ORIGINAL ARTICLE

Dermatopontin Regulates Fibrin Formation and its Biological Activity

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Dermatopontin (DP) is a small extracellular matrix component in the dermis. Fibrin is a major component of a provisional matrix that is formed just after wounding. Previously, we found that DP was present in the provisional matrix, and it interacted with fibrin. Here, we examined the role of DP on fibrin function. DP interacted with both the fibrin monomer and fibrils, and was incorporated into the fibrils during fibrin formation. A DP sequence, PHGQVVVAVRS, was identified as a fibrin-binding site, and a globular D domain of fibrin was the binding site for DP. DP accelerated fibrin fibril formation into structurally modified fibrils. Fibrin fibrils formed in the presence of DP enhanced both endothelial cell attachment and cell spreading. The attached cells developed a more organized cytoskeleton when compared with those that attached to fibrin fibrils only. The main receptor for cell adhesion was identified as αvβ3 integrin, and a cooperating receptor was a β1-containing integrin species, probably α5β1 integrin. These results indicate that DP can modify certain biological functions of fibrin, and thus a another function of this extracellular matrix protein was revealed. In addition, the fibrin–DP complex might become useful for developing an improved artificial matrix for improving wound healing.

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INTRODUCTION

Dermatopontin (DP) is a small acidic extracellular matrix protein with a molecular weight of 22 kDa (Okamoto and Fujiwara 2006; Okamoto *et al.*, 2011). It is an enriched protein within a population of small proteins having molecular weight <40–50 kDa in the dermis. In spite of its abundance in the dermis, knowledge about DP is limited. DP interacts with decorin, an abundant proteoglycan in the dermis (Lewandowska *et al.*, 1991; Okamoto *et al.*, 1996), and it accelerates collagen fibrillogenesis (MacBeath *et al.*, 1993). DP-knockout mice demonstrate an Ehlers–Danlos syndrome phenotype, showing skin fragility, disturbed lateral arrangement of collagen microfibrils, and a modified arrangement of corneal collagen (Takeda *et al.*, 2002; Cooper *et al.*, 2006).

Apart from the structural aspects above, functional analyses of DP are very limited, but we previously found that DP is a potent cell adhesion protein for keratinocytes (Okamoto *et al.*, 2010). In addition, we have found that DP interacts with transforming growth factor-β1, and that DP expression is also induced by this growth factor (Kuroda *et al.*, 1999; Okamoto *et al.*, 1999). DP was found to be overexpressed in the myocardial infarct zone; its expression profile correlated with collagen (Takemoto *et al.*, 2002). These findings suggest that DP affects epidermal cells and promotes wound healing of the skin and other tissues. Recently, we reported that DP is present in the provisional matrix mainly composed of fibrin and fibronectin, and in the serum (Kato *et al.*, 2011). We showed that DP interacts with both fibrin and fibronectin; it induces the formation of fibronectin fibrils by activating it. The activated fibronectin enhanced fibroblast cell adhesion. These findings indicate that DP functions not only in the dermis but also plays important roles in an early phase of wound healing.

Fibrin is the major protein in the provisional matrix (Singer and Clark, 1999; Diegelmann and Evans, 2004). It is formed from fibrinogen by thrombin cleavage of fibrinopeptides A and B. Fibrinogen is a dimeric molecule composed of three paired chains, namely Aα, Bβ, and γ chains, and the amino termini of these chains are connected, forming an E domain, and the carboxyl part of each monomer assembles and forms two D domains (Mosesson, 2005) (schematically explained in Figure 1). Once the vessels are damaged, circulating fibrinogen is locally converted to fibrin, and it forms a provisional matrix together with fibronectin and other components. Then, platelets, leukocytes, endothelial cells, and fibroblasts attach to the provisional matrix, leading to the formation of new vessels and granulation tissue.

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Abbreviations: DP, dermatopontin; HUVEC, human umbilical vein endothelial cell

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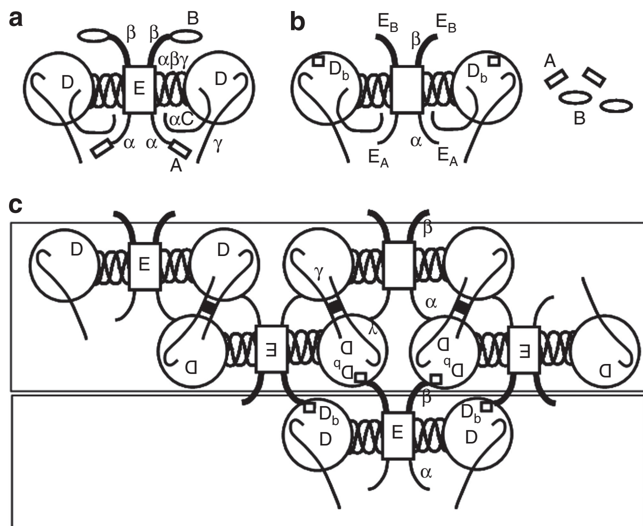

