8 V/cm) under reducing condition. In a dose response experiment, fibrinogen at 300 μg/ml was incubated with increasing concentrations of DP-4 in the same conditions as described above.

2.4. Electron microscopy

Electron microscopy was performed as reported previously [20]. In brief, after the fibrin formation assay and the fibrinogen polymerization assay, the samples were centrifuged and the pellets were fixed with 2% formaldehyde–2.5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.4, containing 3 mM $CaCl_2$. The samples were embedded in Epon resin, sliced as 1 μm sections, stained with 1% OsO_4 and 1% tannic acid, dehydrated, dried, and coated with OsO_4. All the observations were performed with a transmission electron microscope (S-4800, HITACHI, Tokyo, Japan) operated at an acceleration voltage of 15 kV.

2.5. Solid-phase assay for protein interaction

The experiment was performed according to a protocol described previously [12,20]. In brief, fibrinogen or the domains were coated in wells of a 96-well plate (Assist, Tokyo, Japan) at 10 μg/ml overnight. After blocking with BSA, 1 μg/ml of DP was applied alone, or in a mixture with 100 μg/ml of DP-4 deletion peptides, and was incubated overnight. Bound DP was probed with anti-DP antibody. After color development using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), absorbance was measured at 405 nm using a UV spectrophotometer, ELx808 (BioTek, Winooski, VT).

2.6. Separation of higher and lower MW fibrinogen

Higher and lower MW fibrinogens were separated according to a previous report [22]. Briefly, fibrinogen at 5 mg/ml was sequentially precipitated with 19, 22, 24, 26, and 30% ammonium sulfate. The pellets were dialyzed against PBS and the concentrations were determined using a BCA reagent using fibrinogen as a standard.

2.7. Cell adhesion assay

HUVECs were cultured in KJB-210 medium at 37 °C in a humidified 5% CO_2, 95% air atmosphere. The cell adhesion assay

was performed according to a protocol described previously [11,12]. Briefly, 0.5 μg/ml of fibrinogen was mixed with either DP-4 or DP-4S and incubated at room temperature for over 6 h, and then the samples were coated in the wells. After blocking with BSA, HUVECs suspended at a density of 30,000 cells/100 μl in DMEM were inoculated and the cells were allowed to adhere for 1 h. Next, the cells were fixed with glutaraldehyde, stained with crystal violet, and finally the dye was eluted and an absorbance at 595 nm was determined. For inhibition of cell adhesion, fibrinogen at 0.5 μg/ml was incubated with the DP-4 peptide at 10 μg/ml, then the mixture was immobilized. Functional blocking anti-integrin antibodies were used at 10 μg/ml.

2.8. Cytoskeletal staining

Cytoskeletal staining was performed according to our previous reports [12,20]. Briefly, 5 μg/ml of fibrinogen was incubated with or without 5 μg/ml of DP-4 in PBS for 2 h at room temperature. Then, 10 μl of the sample was coated on glass plates (Dako Japan, Kyoto, Japan) overnight. After rinsing and blocking, HUVECs were detached and suspended in DMEM at a density of 15,000 cells/10 μl, and 20-μl aliquots of the suspension were added to the wells. After 1 h incubation at 37 °C, nonadherent cells were removed by rinsing, and the adherent cells were fixed, dehydrated, and permeabilized. Actin was detected using FITC-conjugated phalloidin. Vinculin was detected by anti-vinculin antibody followed by incubation with Alexa Fluor 594-labeled anti-mouse IgG antibody. Finally, the images were acquired using a confocal laser scanning microscope, LSM-710 (Carl Zeiss, Oberkochen, Germany).

2.9. Statistical analysis

A statistical assessments was done by a Student's *t*-test and a *p* value under 0.01 was recognized as significant.

3. Results

3.1. The DP-4 peptide produces fibrinogen fibrils

DP enhanced fibrin fibril formation but did not influence fibrinogen [20]. The DP-4 peptide was previously identified as a functional site in DP [11,20]. First, we examined the effect of DP-4 on fibrin formation. When DP-4 was simply mixed with a fibrinogen solution, the solution became turbid immediately, the absorbance continued increasing during the following 30 min, and then it became stable thereafter (Fig. 1A). The final absorbance was roughly proportional to the DP-4 concentration. In contrast, when DP-4/thrombin mixture was added to the fibrinogen solution, the turbidity increased at 5 min because fibrinogen solution became turbid immediately by DP-4, and the turbidity of fibrin suspension became stable after 30 min. After 30 min, the turbidity of fibrin formed in the presence of DP-4 tended to be greater than that of fibrin only (Fig. 1B). As seen clearly in Fig. 1B, at 5 min, the difference of turbidity in both groups was statistically significant, but when compared at 30, 60, 90 and 120 min, the *p* values of the turbidity difference was just below and above 0.05, thus we decided that the values were not statistically significant in the present study. Finally, these results indicate that the DP-4 does not influence fibrin fibril formation although there was a tendency that DP-4 weakly enhances fibrin fibril formation. Instead, it does promote fibrinogen polymerization. Thus the function of DP-4 for fibrin and fibrinogen fibril formation is different from that of the parental DP protein.

