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Regular Article

Protein S and factor V in regulation of coagulation on platelet microparticles by activated protein C

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ABSTRACT

Introduction: Platelets are the main source of microparticles in plasma and the concentration of microparticles is increased in many diseases. As microparticles expose negatively charged phospholipids, they can bind and assemble the procoagulant enzyme-cofactor complexes. Our aim was to elucidate possible regulation of these complexes on microparticles by the anticoagulant protein C system.
Materials and methods: Platelets were activated with thrombin ± collagen or the calcium ionophore A23187 ± thrombin to generate microparticles. The microparticles were analyzed using flow cytometry and functional coagulation assays to characterize parameters with importance for the activated protein C system.
Results: Activation with A23187 + thrombin was most efficient, fully converting the platelets to microparticle-like vesicles, characterized by high lactadherin and protein S binding capacity. Suppression of thrombin generation by activated protein C in plasma spiked with these microparticles was dependent on the presence of plasma protein S. Experiments with purified components showed that activated protein C inhibited both factor Va and factor VIIIa on the microparticle surface. Inhibition of factor Va was stimulated by, but not fully dependent on, the presence of protein S. In the factor VIIIa-degradation, activated protein C was dependent on the addition of protein S, and exogenous factor V further increased the efficiency.
Conclusions: Protein S is crucial for activated protein C-mediated inhibition of thrombin generation on platelet-derived microparticles in plasma. Moreover, protein S and factor V are synergistic cofactors in the inhibition of factor VIIIa. The results demonstrate that the activated protein C system has the capacity to counterbalance the procoagulant ability of microparticles.

Introduction

Microparticles (MPs) are small membrane-containing vesicles released by numerous cell types upon activation, apoptosis or stress [1–3]. Their features are dependent on the cell origin; hence they can be identified by surface expression of cell specific markers. MPs from platelets have a high surface content of the negatively charged phospholipid phosphatidylserine, and support the activations of factor X (FX) and prothrombin [4–8]. FX is activated by the intrinsic Xase complex where activated factor IX (FIXa), together with its cofactor activated factor VIII (FVIIIa), is bound to negatively charged phospholipids, whereas prothrombin is activated by the prothrombinase (PTase) complex (activated FX (FXa), its cofactor activated factor V (FVa) and the phospholipids). The platelets contain about 20% of the total amount of FV in blood [9]. This platelet-derived FV is released upon activation and bind to the negatively charged activated platelets, thereby contributing to the formation of PTase complexes. The fully assembled complexes are 10^5-10^6 more efficient than the respective enzymes alone [10,11].

The Xase- and PTase complexes are regulated by activated protein C (APC) [12], which inactivates FVIIIa and FVa [13,14]. Protein S serves as an APC-cofactor in these reactions [15,16]. In human plasma, approximately 35% of protein S is free, the remaining being bound to C4b-binding protein (C4BP) [17]; mainly the free form serving as APC cofactor [18]. In the inhibition of FVIIIa, intact FV functions in synergy with protein S as cofactor to APC [19,20]. Platelets contain around 2.5% of the total protein S in blood, and it is released upon platelet activation [21]. The functional importance of protein S and protein C is evident from the increased risk of venous thrombosis affecting individuals with heterozygous deficiency of either protein [22].

Normal plasma contains 0.5-2.8 × 10^6 MPs/mL [23,24], but in several diseases, such as cancer [25], rheumatoid conditions [26], diabetes [27],

Abbreviations: APC, activated protein C; C4BP, C4b-binding protein; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; F(), coagulation factor (number); F()a, activated coagulation factor (number); MPs, microparticles; PSB, platelet storage buffer; PTase, prothrombinase; PWB, platelet wash buffer; TF, tissue factor; TFPI, tissue factor pathway inhibitor; Xase, intrinsic tenase.

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systemic lupus erythematosus [24], atherosclerosis and coronary disorders [28], increased numbers of circulating MPs have been reported. Interestingly, an increased risk of thrombosis is observed in many of these diseases and it has been postulated that the circulating MPs contribute to the thrombosis risk.