Figure 1. Schematic structures of fibrinogen, fibrin, and fibrin fibrils. (**a**) Fibrinogen molecule. Circles represent D domains; a central rectangle represents an E domain. Small rectangles A and ovals B indicate fibrinopeptides A and B, respectively. The α, β, and γ represent the α, β, and γ chains of fibrinogen. αC indicates a carboxyl terminal part of the α chain. (**b**) Fibrin monomer. E_A and E_B represent active sites of the α and β chains exposed by cleavage of fibrinopeptides A and B. Rectangles Db indicate active sites that bind with E_B domains. (**c**) Fibrin fibrils. Covalent bonds between two γ chains are indicated by thick black lines, by which γ chain dimers are formed. Each protofibril is surrounded by a rectangle.

During examination of the DP–fibronectin–fibrin interaction, we found that the interaction between DP and fibrin was potent. Therefore, we closely examined the DP–fibrin interaction and found its another function. We also examined the effects of DP on the biological activity of the fibrin, and based on the effects, we discuss the function of DP and a possible therapeutic use.

RESULTS

Identification of fibrin binding sites on DP

DP bound to immobilized fibrin and to fibrinogen in a dose-dependent manner (Figure 2a). When we examined the inhibition of DP binding to the immobilized fibrin by synthetic DP peptides, only DP-4 peptide specifically inhibited the interaction (Figure 2b). In addition, a biotinylated DP-4 peptide interacted with fibrin, whereas a biotinylated DP-4S peptide, a scrambled DP-4 peptide, did not bind (Figure 2c). These results indicate that the DP-4 peptide directly interacts with fibrin and the activity is sequence specific.

Next, the D and E domains of fibrinogen (Figure 1) were immobilized and their interaction with DP was examined. DP interacted with the D domain as well as fibrinogen but not with the E domain (Figure 2d). Similarly, the DP-4 peptide bound to the immobilized D domain (Figure 2e) but, as expected, the DP-4S peptide did not interact with the D domain (Figure 2e). In addition, the DP-4 peptide did not bind to the immobilized E domain (data not shown). Furthermore, the interaction between DP and the D domain was inhibited

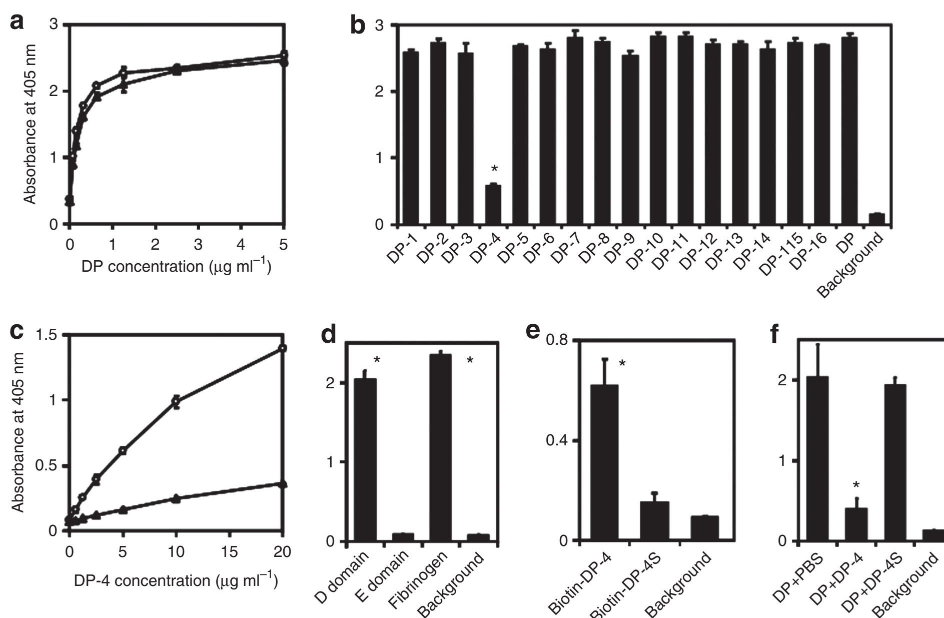

Figure 2. Identification of binding site in dermatopontin (DP). (**a**) A solid-phase assay of interaction between DP and fibrin (○) and fibrinogen (▲). DP at indicated concentrations was added. (**b**) Inhibition of the interaction between DP and fibrin by synthetic DP peptides. (**c**) Interaction between DP-4 peptide and fibrin. Biotinylated DP-4 peptide (○) was added. Biotinylated DP-4S peptide (▲) was used as a control. (**d**) Interaction between DP and the D and E domains of fibrinogen. (**e**) Interaction between DP-4 peptide and the D domain. (**f**) Inhibition of interaction between DP and D domain by DP-4 peptide. DP-4S peptide was used as a control. Data are presented as mean ± SD, *$P < 0.05$.