Electron microscopic observation revealed many fibrils in the turbid sample made by incubating fibrinogen and DP-4 (Fig. 1C, panel 1). Comparing with fibrin fibrils made by incubating thrombin and fibrinogen (Fig. 1C, panel 2), the density of fibrinogen fibrils was greater (Fig. 1C, panel 1). The diameters of the fibrinogen fibrils were around 20 nm and they were much thinner than fibrin fibrils (approx. 100 nm, compare inserts in panels 1 and 2). The fibrinogen fibrils were straighter than the fibrin fibrils, and they did not show evidence of branching as can be seen at the periphery of the fibrin fibrils (inserts in panels 1 and 2). From these results, it was revealed that DP-4 assembles soluble fibrinogen molecules into insoluble fibrinogen fibrils that differ in size and in structure from fibrin fibrils made in the presence of thrombin.

3.2. DP-4 specifically polymerizes fibrinogen

When DP-4 was incubated with fibrinogen, most of the fibrinogen precipitated and was collected as pellet (Fig. 2A, lane 2). Only a minor fraction of fibrinogen remained in the supernate after centrifugation and SDS-PAGE (Fig. 2A, lane 1), as compared with control lanes (lanes 9 and 10). On the other hand, an overlapping DP-5 peptide and a scrambled DP-4S peptide, and DP protein did not induce fibrinogen polymerization, leaving most of the fibrinogen in the supernate (Fig. 2A, lanes 3–8).

To confirm that the pellet is composed of fibrinogen and not fibrin, the pellet was analyzed by SDS-PAGE under reducing conditions, and the patterns were compared with both fibrinogen and fibrin. As shown in Fig. 2B, the pellet formed by incubation of fibrinogen with DP-4 (lane 2) was composed of three bands. The MWs of these three bands were identical to the Aα, Bβ, and γ chains of standard fibrinogen, respectively (lane 3). The MWs of these Aα and Bβ chains were clearly larger than those of the A and B chains of fibrin (lane 1). These findings clearly indicate that the pellet is composed of fibrinogen, but not fibrin.

Next, in order to know a minimum ratio of DP-4 to fibrinogen for polymerization, fibrinogen was incubated with various concentrations of DP-4 (0–1400 μg/ml) (Fig. 2C). Fibrinogen polymerized in proportion to the DP-4 concentration, and the half-maximal concentration of DP-4 peptide for fibrinogen polymerization was about 100 μg/ml. When the DP-4 peptide concentrations were greater than 350 μg/ml, all the fibrinogen pelleted (Fig. 2C, right side of the panel). These results suggest that the DP-4 peptide is a functional site in the DP molecule and the weight ratio of DP-4 peptide to fibrinogen for a half-maximal polymerization was 1:3.

3.3. Determination of the essential sequence in the DP-4 for interacting with fibrinogen

To determine the minimum active amino acid sequence in the DP-4 peptide, deletion peptides of DP-4 (DP-4a–i) were used to inhibit the DP-D domain interaction (Table 1). As shown in Fig. 3A, a deletion peptide, DP-4c, which lacks four amino-terminal amino acids, did not inhibit the DP-D domain interaction, whereas the interaction was maintained in the presence of DP-4b peptide that lacks two amino acids (Fig. 3A). Accordingly, the DP-4b peptide could induce an insoluble fibrinogen polymer, whereas the DP-4c peptide could not (Fig. 3B). These results suggest that a minimal functional peptide requires the histidine at the amino terminus.

Next, carboxyl terminal deletion peptides were examined. DP-4f lacks two carboxyl terminal amino acids and could still inhibit the DP-D domain interaction, whereas the inhibition became weaker when DP-4g that lacks three amino acids was used (Fig. 3A). Accordingly, DP-4f could make fibrinogen insoluble polymer, whereas DP-4g did not (Fig. 3B). Here, deletion peptides DP-4g, h, and i also inhibited the DP-D domain interaction significantly (Fig. 3A). However, the inhibition was not complete when compared with DP-4e and f, and moreover, the DP-4g, h, i

