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Full Length Article

Effect of fibrinogen, fibrin, and fibrin degradation products on transendothelial migration of leukocytes

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ABSTRACT

In spite of numerous studies on the involvement of fibrinogen in transendothelial migration of leukocytes and thereby inflammation, there is still no clear understanding of which fibrin(ogen) species can stimulate leukocyte transmigration. Although we have previously proposed that interaction of fibrin with the VLDL receptor (VLDLR) promotes leukocyte transmigration, there is no direct experimental evidence for the involvement of fibrin in this process. To address these questions, we performed systematic studies of interaction of VLDLR with fibrinogen, fibrin, and their isolated recombinant BβN- and βN-domains, respectively, and the effect of various fibrin(ogen) species on transendothelial migration of leukocytes. The results obtained revealed that freshly purified fibrinogen does not interact with VLDLR in solution and has practically no effect on leukocyte transmigration. They also indicate that the VLDLR-binding site is cryptic in fibrinogen and becomes accessible upon its adsorption onto a surface or upon its conversion into fibrin. We also found that the D-D:E_1 complex and higher molecular mass fibrin degradation products, as well as soluble fibrin and fibrin polymers (clots) anchored to the endothelial monolayer, promote leukocyte transmigration mainly through the VLDL receptor-dependent pathway. Thus, the results of the present study suggest that fibrin degradation products and soluble fibrin that may be present in the circulation *in vivo*, as well as fibrin clots that may be deposited on the surface of inflamed endothelium, promote leukocyte transmigration. These findings further clarify the molecular mechanisms underlying the fibrin-VLDLR-dependent pathway of leukocyte transmigration and provide an explanation for a possible (patho)physiological role of this pathway.

1. Introduction

Fibrinogen is the major plasma protein that plays an important role in hemostasis. Fibrinogen molecule consists of two identical sub-units each of which is composed of three non-identical polypeptide chains, Aα, Bβ, and γ [1]. The N-terminal portions of all six chains linked together by a number of disulfide bonds form the central part of the molecule, which was originally termed the N-terminal disulfide knot (NDSK) [2]. The central part of fibrinogen, also called the E region [3], is released from the molecule upon digestion with plasmin or CNBr in the form of E fragment or NDSK fragment, respectively [1]. Digestion of fibrinogen with plasmin also produces the D fragment derived from two identical terminal D regions of the molecule [1,3]. The N-terminal portions of the Aα and Bβ chains contain fibrinopeptides A and B including amino acid residues 1-16 and 1-14, respectively [1,3]. Upon vascular injury, when the blood coagulation cascade is activated resulting in generation of thrombin, the later converts fibrinogen into fibrin by proteolytic removal of its fibrinopeptides. Fibrin molecules spontaneously polymerize through the interaction between their newly exposed polymerization sites of the central region including knobs “A” and “B” and complementary holes “a” and “b” of the D regions [3]. The resultant fibrin polymer, which is reinforced by covalent crosslinking with factor XIIIa [4], seals damaged vasculature thereby preventing the loss of blood. Crosslinked fibrin polymers (clots) can be dissolved by proteolytic cleavage with plasmin resulting in soluble fibrin degradation products.

Besides its prominent role in hemostasis, fibrinogen participates in various physiological and pathological processes including inflammation. For example, it was shown that fibrinogen is required for efficient inflammatory responses *in vivo* [5], fibrin deposition contributes to the pathogenesis of intraabdominal abscess formation [6], and fibrin(ogen) mediates acute inflammatory response to implanted biomaterials [7].