While the procoagulant functions of platelet-derived MPs have been extensively investigated, few reports focus on the anticoagulant properties of MPs. Several studies have shown that APC-mediated inactivation of FVa on activated platelets is hampered and that platelet-derived FVa is less susceptible to APC-mediated degradation than plasma-derived FVa [29–31]. Degradation of FVa by APC on the surface of ionophore-activated platelets (MPs) has been shown to be more efficient than on platelets activated by thrombin, however compared to phospholipid vesicles, the degradation rate was still low [5,32]. The APC-mediated degradation of FVIIIa on MPs has not been studied, but platelet-derived MPs can bind FVIIIa and FIXa [6,7].

It has previously been shown that free, but not C4BP-bound, protein S specifically binds to platelet-derived MPs but not to resting or activated platelets [33]. To investigate whether the binding of protein S to the MPs renders them less procoagulant, the ability of protein S and FV to function as cofactors to APC on MPs was investigated.

Materials and Methods

Reagents

Antibodies were from BD Biosciences, Franklin Lakes, NJ, USA (anti-CD41a-PerCPcy5.5 and mouse IgG1-PerCPcy5.5) or Beckman Coulter, Brea, CA, USA (anti-CD61-PE and mouse IgG1-PE). Rabbit-anti-protein S (A0384 DAKO, Glostrup, Denmark) was labeled with Alexa488 using the Microscale Protein Labeling kit (A30006), (Life Technologies, Invitrogen, Carlsbad, CA, USA). FIXa and lactadherin-FITC were from Haematologic Technologies Inc, Essex Junction, VT, USA. Protein S and FXa were from Enzyme Research Laboratories (ERL, South Bend, IN, USA). Ionophore A23187 (calcimycin) was from Life Technologies, Invitrogen, FVIII was from Octapharma, Lachen, Switzerland. Human FV was purified from plasma as described [34], with minor modifications [5]. Bovine FX [35], and human prothrombin [36] were purified from plasma. Human APC was obtained from recombinant protein C expressed, purified and activated, as described [37]. Human α-thrombin was prepared from purified prothrombin, as described [38]. Hirudin was from Pentapharm, Basel, Switzerland. Collagen (native fibrils type 1 form equine tendons) was from Chrono-Log Corp. Haverton, PA, USA. Bovine serum albumin (BSA, #A7030) was from Sigma-Aldrich (St Louis, MO, USA). Natural phospholipids phosphatidylserine (brain extract) and phosphatidylcholine (egg extract) were from Avanti Polar Lipids Inc. (Alabama, USA). Ready gels (4-15% TGX) and stacks for SDS-Page and western blotting were from (Bio-Rad, Hercules, CA, USA).