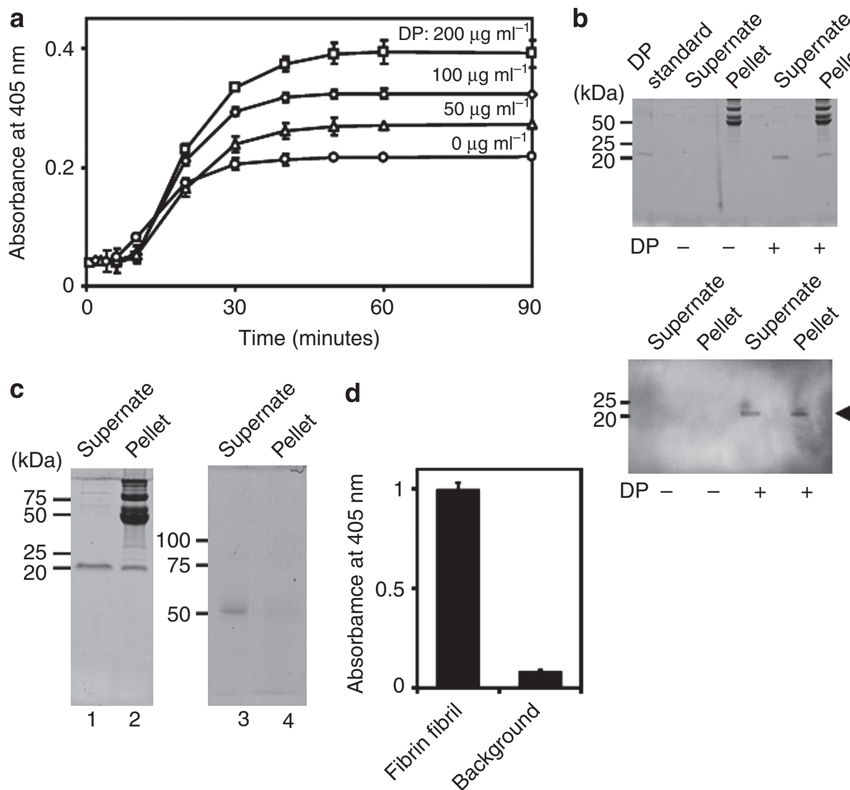

Figure 3. Effects of dermatopontin (DP) on fibrin fibril formation. (**a**) Fibrin formation assay. Fibrin was formed with DP, and the absorbance at 405 nm was monitored. Coincubated DP concentrations are shown in the graph. (**b**) DP is incorporated in the newly formed fibrin fibrils. The supernate and pellet of the fibrin fibril suspension made as above were analyzed. Coomassie stain is shown in the upper panel, and detection of DP by western blotting is shown in the lower panel. (**c**) DP interacts with preformed fibrin fibrils. Liquid-phase assay. Fibrin fibrils were preformed and DP (lanes 1 and 2) or BSA (lanes 3 and 4) were incubated and were analyzed. BSA was analyzed with a conventional 10% gel in nonreducing conditions. (**d**) Solid-phase assay. The left lane indicates DP interaction with fibrin fibrils and the right lane is the background.

by the DP-4 peptide but not by the DP-4S peptide (Figure 2f). These results suggest that DP interacted with the fibrinogen D domain through the DP-4 sequence.

DP affects fibrin formation

Next, we examined the effect of DP on fibrin fibril formation. When fibrinogen is incubated with thrombin, the resulting fibrin molecules polymerize and the turbidity of the solution increases (Wolberg, 2007). When fibrinogen alone was incubated with thrombin, the turbidity increased after a short lag time and then reached a plateau at ∼30 minutes (Figure 3a). In the presence of DP, the final turbidity became greater in proportion to the DP concentration (Figure 3a). In contrast, when DP and thrombin, or DP and fibrinogen, were coincubated, no apparent turbidity change was observed (data not shown). DP did not influence the thrombin activity as assessed by a chromogenic substrate S-2238 (data not shown). Crosslink formation, as assessed by the γ-chain dimer (Figure 1), was not affected by DP (data not shown). These data indicate that DP directly accelerates fibrin fibril formation.