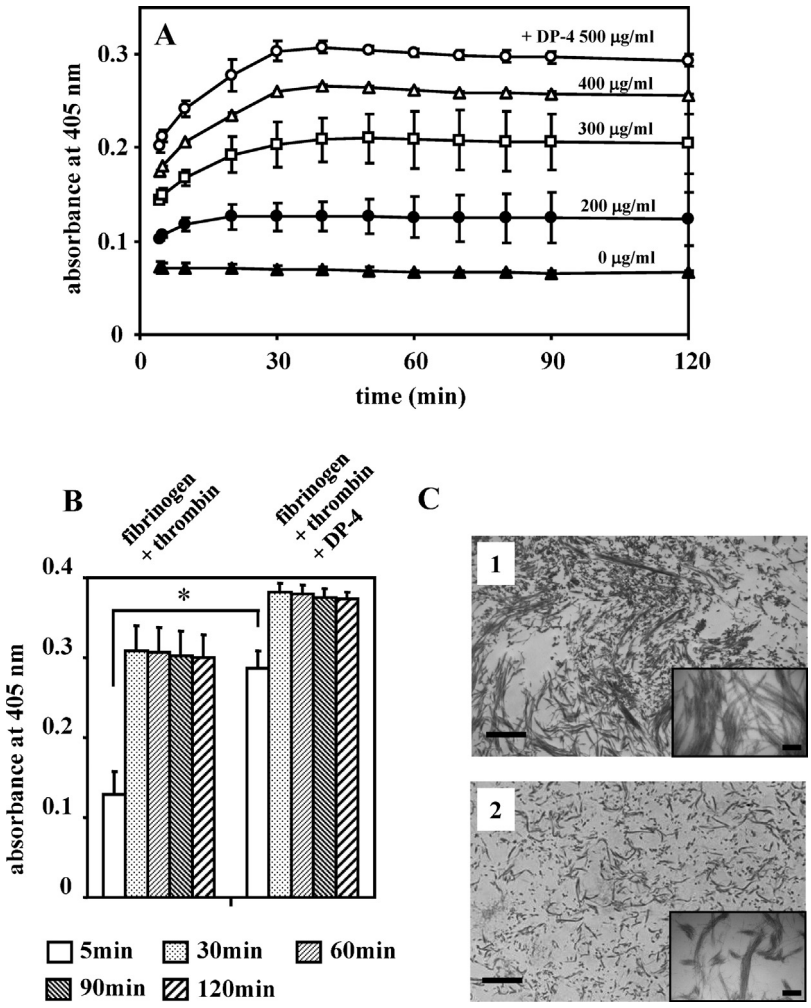

Fig. 1. Kinetics of fibrinogen fibril formation and structure of the fibrils. (A) Kinetics of fibrinogen fibril formation. Fibrinogen solution in wells was mixed with DP-4 solution and turbidity changes at 405 nm were monitored during 2 h. DP-4 concentrations are indicated by each line. (B) Fibrin formation in the presence of DP-4. Fibrin was formed in the absence and presence (indicated on the top of panel) of DP-4 at 500 μg/ml. Incubation time of each bar is indicated at the bottom. An asterisk indicates $p < 0.01$. In A and B, error bars indicate means ± SD of triplicate determinations. (C) Ultrastructure of fibrinogen pellet and a comparison with fibrin fibrils. Fibrinogen pellet was formed by incubation of 1.6 mg/ml fibrinogen and 400 μg/ml DP-4 for 2 h. Then, the pellet was collected and subjected to electron microscopy. Fibrin fibrils were formed by incubating fibrinogen and thrombin, and the pellet was collected. Panel 1: fibrinogen fibrils. Panel 2: fibrin fibrils. Photos were taken at a magnification of ×10,000. Bars indicate 2 μm. In inserts in panels 1 and 2, the magnification was ×100,000. Bars indicate 200 nm.

failed to produce fibrinogen fibril formation. Hence, the two carboxyl terminal amino acids are not necessary for interaction with D domain and for making fibrinogen polymer. Taking these results together, an essential amino acid sequence of DP-4 peptide was determined to be GQVVVAV.

3.4. Fibril formation of lower MW fibrinogen is less than that of higher MW fibrinogen

It is known that a fibrinogen molecule lacking the carboxyl terminal αC region is present in a small amount within a conventional fibrinogen fraction [22]. This type of lower MW fibrinogen is precipitated at 30% ammonium sulfate, whereas a fraction precipitating at 19% contains only a higher MW fibrinogen that retains an intact Aα chain [22]. Before enrichment, a second major band (arrowhead) is visible just under a major dense band (arrow) under a non-reducing condition (Fig. 4A, lane 1), whereas after enrichment, in the higher MW fibrinogen fraction, the second band became less (lane 2). In the lower MW fibrinogen fraction, the second band became major and some higher MW fibrinogen was contaminated (lane 3). Accordingly, under reducing conditions, the amount of Aα chain in lower MW fibrinogen fraction was about 60% of that in higher MW fibrinogen by densitometry (Fig. 4A, lane 6). Thus, the fraction precipitated at 30% ammonium sulfate contains about 40% of fibrinogen lacking the carboxyl terminal αC region of Aα chain.

DP-4-induced pellet formation was compared between these higher and lower MW fibrinogen species. Most of the higher MW fibrinogen formed a pellet in the presence of DP-4 (Fig. 4B, lanes 1 and 2). In contrast, half of the fibrinogen from the lower MW fibrinogen-rich fraction, became a pellet at the same DP-4 concentration (Fig. 4B, lanes 3 and 4). These findings indicate that fibrinogen lacking the carboxyl terminal αC region of the Aα chain has a poorer tendency to become a pellet, and also that an αC region is involved in DP-4-induced fibrinogen fibril formation.