Platelets

Platelets were obtained from fresh citrated blood (Vacutainer citrate 4.5 mL tubes, BD, Franklin Lakes, NJ, USA) collected from healthy volunteers after informed consent (ethical permission 2012/202, Regional Ethical Review Board, Lund, Sweden. Whole blood was centrifuged (Hermle Labortechnik, Wehnigen, Germany) at 250 g, 15 min, and the obtained platelet rich plasma was pooled into new tubes (10 mL/tube) containing 1 mL platelet wash buffer (PWB, 100 mM Tri-Na-citrate, 10 mM citrate, 150 mM glucose, pH 6.5, supplemented with 2 mM adenosine (Sigma-Aldrich) and 7.5 mM theophylline (Sigma-Aldrich), freshly prepared) and centrifuged 1 000 g, 10 min. The platelet pellet was carefully resolved in PWB and transferred to new tubes containing 10 mL PWB and centrifuged as before (repeated once). Platelet storage buffer (PSB) (140 mM NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 10 mM Hepes (4-(2-Hydroxyethyl-1)-1-piperazineethanesulfonic acid), 0.5 mM NaH_2PO_4, 5.5 mM glucose, 10 mM HCO_3, pH 7.4) was carefully laid over the pellet and removed, followed by resuspension of the platelets in 1.5 mL PSB. Washed platelets were counted (phase contrast inverted microscope, Olympus IMT2-RFL, LRI Instrument AB, Lund, Sweden) using Bürker C-chip (Digital Bio, NanoEnTek, Seoul, Korea). The platelets were diluted to 75×10^6/mL in PSB for further processing. Buffers were room tempered and filtered (0.20 um), low affinity transfer pipets (7.5 mL, 15.5 cm, VWR, Radnor, PA, USA) were used to resolve platelet pellets and the platelets were handled in non-adhesive tubes (#347708 Nunc, Thermo Fisher Scientific), to avoid activation. Washed platelets (1.5 mL, 75×10^6/mL) were activated using 0.5 or 5 U/mL thrombin and 2 mM $CaCl_2$, or 0.5 or 5 U/mL thrombin plus 25 μg/mL collagen and 4 mM $CaCl_2$, or 5 μM calcium ionophore A23187 and 4 mM $CaCl_2$, at 37 °C, 15 minutes. Some batches of A23187-activated platelets were further treated with 0.5 or 5 U/mL of thrombin (37 °C, 10 min). Thrombin activity was inhibited by addition of double concentration of hirudin. Experiments were performed within 6 hours from blood collection, and 3 hours from activation time and were kept at room temperature meanwhile. The platelets/MPs concentrations will be referred to as the concentration equivalent to the concentration of platelets before activation. Platelets from different donors were used.

Flow Cytometry

Two flow cytometers; FC500 and Gallios (Beckman Coulter, Brea, CA, USA) were used. IsoFlow™ Sheath Fluid (Beckman Coulter) was used as fluid phase and the laser was set at 488 nm. In the Gallios, the laser setting W^2 was used. Thresholds for forward scatter and side scatter were set to 2. Forward scatter, side scatter and fluorescence channels were set at logarithmic gain. The flow cytometry data were analyzed using FlowJo 8.7.1 (Tree Star, Inc., Ashland, OR, USA). The platelet gate was set using resting platelets and MPs were defined based on phosphatidylserine exposure and protein S binding capacity. To verify the MP gate, washed platelets were incubated with A23187 and centrifuged (1 000 g, 10 min) to pellet platelets. The forward/side scatter properties of the MPs in the supernatant coincided with the populations defined as MPs by phosphatidylserine exposure (lactadherin binding) and protein S binding. Centrifugation of resting platelets led to almost complete loss of events in the supernatant.

Counting of Platelets/MPs Using Flow Cytometry

After incubation (15-20 min RT, dark) of platelets/MPs (7.5×10^6/mL, 5 μL) with mouse-anti-CD41a-PerCPCy5.5 (5 μL, 25 μg/mL) and lactadherin-FITC (5 μL, 1.6 μM) in a total volume of 50 μL PSB, 50 μL calibration beads (Flow Count®, Beckman Coulter) were added and the mixture was diluted to 500 μL with PSB. CD41-positive platelets/MPs (FL4) were collected in flow cytometer FC500 and compared to the known concentration of calibration beads.

Phosphatidylserine Exposure Measured by Lactadherin Binding

Approximately 0.375×10^6 platelets/MPs were incubated for 15-20 min, RT, with 5 μL lactadherin-FITC (1.6 μM) and 5 μL mouse anti-CD61-PE (25 μg/mL) in a total volume of 50 μL PSB. After labeling, the platelets/MPs were diluted to 500 μL in PSB and the analyzed in Gallios. CD61-positive events were collected as identified in FL2. Lactadherin-FITC-binding (detected in FL1) was used to estimate activation state due to exposure of phosphatidylserine [39].

Protein S Binding Measured in by Flow Cytometry

Platelets/MPs (0.375×10^6) were incubated for 15-20 min, RT with 5 μL protein S (125 μg/mL), 5 μL mouse anti-CD61-PE (25 μg/mL) in a total volume of 50 μL PSB containing 4 mM $CaCl_2$. Rabbit-anti-protein S (alexa488-labeled) was added (5 μL 90 μg/mL) to detect protein S binding. After incubation as above, the platelets/MPs were diluted