Next, we examined whether DP is incorporated into fibrin fibrils. Fibrinogen was coincubated with DP and thrombin, and the resulting insoluble fibrils were separated by centrifugation. Figure 3b shows a clear 22-kDa band in the pellet, and the band was not seen in that formed in the absence of DP. The identity of the band as DP was confirmed by western blotting (Figure 3b). Thus, it was proven that DP not only interacts with preformed fibrin monomers, but also is incorporated into the fibrin fibrils.

Because DP was incorporated in fibrin fibrils, the interaction between fibrin fibrils and DP was examined. When DP was incubated with preformed fibrin fibrils in solution, DP coprecipitated with the fibrin fibrils after centrifugation (Figure 3c, lane 2). Coprecipitated BSA was also present but in a trace amount (Figure 3c, lane 4). When preformed fibrin fibrils were immobilized on a solid phase, DP also showed a clear interaction (Figure 3d). Thus, DP interacts with fibrinogen, fibrin monomer, and fibrin fibrils, and these results support the incorporation of DP in fibrin fibrils as shown in Figure 3b.

DP affects the structure of fibrin fibrils

The increase in the turbidity by DP during fibrin fibril formation was reminiscent of certain structural changes of the fibrin fibrils. Therefore, we compared the ultrastructure of fibrin fibrils formed in the presence and absence of DP. Fibrin alone formed fibrils with diameters of ∼50 nm

(Figure 4a and b). Thinner fibrils of several nm in diameter surrounded the thick fibrils and often merged into them (Figure 4b). In contrast, fibrin in the presence of DP formed fibrils thicker in diameter (50–100 nm; Figure 4c and d). Furthermore, there were fewer observable thinner fibrils around the thicker fibrils, and these thicker fibrils appeared more solid. No fundamental difference of fibrinolysis of these fibrin fibrils was seen (data not shown).

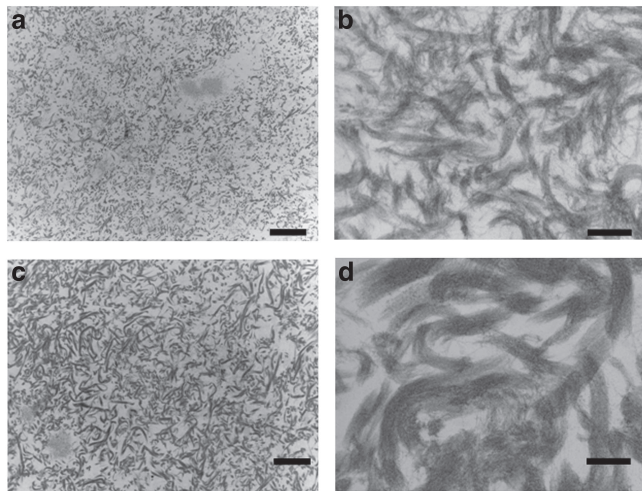

Figure 4. Morphological change of fibrin fibrils by dermatopontin (DP). (**a**, **b**) Fibrin fibrils formed in the absence of DP. (**c**, **d**) Those formed in the presence of 200 μg ml^{-1} of DP. Scale bar = 2 μm (for **a**, **c**; original magnification ×10,000) and 200 nm (for **b**, **d**; original magnification ×100,000).

DP enhances adhesion and spreading of HUVECs on fibrin

Next, we examined the effect of DP on the biological activity of fibrin. Human umbilical vein endothelial cell (HUVEC) adhesion to fibrin was examined (Figure 5). HUVECs adhered only weakly to DP when immobilized solely at high concentration (data not shown), and apparent adhesion was not seen with the concentrations used in this experiment (Figure 5a). On the other hand, HUVECs adhered to fibrin in a dose-dependent manner (data not shown). When HUVECs were inoculated on fibrin made in the presence of DP, the number of attached cells was enhanced in a dose-dependent manner (Figure 5a). The enhancement reached a plateau at ~2.5 μg ml^{-1} of DP. Accordingly, cell spreading was clearly enhanced as a function of the coincubated DP concentrations (Figure 5b and c). The enhancement of cell adhesion was strongly inhibited by anti-$\alpha v\beta 3$ integrin antibody and weakly by anti-αv-subunit antibody (Figure 5d). A complete inhibition was achieved with a mixture of anti-$\alpha v\beta 3$ and $\beta 1$-subunit antibodies. An almost identical inhibition profile was obtained in the cell adhesion assay to fibrin only (data not shown). These data suggest that structurally modified fibrin fibrils by DP have an enhanced cell adhesion activity, and that the main cellular receptor is an $\alpha v\beta 3$ integrin.

