Thrombosis Research 135 (2015) 1025–1030

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

A recombinant fragment of von Willebrand factor reduces fibrin-rich microthrombi formation in mice with endotoxemia

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ARTICLE INFO

Article history:
Received 25 September 2014
Received in revised form 24 February 2015
Accepted 25 February 2015
Available online 3 March 2015

Keywords:
microvascular thrombosis
endotoxemia
disseminated intravascular coagulation (DIC)
platelet adhesion
and fibrin

ABSTRACT

Introduction: Disseminated fibrin deposition in the microvasculature such as in disseminated intravascular coagulation (DIC) arises from uninhibited activated coagulation secondary to sustained systemic inflammation. Currently there is no treatment for DIC. Treating the underlying trigger and supportive care are the current recommendations to manage DIC. This study aims at using recombinant von Willebrand factor (VWF) A2 domain polypeptide to inhibit VWF–mediated platelet adhesion to fibrin and prevent DIC.
Materials and Methods: We use flow chamber assay to test the capacity of purified A2 protein to inhibit platelet adhesion to immobilized fibrin(ogen) and platelet-fibrin clot formation. We use a murine model of lipopolysaccharide-induced DIC to examine the effect of A2 protein on DIC.
Results: The A2 protein blocked flow-dependent platelet adhesion to fibrin, delayed fibrin polymerization, and inhibited platelet-fibrin clot formation *in vitro*. The infusion of the purified A2 protein to the endotoxin-treated mice prevented fibrin-rich microthrombi formation in brain, lung, kidney, and liver. It also attenuated levels of inflammatory mediators, and markedly reduced mortality rates at 96 hours.
Conclusions: The A2 protein inhibited platelet interaction with fibrin(ogen). Furthermore, A2 prevented disseminated fibrin-rich microthrombi and decrease mortality in a lipopolysaccharide-induced DIC murine model. A2 could provide a novel therapeutic approach in critically ill patients with uninhibited activated coagulation and disseminated fibrin deposition such as DIC.

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Introduction

Most of critically ill patients have some evidences of activated coagulation. However, with sustained systemic inflammation the activated coagulation may become uncontrollable and cause tissue damage. Uninhibited activated coagulation will lead to thrombotic microangiopathy, which is a family of syndromes associated with disseminated microvascular thromboses. Disseminated intravascular coagulation (DIC) is an entity in the spectrum of thrombotic microangiopathy which can contribute to multiple organ dysfunction syndrome and death [1]. DIC can occur in 50-60% of septic patients [2,3], in 14-40% of new onset thrombocytopenic critically ill patients [4–6], and in 8% of all critically ill patients [7]. Conditions associated with triggering DIC include sepsis, trauma, burn, vasculitis, obstetric complications, and toxin exposure. Autopsies performed in patients who died from DIC reveal fibrin-rich microthrombi in small and mid-size vessels in all organs [8–10]. Reported mortalities associated with DIC range from 22-75% [2–4,7]. Currently, the recommended managements for DIC are 1) treat the underlying trigger and 2) provide supportive care [11]. Multiple mono-therapeutic agents have been tried to treat DIC without conclusive success including heparin [12–14], antithrombin III [15], recombinant tissue factor pathway inhibitor [16], recombinant human activated protein C [2,17,18], protein C concentrate [19,20], and recombinant human soluble thrombomodulin [21].

DIC is characterized by a wide spread fibrin deposition, which may contribute in mediating platelet adhesion and thrombus formation. The interaction between circulating platelets and the deposited fibrin is primarily via the fibrinogen receptor glycoprotein (GP)IIb/IIIa [22,23], and the secondary mechanism via the von Willebrand factor

Abbreviations: von Willebrand factor, VWF; glycoprotein, GP; enzyme-linked immunosorbent assay, ELISA; lipopolysaccharide, LPS.

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http://dx.doi.org/10.1016/j.thromres.2015.02.033
0049-3848/Published by Elsevier Ltd.

(VWF)-GPIbα interaction [24–26]. The mature VWF consists of a 2,050-residue subunit that contains multiple copies of A, C, and D type domains [27]. The central portion of the VWF subunit contains three homologous A domains. While characterizing the isolated A2 domain of human VWF in our laboratory [28], we noticed that this recombinant A2 domain (A2 protein) had a significant binding activity for fibrin. Based on this novel observation, we proposed to examine the significance of this interaction *in vitro* under shear conditions, and *in vivo* using in a mouse endotoxemia-induced DIC model.

Materials and Methods

Antibodies and Reagents

Antibody 6D1 was a gift from Dr. Barry Coller (The Rockefeller University, New York, NY). Human fibrinogen was obtained from Calbiochem (Gibbstown, NJ), D-dimer and fragment E were purchased from Hyphen Biomed (Mason, Ohio). Lipopolysaccharide (LPS, 0111:B4) was obtained from Sigma. Fibrin monomer was prepared as previously described [29]. Purified plasma VWF, A domain proteins ($A2_{1481\text{-}1668}$, and $A3_{1671\text{-}1874}$) were obtained as previously described [30,31].