3.5. Cell adhesion is enhanced by fibrinogen fibrils

As shown above, because the biochemical features and ultrastructures of fibrinogen were modified by DP-4, we next questioned if certain functions of fibrinogen are changed by DP-4. In this study, a HUVEC adhesion activity was used to determine

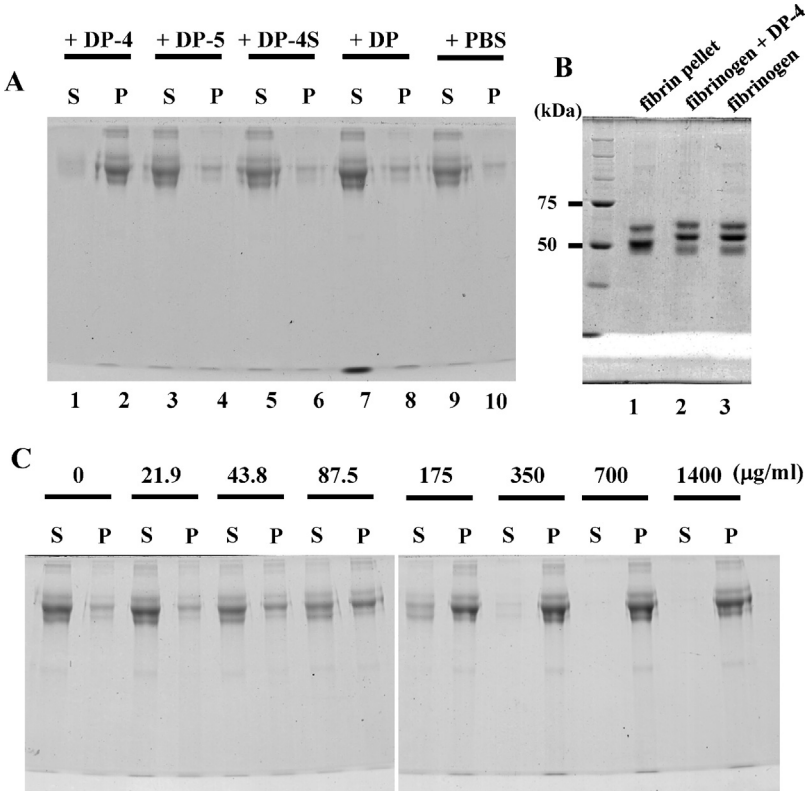

Fig. 2. DP-4 specifically induces fibrinogen fibrils. (A) Fibrinogen was incubated with peptides indicated on the top of the panel. After incubation, the supernate (S) and the pellet (P) were separated, and analyzed by electrophoresis under non reducing conditions. (B) Determination of the pellet as fibrinogen. The pellet was analyzed by electrophoresis under reducing conditions, and the patterns were compared with fibrin and fibrinogen as indicated on the top of the panel. Note that the patterns are identical to those of fibrinogen, not fibrin. MW standard and the MWs of representative bands are shown in the left of the panel. (C) Determination of the minimal active DP-4 concentration. Fibrinogen was incubated with increasing concentrations of DP-4 as indicated on the top of the panel, and the supernate (S) and pellet (P) were separated and analyzed under non reducing conditions.

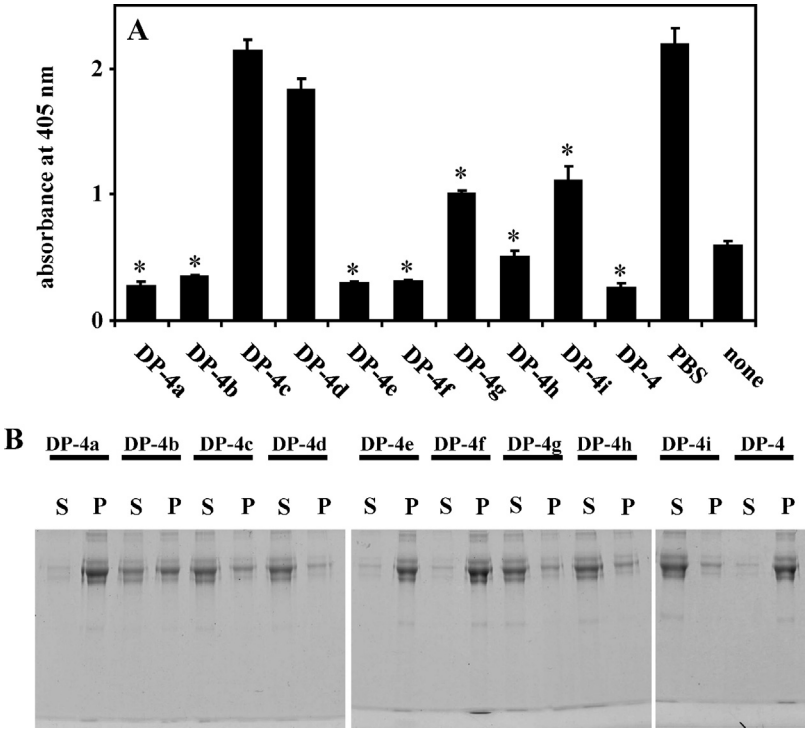

Fig. 3. Identification of the minimal active sequence of DP-4. (A) Inhibition of the interaction between DP and a D domain by deletion peptides shown in Table 1. DP was mixed with deletion peptides indicated at the bottom of the panel and the mixture was applied to immobilized D domain, followed by detection described in Section 2. Error bars indicate means ± SD of triplicate determinations. Asterisks indicate $p < 0.01$. (B) Fibrinogen polymerization by deletion peptides. Fibrinogen was incubated with the deletion peptides indicated on the top of the panels, and the supernate (S) and pellet (P) were separated, and analyzed by electrophoresis under non reducing conditions.

