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

Weimin Wu [a], Osamu Okamoto [b,*], Aiko Kato [a], Noritaka Matsuo [c], Jun Kumai [d], Motoyoshi Nomizu [d], Sakuhei Fujiwara [b]

[a] Department of Plastic Surgery, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu-shi, Oita 879-5593, Japan
[b] Department of Dermatology, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu-shi, Oita 879-5593, Japan
[c] Department of Biochemistry, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu-shi, Oita 879-5593, Japan
[d] Laboratory of Clinical Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1, Horinouchi, Hachioji, Tokyo 192-0392, Japan

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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.

* Corresponding author. Tel.: +81 97 586 5882; fax: +81 97 586 5889.
E-mail address: ookamoto@oita-u.ac.jp (O. Okamoto).

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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