Fibrin–DP complex supports HUVEC cytoskeletal organization

Then, we investigated the effect of DP on the cytoskeletal formation of HUVECs adhering to fibrin. HUVECs adhered to

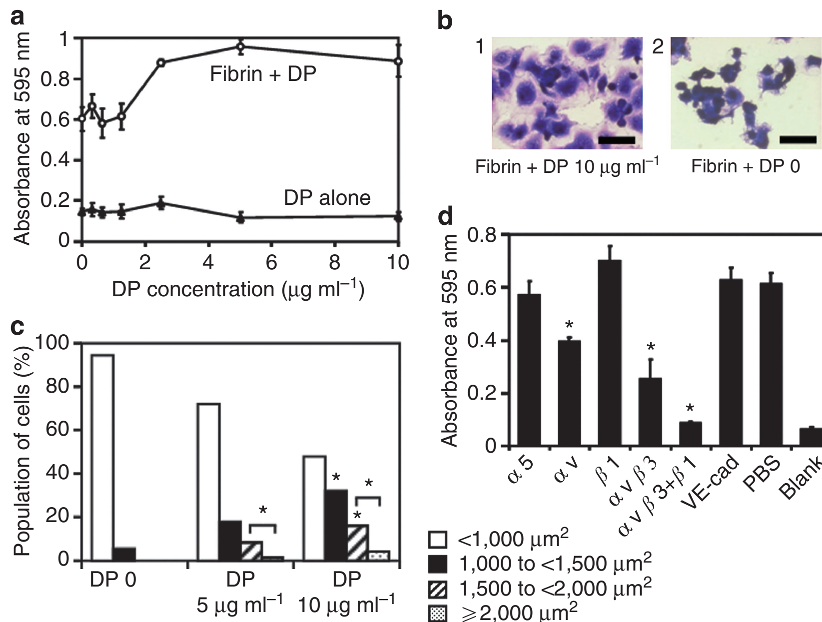

Figure 5. Effect of dermatopontin (DP) on human umbilical vein endothelial cell (HUVEC) adhesion to fibrin. (**a**) Fibrin fibrils were formed in the presence of DP and HUVEC adhesion was quantified. A horizontal line indicates DP concentrations. Data are presented as mean ± SD. (**b**) Morphologies of the HUVECs adhering to the fibrin fibrils formed with DP. Scale bars = 50 μm. (**c**) Quantification of cell spreading. The area of each adhered cell was measured, and histograms were made. Classification of each bar is explained at the bottom. Population bars that significantly differed from those in the control experiment ("DP 0") are marked by asterisks. *$P<0.05$. (**d**) Inhibition of cell adhesion to fibrin–DP substrate by antibodies. Antibodies are indicated in the horizontal line. *$P<0.05$. PBS, phosphate-buffered saline; VE-cad, vascular endothelial–cadherin.