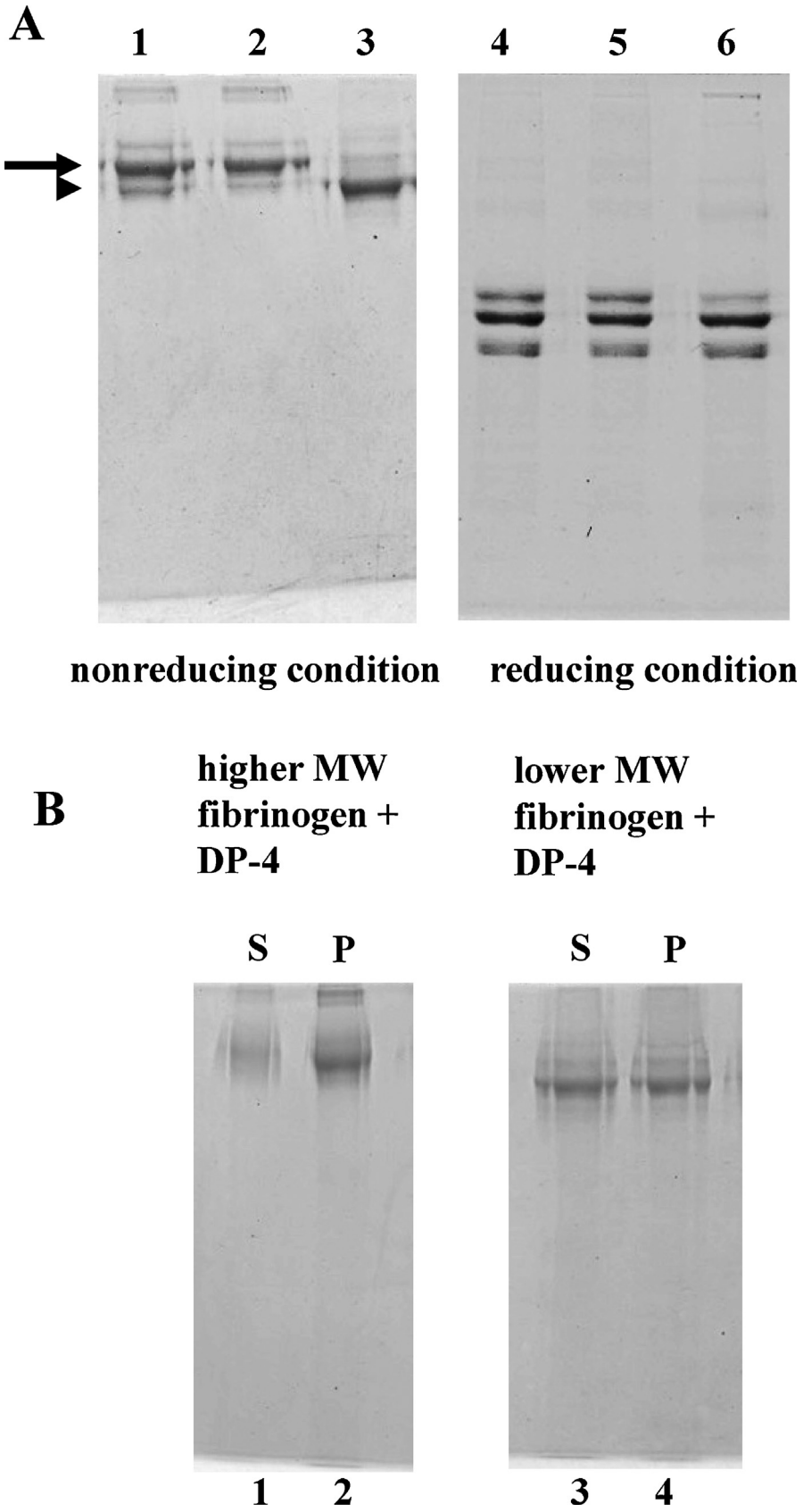

Fig. 4. Enrichment of fibrinogen molecule lacking αC region, and its fibril formation property. (A) Higher MW fibrinogen, and lower MW fibrinogen-rich fractions. Lanes 1 and 4: Starting material of fibrinogen before enrichment. An arrow indicates the higher MW fibrinogen and an arrowhead the lower MW fibrinogen. Lanes 2 and 5: Higher MW fibrinogen fraction precipitating at 19% ammonium sulfate. Lanes 3 and 6: Lower MW fibrinogen-rich fraction precipitating at 30% ammonium sulfate. Lanes 1–3: non reducing conditions, 5% gel. Lanes 4–6: reducing conditions, 10% gel. (B) Fibril formation assay. Three hundred μg/ml of each fraction was incubated with 175 μg/ml of DP-4, and the supernate and pellet were separated, and analyzed by 5% gel. Lanes 1 and 2: Higher MW fibrinogen. Lanes 3 and 4: Lower MW fibrinogen-rich fraction. Lanes 1 and 3 indicate the supernate (S), and lanes 2 and 4 the pellet (P).

the function of fibrinogen. In fact, the cell adhesion to DP-4-induced fibrinogen fibrils was enhanced in a dose-dependent manner (Fig. 5A). On the other hand, no adhesion enhancement was seen when fibrinogen was incubated with a scrambled peptide, DP-4S (Fig. 5A). The DP-4 peptide by itself showed quite limited cell adhesion when compared with the enhancement described above (data not shown). The adherent cells to DP-4-induced fibrinogen fibrils showed somewhat enhanced cell spreading compared with those adhering to immobilized fibrinogen (Fig. 5A, right panels 1 and 2).