Binding Assays

The analyses of the interaction of A2, or A3 protein with fibrin monomer or fibrin(ogen), which exposes fibrin-specific sequences upon surface adsorption, were performed by enzyme-linked immunosorbent assay (ELISA) as described elsewhere [30,31]. Briefly, the wells of microtiter plate were coated with either fibrin monomer or fibrinogen (5 μg/ml) in 50 mM carbonate buffer, pH 9.6, and blocked with 3% (w/v) bovine serum albumin (BSA). Following incubation and washing, the bound A2, or A3 protein was detected by using monoclonal anti-histidine-horseradish peroxidase conjugate (Sigma). In other assays, microtiter wells were coated with the A2 protein (5 μg/ml) and increasing concentrations of fibrin monomer, fibrinogen, D-dimer, or fragment E were added to the wells. The fibrinogen-related proteins were detected using anti-human fibrinogen antibody (Dako). Surface Plasmon Resonance (SPR) binding studies were performed similar to our previous studies with some modifications using a BIAcore 3000 system (BIAcore, Piscataway, NJ)[32,33]. The human fibrinogen (50 μg/ml in 50 mM sodium acetate pH-5.0) was covalently coupled via amine coupling to sensor chip CM5 as directed by the supplier. The binding assays were performed in 10 mM Tris-HCl, 150 mM NaCl, 0.001% (v/v) Tween-20, pH-7.4 at 25 °C at a flow rate of 10 μl/min. The binding of A domain proteins to fibrinogen was corrected for non-specific binding to the control channel. Fibrinogen binding at equilibrium was determined at different protein concentrations (50 - 2000nM). As previously described [32], the equilibrium dissociation constant (K_D) were calculated by curve fitting with the BIAevaluation software (version 4.1.1) supplied by the manufacturer. A 1:1 Langmuir interaction model was used. After measuring the A2 binding to fibrinogen, the chip was regenerated by injection of 10 mM Glycine, pH-3.0, and 1 M NaCl.

Fibrin Polymerization

Polymerization of fibrin was evaluated by measuring change in absorbance at λ390 nm using a spectrophotometer (SynergyMX (BioTek Instruments) in a 96 well microtiter format. Polymerization was initiated by the addition of thrombin (0.1 U/mL) at time 0 to the reaction mixture containing fibrinogen (0.1 mg/mL or 0.3 μM) and A2 or A3 protein (0.13 mg/ml or 4.5 μM) in a pH 7.5 buffer containing 10 mM Tris, 0.15 M NaCl, 1 mM $CaCl_2$. Measurements were made at room temperature and at interval of 20 seconds.

Preparation of Protein-coated Surfaces

Dishes coated with fibrinogen were prepared as we previously described [31]. Fibrinogen was diluted to 100 μg/ml in 65 mM sodium phosphate buffer, pH 6.5, and incubated for 1 h at 37 °C. After washing with phosphate-buffered saline, pH-7.4 (PBS) the dishes were blocked with 3% BSA in PBS before using in the flow assays.

Flow Assays

Citrated blood from healthy volunteers was obtained by venipuncture following an informed consent approved by the committee for the protection of human subjects at the Baylor College of Medicine. Perfusion assays were carried out as we described elsewhere [32]. One ml of citrated whole blood was perfused over the fibrin(ogen)-coated coverslip at a shear stress of 1,500 s^{-1} and tethered platelets were observed with phase contrast objectives, recorded by video-microscopy, and analyzed as previously described [31]. Experiments were performed in triplicate using different blood donors. In some experiments, fibrinogen-coated dish was pre-incubated with A2, A3 protein [5.0 μM] or buffer before perfusion. In some experiments, A2 protein [0.4 μM] and fibrin monomer (20 μg/ml) were added to blood before the perfusion over the fibrinogen surface. In experiments with blood containing fibrin monomer, the platelet-clot formed during the perfusion was instantly arrested to the surface, and several view fields (~10-12) were recorded.

Mice

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Mice (C57BL/6, 10-12 weeks old) were injected with LPS (25-40 mg/kg) via intraperitoneally (I.P.) to maximize the manifestations of endotoxin. The A2 (4 mg/kg) or saline was injected via I.P. 1.5 hour after the LPS injection. In some experiments, saline was substituted by the A3 protein (4 mg/kg).

Histology

As described before [34], mice were perfused with phosphate-buffered formaldehyde followed by the removal of brain, liver, kidneys, and lungs after 24 hours of LPS injection in sham and LPS- A2 treated mice. These organs were processed using the services of the Comparative Pathology Laboratory (CLP) of Baylor College of Medicine. Microvascular fibrin-rich thrombi in paraffin embedded brain, liver, kidney, and lung tissues were analyzed with polyclonal fibrinogen antibody (Dako, Carpinteria California). In addition, the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and urea were determined by using the services of the CPL. Lastly, Bio-Plex Multiplex Immunoassay kit (BioRad) was use to measure the levels of cytokines and chemokines in mice. Bleeding time of mice treated with A2 or saline was performed as previously described [35]. P-values were calculated with student's t-test.

Results

Recombinant A2 Domain of Von Willebrand Factor has Binding Activity for Fibrin(ogen)

The A2 protein effectively bound to fibrin(ogen) in saturable manner with half-maximal binding at 90 ± 20 nM (K_D of 125 ± 30 nM by SPR), while the homologous A3 domain did not have any significant binding (Fig. 1). Same result was obtained when the A2 protein bound to immobilized fibrin monomer on microtiter wells (half-maximal binding at 60 ± 10 nM, Fig. S1C). Since immobilized fibrinogen exposes the neoepitopes of fibrin, we analyzed the binding of soluble fibrin