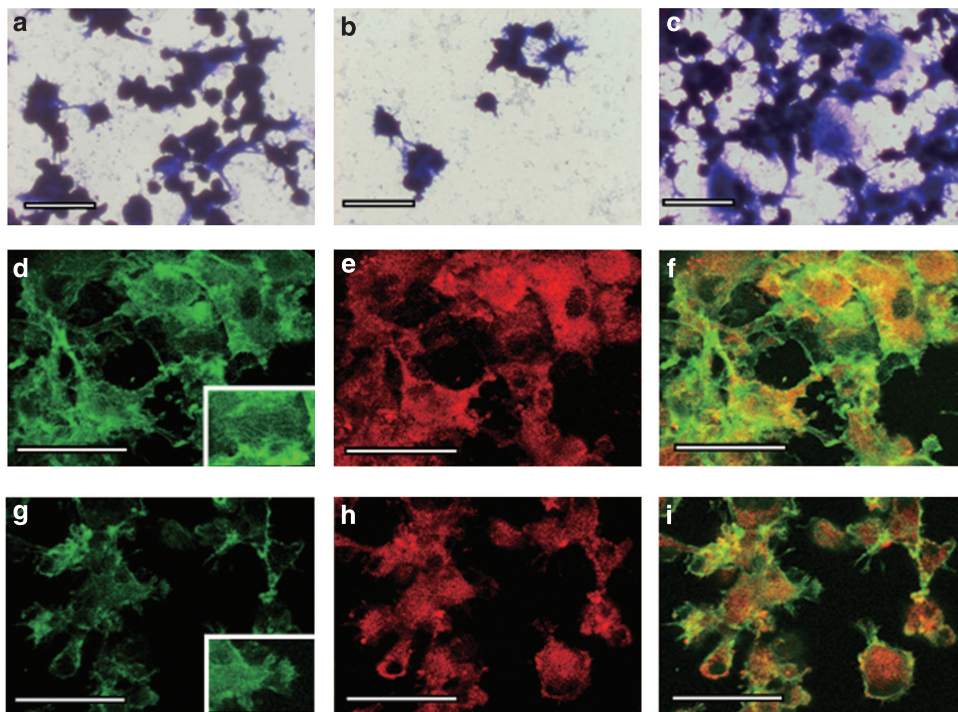

Figure 6. Appearance of the cytoskeleton of human umbilical vein endothelial cells (HUVECs) adhering to fibrin–dermatopontin (DP) matrix. (**a**–**c**) Crystal violet stains of HUVECs that adhered to (**a**) fibrin fibrils alone, (**b**) DP alone, and (**c**) fibrin fibrils made with DP, respectively. (**d–f**) HUVECs that adhered to the fibrin fibrils made in the presence of DP. (**g–i**) HUVECs that adhered to the fibrin fibrils alone. (**d**, **g**) Phalloidin staining, (**e**, **h**) vinculin staining, and (**f**, **i**) merged images. Inserts in **d** and **g** are close-up views of the cytoskeleton. Bar = 50 μm.

immobilized fibrin (Figure 6a), but the spreading was poor as visualized by crystal violet stain, and a fluorescent analysis demonstrated limited cytoskeleton formation (Figure 6g–i). DP alone promoted weak cell adhesion, and minimal cell spreading (Figure 6b). On the other hand, spreading of the cells that adhered to the fibrin–DP complex was enhanced (Figure 6c), as was observed in the 96-well plate. Similarly, the formation of the actin fibers and accumulation of vinculin in these cells were enhanced (Figure 6d–f) when compared with those formed on immobilized fibrin alone (Figure 6g–i).

DISCUSSION

Previously, we reported that DP interacts with fibrin, and the interaction of DP with fibrinogen was weaker than that with fibrin (Kato *et al.*, 2011). However, in this study, we found that DP also interacts with fibrinogen at a quite similar level as with fibrin. In this study, the results are highly reproducible and the reason why the discrepancy occurred might be because of poor optimization of the reaction conditions of the previous DP–fibrinogen interaction experiment.

These data indicate that DP interacts not only with the fibrin monomer but also with the higher-order structure of fibrin fibrils. We showed that the D domain of fibrin binds to DP, but the isolated α, β, and γ chains of D domain did not interact with DP (data not shown). Therefore, DP recognized a certain three-dimensional structure in the D domain. On the other hand, a DP-4 peptide was identified as an interaction site with the D domain. The DP-4 peptide has also been indentified as a syndecan-binding site in our previous study (Okamoto *et al.*, 2010). The DP-4 peptide has multiple functions for cell adhesion and for interaction between the extracellular matrix proteins. However, it still remains unknown if the DP-4 peptide can reproduce the major functions of DP. If DP-4 peptide functions like DP, it would suggest an attractive possibility for controlling certain biological events by this peptide.

Newly generated fibrin monomers first form linear protofibrils. Then the fibrils are laterally connected, making a network recognized as the fibrin clot (Mosesson, 2005). It is believed that the lateral association of the protofibrils reflects the turbidity change (Figure 1) (Wolberg, 2007). Therefore, the DP-induced turbidity increase during fibrin fibril formation indicates that DP accelerates the lateral association of fibrin protofibrils. This observation was supported by the ultrastructure of the fibrin fibrils formed in the presence of DP, where the diameters of the fibrils are increased. The lateral association of the protofibrils is generally believed to occur via the interaction of an E_B site of β chain with a Db site of the D domain of another fibrin monomer (Figure 1) (Medved *et al.*, 1993; Everse *et al.*, 1998; Betts *et al.*, 2006). As DP interacts with the D domain, it is feasible that DP affects the interaction between E_B and Db sites.

Because DP remained in the newly formed fibrin fibrils, it is likely that DP functions as a connecting molecule between

adjacent fibrin molecules. The resulting structural change of fibrin fibrils would enhance both HUVEC adhesion and spreading. Several cell surface receptors have been identified for adhesion to fibrin (Sakura *et al.*, 1997; Bach *et al.*, 1998). As expected, $\alpha v\beta 3$ integrin was identified as a main receptor involved in the adhesion of HUVECs to the fibrin–DP complex. In this study, we also confirmed that the $\alpha v\beta 3$ integrin is a main receptor for adhesion to fibrin (data not shown). Thus, within the fibrin–DP complex, the cells adhere to fibrin. In addition, $\beta 1$-containing integrin cooperated with $\alpha v\beta 3$ integrin for cell adhesion. As $\alpha 5\beta 1$ integrin has been identified as a fibrinogen receptor (Suehiro *et al.*, 1997), this integrin species is supposed to contribute to the cooperation. The receptors interact with fibrin at the αC region of the $A\alpha$ chain that extends from a D domain (Figure 1a and b) (Cheresh *et al.*, 1989; Felding-Habermann *et al.*, 1992), and hence it is supposed that after binding of DP with the D domain, the conformation of the adjacent αC region changes so that it is more accessible for the cells.