Next, cytoskeleton formation was examined on the cells adherent to the fibrinogen fibrils immobilized on a glass surface. When immobilized on a glass surface, the cells adhering to the DP-4-induced fibrinogen fibrils exhibited better cell spreading comparing with those adhering on a plastic surface (compare Fig. 5A upper right panel and B panel 1). The number of the cells adhering to DP-4-induced fibrinogen fibrils was more than that of the cells adhering to fibrinogen, and the number of larger cells was greater (Fig. 5B panels 1 and 5). The cells adhering to fibrinogen fibrils demonstrated clear linear actin staining and a punctuate vinculin pattern (Fig. 5B panels 2–4) compared to those adhering to fibrinogen only (Fig. 5B panels 6–8).

3.6. Identification of cell surface receptor for DP-4 peptide-induced fibrinogen fibrils

HUVEC adhesion to the fibrinogen fibrils was clearly inhibited by EDTA as well as by heparin (Fig. 6A and B), indicating that cell surface receptors belong to both integrin and heparan sulfate proteoglycan-type families. We examined the inhibition of cell adhesion by a panel of anti-integrin subunit antibodies, and found a weak inhibition only by αvβ3 integrin antibody, and that a β1 antibody did not work by itself. However, when these antibodies were used as a mixture, an almost complete inhibition of the cell