Abbreviations: VLDLR, very low density lipoprotein receptor; FDP, fibrin degradation products; HMM-FDP, high molecular mass fibrin degradation products; FpB, fibrinopeptide B; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; RAP, receptor-associated protein

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Recruitment of leukocytes from the circulation to sites of inflammation is an integral part of the inflammatory response and transendothelial migration of leukocytes is a key step in such recruitment [8–10]. Numerous data suggest that fibrin(ogen) plays a prominent role in leukocyte transmigration and thereby inflammation; however, the proposed mechanisms of fibrin(ogen)-mediated leukocyte transmigration raise a number of questions. For example, it was suggested that binding of fibrinogen to vascular cell receptors mediates a specific pathway of cell-to-cell adhesion by bridging together leukocytes and endothelial cells [11]. Further, it was proposed that such fibrinogen-mediated intercellular bridging, which occurs through the interaction of fibrinogen with the leukocyte receptor Mac-1 and endothelial cell receptor ICAM-1, promotes transendothelial migration of leukocytes [12,13]. However, subsequent studies revealed that Mac-1 binding sites are cryptic in soluble fibrinogen [14,15]. This raises a question of how fibrinogen in the circulation may interact with Mac-1 and promote leukocyte transmigration in the proposed (Mac-1)-fibrinogen-(ICAM-1)-dependent intercellular bridging pathway.

Another suggested mechanism of fibrinogen-dependent leukocyte transmigration involves fibrin-derived products [16]. Specifically, it was shown in the *in vitro* experiments that fibrin-derived NDSK-II fragment, which corresponds to the central part of the fibrin molecule, promotes leukocyte transmigration and was proposed that NDSK-II bridges leukocytes to the endothelium through the interaction with endothelial receptor VE-cadherin and leukocyte integrin receptor CD11c [16]. It was also suggested that *in vivo* leukocyte transmigration is stimulated by fibrin degradation product E_1 fragment, which is an analog of the NDSK-II fragment [16,17]. Thus, the proposed (CD11c)-E_1 fragment-(VE-cadherin)-dependent intercellular bridging pathway of leukocyte transmigration utilizes the ability of the E_1 fragment to interact simultaneously with CD11c and VE-cadherin through its N-terminal α chain sequence Gly17-Pro-Arg and β chain sequence 15–42, respectively [16,17]. However, upon fibrin degradation, the E_1 fragment is released only in a complex with the D-D fragment (D-D:E_1 complex) [18,19] in which the αGly17-Pro-Arg sequences are not accessible. This raises a question of whether such D-D:E_1 complex or larger soluble FDP can promote leukocyte transmigration through the proposed (CD11c)-E_1 fragment-(VE-cadherin)-dependent bridging pathway.

A third suggested pathway of fibrinogen-dependent leukocyte transmigration involves fibrin. Namely, we found that fibrin interacts with the very low density lipoprotein receptor (VLDL receptor, VLDLR) through a pair of fibrin βN-domains, each consisting of the β chain residues 15-66, and demonstrated that RAP, an antagonist of ligand binding of VLDLR and other low density lipoprotein (LDL) receptor family members, inhibits transendothelial migration of leukocytes [20]. Based on these and some other findings, we proposed that fibrin promotes leukocyte transmigration through a fibrin-VLDLR-dependent pathway [20]. However, to stimulate leukocyte transmigration in our *in vitro* experiments we used NDSK-II as a simple soluble model of fibrin [20,21], which, in fact, resembles more closely fibrin degradation product E_1 fragment. Therefore, there is still no direct experimental evidence that fibrin can stimulate leukocyte transmigration through the proposed fibrin-VLDLR-dependent pathway. Furthermore, a well-established fact that fibrin βN-domains contain secondary polymerization sites (knobs “B”) that interact with the complementary sites (holes ‘b”) of the D regions upon fibrin polymerization [3] raises a question of whether their VLDLR-binding sites are accessible in fibrin polymers. Moreover, our recent study revealed that the VLDLR-binding sites are mainly located in the C-terminal halves of fibrin βN-domains [22], i.e. they may be distant from fibrinopeptides B that are removed upon conversion of fibrinogen into fibrin. This raises another question of whether these sites are accessible for interaction with VLDLR in the fibrinogen molecule.

Thus, in spite of numerous studies on the involvement of fibrin (ogen) in transendothelial migration of leukocytes, there is still no clear evidence that fibrinogen or fibrin can promote leukocyte transmigration. It is also unclear which fibrin degradation product(s) may play a role in this process. To address the questions mentioned above, we performed systematic studies of interaction of fibrinogen, fibrin, and fibrin degradation products with the VLDL receptor, as well as the effect of these fibrin(ogen) species on transendothelial migration of leukocytes.