Fibrin serves as a scaffold for endothelial cells in the initial phase of the wound healing for allowing angiogenesis (Lamalice *et al.*, 2007). The fibrin structure changes depending on diseases or on chemical conditions, and its function can be modified (Laurens *et al.*, 2006). Accordingly in this study, DP promoted the formation of thicker fibrin fibrils and enhanced cell adhesion and spreading. In a previous report, thick fibrin fibrils suppressed capillary tube formation (Nehls and Herrmann, 1996). On the other hand, in another report, the ingrowth of tubular structures is accelerated by thick fibrin fibrils (Collen *et al.*, 1998). Hence the behavior of the endothelial cells on the structurally modified fibrin fibrils may vary depending on the assay used. Thus, at present, the fate of the enhanced cell adhesion and spreading by a simple combination of DP and fibrin remains uncertain. Further investigation of the modification of the cell behavior by fibrin–DP complex will be required.

In this study, DP was used in a concentration range between several $\mu g\,ml^{-1}$ and several hundred $\mu g\,ml^{-1}$. Approximately 50 mg of DP can be purified from 1 kg of the dermis (Okamoto *et al*, 2011), and hence the physiological DP concentration in the dermis can be estimated to be $\sim 50\,\mu g$ per g wet weight. The DP concentration in the fibrin provisional matrix has not been determined, but considering from its concentration in the surrounding dermis, the concentration would be much less than the dermal level. Thus, in some experiments in this study, the DP concentrations exceeded these physiological concentrations. Therefore, the observed effects of DP on fibrin fibril formation and, possibly, cell adhesion may not always reflect the *in vivo* state.

The effects of DP on fibrin functions observed in this study imply a possibility that certain biological events, such as wound healing, are regulated by externally adding DP-loaded artificial fibrin provisional matrix. The authentic provisional matrix does not comprise fibrin only, but it contains fibronectin as a major nonfibrin component (Clark *et al.*, 1982). In our previous report, DP was found to activate fibronectin (Kato *et al.*, 2011), and the activated fibronectin demonstrated a pronounced cell adhesion activity (Morla *et al.*, 1994). Taking fibronectin activation and the enhancement of cell adhesion to fibrin into account, DP is a likely factor for positively regulating wound healing by combining with these provisional matrix components. By adjusting the composition of these components, it is expected that an improved artificial provisional matrix for controlling wound healing might be developed in the future.

MATERIALS AND METHODS

Materials

DP was purified and an anti-DP carboxyl terminal peptide antibody was produced as reported previously (Okamoto *et al.*, 1996). HUVECs were purchased from DS Pharma Biomedical (Osaka, Japan). Bovine fibrinogen and human D and E fragments were purchased from Merck-Calbiochem Japan (Tokyo, Japan). Function-blocking anti-integrin subunit antibodies (LM609 for integrin $\alpha v\beta 3$, P1D6 for $\alpha 5$, and 6S6 for $\beta 1$) were from Chemicon (Temecula, CA), and BV9 for vascular endothelial–cadherin was from Hycult Biotech (Plymouth Meeting, PA). An anti-vinculin antibody was from Enzo Life Sciences International (Plymouth Meeting, PA), and an Alexa Fluor 594–labeled anti-mouse IgG antibody was from Invitrogen (Carlsbad, CA). Overlapping peptides of DP were synthesized as described previously (Okamoto *et al.*, 2010). The nomenclature and the amino acid sequences are the same as those described in the report.

Solid-phase assay for protein interaction

The experiment was done according to a protocol described previously (Kato *et al.*, 2011). In brief, fibrinogen, D and E domains were coated in wells of a 96-well plate at $10\,\mu g\,ml^{-1}$. When necessary, the fibrinogen was converted to fibrin by thrombin. This was recognized as fibrin monomer. After blocking, DP was added to the wells and was incubated overnight. Bound DP was probed with anti-DP antibody. For assessing the interaction between fibrin fibrils and DP, fibrinogen solution at $5\,\mu g\,ml^{-1}$ in 50 mM HEPES buffer (0.14 M NaCl and 20 mM $CaCl_2$, pH 7.5) was mixed with thrombin at $1\,U\,ml^{-1}$, the mixtures were incubated overnight at room temperature, and the generated fibrin fibrils were immobilized. The DP concentration was $1\,\mu g\,ml^{-1}$.