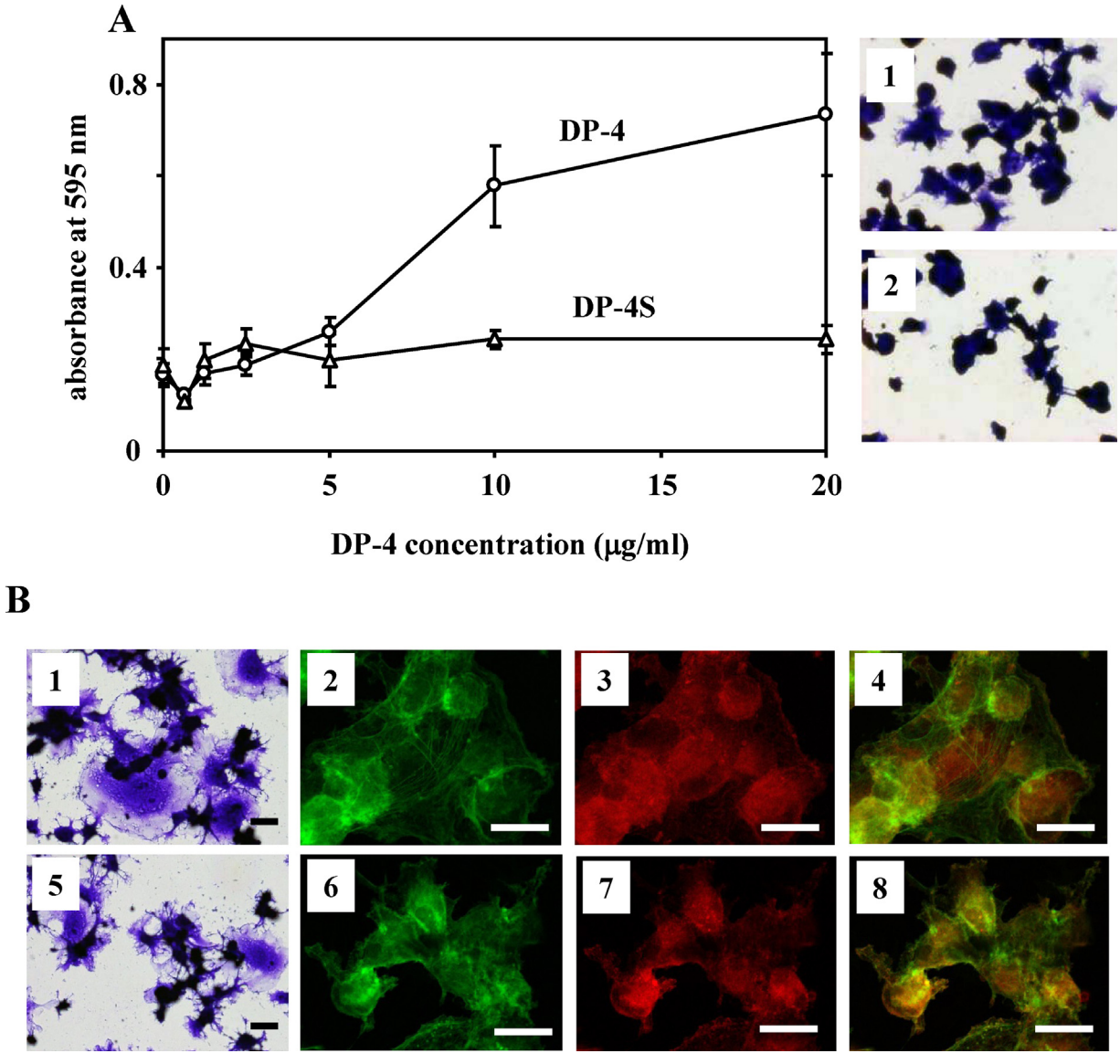

Fig. 5. Enhancement of cell adhesion to DP-4-induced fibrinogen fibrils. (A) Cell adhesion assay. Fibrinogen was incubated with DP-4 at the indicated concentrations, and the mixtures were immobilized. Then, a HUVEC adhesion assay was done. Circles (○) indicate DP-4 peptide, and triangles (△) DP-4S peptide as a negative control. Error bars indicate means ± SD of triplicate determinations. Panel 1: Crystal violet staining of cells that adhered to fibrinogen incubated with 20 μg/ml DP-4. Panel 2: Same staining of cells adhering to fibrinogen incubated with 20 μg/ml DP-4S. (B) Cytoskeleton staining. Panels 1–4: HUVECs that adhered to DP-4-induced fibrinogen fibrils. Panels 5–8: Cells that adhered to fibrinogen alone. Panels 1 and 5: Crystal violet staining. Panels 2 and 6: Actin staining by phalloidin. Panels 3 and 7: Vinculin staining. Panels 4 and 8: Merged images of panels 2, 3 and panels 6, 7, respectively. Bars indicate 20 μm.

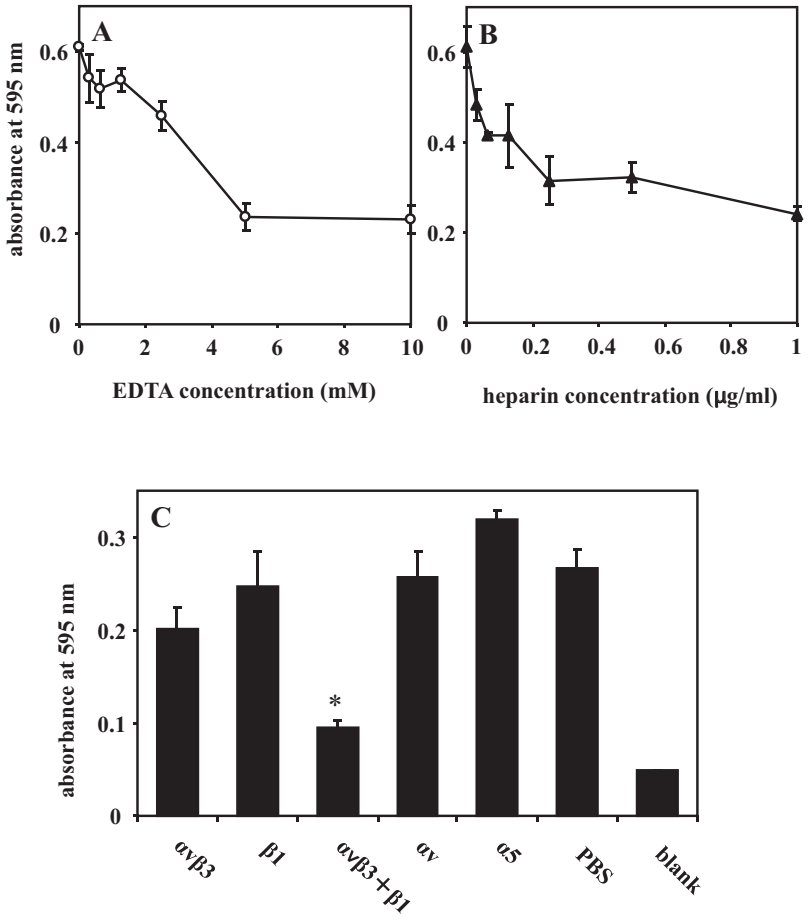

Fig. 6. Inhibition of HUVEC adhesion to immobilized fibrinogen fibrils. (A) Inhibition by EDTA. (B) Inhibition by heparin. In A and B, horizontal lines indicate EDTA and heparin concentrations, respectively. (C) Inhibition of cell adhesion by anti-integrin subunit antibodies. Each antibody is shown at the bottom of the panel. In (A)–(C), error bars indicate means ± SD of triplicate determinations. An asterisk indicates $p < 0.01$.

adhesion was achieved (Fig. 6C). Because syndecan-1 has already been identified as a heparan sulfate proteoglycan-type receptor for DP-4 [11], further examination was not done.

4. Discussion

In a previous publication, we reported that DP bound fibrinogen and fibrin, accelerated fibrin fibril formation, and changed the fibrin ultrastructure [20]. The DP-4 peptide was identified as a functional peptide of DP [20]. In this study, we examined if the DP-4 could reproduce the effects of DP on fibrin formation. However, DP-4 did not apparently enhance fibrin formation, instead, the peptide promoted the formation of insoluble fibrinogen fibrils. Therefore, DP-4 could not reproduce the function of DP, but it demonstrated an unexpected novel phenomenon. These findings suggest that additional peptide sequences are necessary to reproduce the function of parental DP protein.

Formation of fibrinogen fibrils by a peptide is a novel finding. It has been known that not only fibrin but also fibrinogen forms fibrils [23–25]. Fibrinogen forms striated fibrils in the presence of certain chemicals, such as protamine sulfate or adenosine triphosphate, and it even forms crystals by adjusting the physico-chemical conditions [25]. Previous studies reported that fibrinogen also formed fibrils on solid surfaces [24,26,27], and among the solid surfaces, a hydrophobic surface was preferable for inducing fibrinogen fibril formation [27]. The formation of fibrinogen fibrils begins at as early as several minutes and discrete fibrils form after longer incubation [27].