2. Materials and methods

2.1. Proteins, antibodies, and reagents

Human fibrinogen depleted of plasminogen, von Willebrand factor and fibronectin (FIB 3), human plasmin, human thrombin, and HRP-conjugated sheep anti-human fibrinogen antibodies were purchased from Enzyme Research Laboratories. The soluble form of human VLDL receptor, sVLDLR, was prepared using the *Drosophila* Expression System (Invitrogen) as previously described [23]. The recombinant fibrin (ogen) $(Bβ1\text{-}66)_2$ and $(β15\text{-}66)_2$ fragments were produced in *E. coli* and purified as we described earlier [23]. Human receptor-associated protein (RAP) was expressed in *E. coli* and purified as described in [24]. Anti-VLDL receptor monoclonal antibodies mAb 5F3 and mAb 1H10 were purified from hybridoma supernatants by affinity chromatography on Protein A-Sepharose (Sigma-Aldrich) as we described earlier [21]. Goat secondary anti-mouse polyclonal antibodies conjugated with HRP and HRP substrate SureBlue TMB were from KPL. Calcein AM fluorescent dye and phorbol 12-myristate 13-acetate (PMA) were obtained from BD Biosciences and Promega, respectively, and Gly-Pro-Arg-Pro peptide and N-formyl-Met-Leu-Phe (fMLP) were from Sigma-Aldrich.

2.2. Preparation of acidic fibrin monomer, soluble fibrin, and fibrin(ogen) degradation products

Acidic fibrin monomer was prepared by dissolving non-crosslinked human fibrin in 0.125% acetic acid as described previously [25–27]. To prepare Alexa 488-labeled fibrin monomer, fibrinogen was mixed with Alexa Fluor 488 dye (ThermoFisher Scientific) at 1 to 20 molar ratio, as recommended by the manufacturer, and incubated for 1 h at room temperature. Unconjugated dye was removed on NAP-25 column Sephadex G-25 (GE Healthcare) and Alexa 488-labeled fibrinogen was converted into acidic Alexa 488-labeled fibrin monomer as described above. Soluble fibrin was prepared by rapid dilution of acidic fibrin monomer at 20 μM with Iscove's modified Dulbecco's medium (IMDM, Invitrogen) containing fibrinogen-derived D_1 fragment to final fibrin concentration of 1.5 μM and final fibrin to D_1 molar ratio of 1 to 4.

Fibrinogen-derived D_1 and E_3 fragments were prepared from plasmin digest of fibrinogen as described earlier [28]. The NDSK-II fragment was prepared from CNBr digest of fibrinogen followed by cleavage of its fibrinopeptides from NDSK with thrombin-agarose as described [29,30]. Fibrin-derived D-D:E_1 complex and dimeric D-D fragment were prepared from plasmin digest of crosslinked fibrin using procedures described earlier [28,31]. The high molecular mass fibrin degradation products (HMM-FDP) were prepared from plasmin digest of crosslinked fibrin using the same procedures [28,31] with some modifications. Briefly, plasmin was added to crosslinked fibrin at 1 to 2000 molar ratio in Tris-buffered saline (TBS) containing 10 mM $CaCl_2$ and incubated for 1 h at room temperature. The reaction was terminated by PMSF and aprotinin, and plasmin was removed from the digest on Lys-Sepharose column (GE Healthcare). SDS-PAGE analysis of the early plasmin digest of crosslinked fibrin polymers revealed that the major degradation products were the E_1 fragment, D-D dimer, and HMM-FDP (Fig. 1, inset). Subsequent fractionation of this digest by size-exclusion chromatography on Superdex-200 column revealed two major peaks, peak 1 was eluted with the free volume before fibrinogen and peak 2 was eluted between fibrinogen and the D-D fragment (Fig. 1). SDS-PAGE analysis of each of these peaks revealed the presence