Biotinylation of DP-4 peptide

DP-4 peptide truncated by one amino-terminal proline (HGQVVVAVRS) and a scrambled peptide DP-4S (VRVHVPVQGSA) were dissolved in 0.1 M phosphate buffer (0.15 M NaCl, pH 8.0). Sulfo-NHS-LC-biotin was added and the mixtures were incubated for 3 hours at 4 °C. The reaction was terminated by an addition of Tris/HCl, pH 7.5, at a final concentration of 50 mM and the mixtures were incubated for 1 hour at 4 °C. The mixture was placed on a Biogel P4 (Bio-Rad, Hercules, CA) column equilibrated with 0.1% trifluoroacetic acid/30% acetonitrile. Void fractions were collected and were lyophilized and the samples were dissolved in phosphate-buffered saline. The biotinylated truncated DP peptide is termed biotinylated DP-4 peptide in this study.

Fibrin formation assay

Fibrinogen dissolved in HEPES buffer was added to wells of a 96-well plate, and a mixture of thrombin and DP was added to the wells. The turbidity change of the solution was monitored at 405 nm. The

final concentration of fibrinogen and thrombin was 1.6 mg ml^{-1} and 1 U ml^{-1}, respectively. An aliquot of the samples was taken and analyzed by conventional 10% gel, or 12% T, 0.3% C tricine gel in reducing conditions (Schägger and von Jagow, 1987).

Liquid-phase assay for DP–fibrin fibril interaction

Fibrin fibrils were formed, and then Tween-20 was added at a final concentration of 0.05%. DP or BSA was added and incubated overnight. The initial concentration of fibrinogen was 800 μg ml^{-1}, and of DP and BSA were 100 μg ml^{-1}. After centrifugation, the pellet was washed, and analyzed by SDS-PAGE.

For detection of DP, the electrophoresed proteins were western blotted to polyvinylidene fluoride membranes, followed by incubation with an anti-DP rabbit antibody, then with a horseradish peroxidase-conjugated anti-rabbit IgG antibody. Images were visualized using an enzyme chemiluminescence reagent.

Electron microscopy

After the fibrin formation assay, the samples were transferred to microtubes and were centrifuged, and the pellet was fixed with 2% formaldehyde (2.5% glutaraldehyde), 50 mM cacodylate buffer, pH 7.4, containing 3 mM $CaCl_2$. The samples were embedded in Epon resin, sliced as 1 μm sections, were stained with 1% OsO_4 and 1% tannic acid, dehydrated, dried, and coated with OsO_4. All the observations and photographs were performed at an acceleration voltage of 15 kV.

Cell adhesion assay

HUVECs were cultured in KJB-210 medium at 37 °C. The cell adhesion assay was performed according to the protocol described previously (Kato *et al.*, 2011). Briefly, 2.5 μg ml^{-1} of fibrinogen was incubated with 1 U ml^{-1} of thrombin in the presence and absence of DP for 2 hours, and the samples were coated in the wells. HUVECs suspended at 30,000 cells per 100 μl in DMEM were inoculated and cell adhesion was assessed after 1 hour. The dried stained cells in the center of the wells were photographed and the cell surface areas were calculated. Finally, the dye was eluted and an absorbance at 595 nm was determined.

For an assessment of receptors for cell adhesion, fibrin was made using 2.5 μg ml^{-1} of fibrinogen and 5 μg ml^{-1} of DP, and the cell suspension was incubated with function-blocking antibodies at 10 μg ml^{-1}.

Cytoskeletal staining

A total volume of 20 μg ml^{-1} of fibrinogen was incubated with 1 U ml^{-1} of thrombin in the absence or presence of 40 μg ml^{-1} of DP for 2 hours. Then, 10 μl of samples was coated on glass plates (Dako Japan, Kyoto, Japan) overnight. After blocking with 1% BSA, HUVECs were suspended in DMEM at a density of 10,000 cells per 10 μl, and the cells were inoculated. After adhesion for 1 hour at 37 °C, the attached cells were fixed with 3.7% formaldehyde, dehydrated in acetone, followed by rehydration in phosphate-buffered saline, and permeabilized with 0.1% Triton X-100. 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 microscopy, PASCAL (Carl Zeiss, Oberkochen, Germany).

Statistical analyses

A statistical assessment for protein interaction was done using *t*-test, and a *P*-value of <0.05 was recognized as significant. The relevant experiments were repeated more than three times in triplicate. For an assessment of cell spreading, surface areas of the attached cells were measured. The measured areas were classified into four populations as shown in Figure 5. Then, a population with the smallest cell surface area and another population was selected from both control and experimental groups, respectively, and a 2×2 χ^2 test was performed.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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