A recent study indicated that an interaction between fibrinogen molecules *via* the αC regions is largely responsible for fibrinogen fibril formation. The αC region is a 40 kDa peptide that is located at the carboxyl-terminus of the α chain. These domains usually interact with a central E domain, but they dissociate from the E domain and interact with other αC regions depending on the surrounding situations, such as thrombin, or contact with hydrophobic surfaces [16,28,29]. Accordingly, in the present study, fibrinogen from a fraction rich in the molecule lacking the αC region, showed a poorer pellet formation by DP-4. Therefore, the αC regions of the Aα chains are involved in DP-4-induced fibrinogen fibril formation. Although DP-4 and a scrambled peptide, DP-4S, have the same hydrophobic feature, DP-4S does not promote fibrinogen fibril formation. Therefore, the mechanism is not simply explained by the hydrophobicity, and specific sequence information of the peptide is required for fibrinogen fibril formation.

In contrast to DP-4, a parental DP protein did not produce fibrinogen fibrils. The finding indicates that events following the DP binding with the fibrinogen D domain are different from DP-4 binding. We suppose that by binding of DP-4 with a D domain, DP-4 dislocates αC regions so that the interaction between αC regions of the adjacent fibrinogen molecule becomes favorable. On the other hand, when DP binds a D domain, DP-4 is engaged in the interaction, but because of the additional mass of the DP molecule, the positions in the αC regions do not become favorable for interaction with other αC regions in an adjacent fibrinogen molecule. However, when fibrinogen is converted to fibrin by

thrombin, the amino-termini of the α and β chains serve as the major interaction sites between fibrin monomers. Finally, the attached DP is incorporated in the fibrin fibrils and affects the structure as reported previously [20], whereas DP-4 is so much smaller that it cannot affect the structure. Overall, the mechanisms of forming fibrinogen fibrils remain to be elucidated.

Cell adhesion to immobilized fibrinogen fibrils was inhibited by a mixture of anti-αvβ3 and anti-β1 subunit antibodies. These types of integrins are receptors for fibrin/fibrinogen [30,31], suggesting that fibrinogen as individual molecules and as fibrils interact with the same cell surface receptors. The β1 subunit-containing integrin on endothelial cells is known to be an α5β1 integrin [31], therefore cell adhesion is mediated by a cooperation of αvβ3 and α5β1 integrin. A cell surface receptor for DP-4 has been determined to be syndecan-1 [11]. It is compatible that the cell adhesion to DP-4-induced fibrinogen fibrils was inhibited by heparin.

The biological significance of fibrinogen fibrils is not clear. Nevertheless, there is some information on fibrinogen fibrils. One is that fibrinogen can be incorporated in the ECM. Not only hepatocytes, but also alveolar epithelial cells produce fibrinogen under certain inflammatory conditions, and fibrinogen is incorporated into the lung ECM in a fibrillar pattern *in vivo* [32–34]. Moreover, when fibrinogen is added to cultured fibroblasts, the fibroblasts assemble the fibrinogen into fibrils together with fibronectin [34,35]. The formation of fibrinogen fibrils by fibroblasts is an *in vitro* phenomenon, and it has not been demonstrated *in vivo*. The fibrinogen-fibronectin hybrid fibrils are reported to enhance fibroblast migration and proliferation [35]. Similarly, in our system, fibrinogen incubated with DP-4 enhanced endothelial cell adhesion. The cell adhesion enhancement may reflect a part of the reported hybrid fibrinogen fibril function.

Another piece of information and one potentially intriguing function of fibrinogen is that fibrinogen coated on a surface prevents blood cell adhesion [29,36,37]. On a solid surface, fibrinogen is adsorbed in a multilayered fashion [29,37]. When there are one or two layers, immobilized fibrinogen induces blood cell adhesion. However, when the layer exceeds three or four, fibrinogen molecules interact together *via* the αC regions and form extensible polymers. Such extensible polymers prevent blood cell adhesion [29]. In an *in vivo* condition, it is known that fibrinogen coats the surface of a fibrin clot and there is no evidence that blood cells adhere to the fibrinogen-coated fibrin clot [38–40]. Fibrinogen accumulating on the surface of a fibrin clot is supposed to form multilayered extensible polymers. Therefore, multilayered fibrinogen is used for an artificial surface, such as a stent in blood vessels, to prevent unwanted blood cell accumulation. The diameter of fibrinogen fibrils made in the present study is about 20 nm, and the fibrils would be equivalent to a multilayered polymer (16–18 layers) [29]. The effect of fibrinogen fibrils reported in this study on the adhesion of blood cells will be examined as part of our next project.

In this report, the DP-4 peptide, which was determined as a functional peptide of DP, could not reproduce the original function of DP. Instead, an unexpected function of the DP-4, that is, fibrinogen fibril formation, was found in this study. Considering from the present knowledge, and by extending the functional analyses, it is expected that the fibrinogen fibrils may have therapeutic uses.

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