Biochimica et Biophysica Acta 1840 (2014) 1188–1193

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Platelets do not express the oxidized or reduced forms of tissue factor

Beth A. Bouchard, Matthew T. Gissel, Matthew F. Whelihan, Kenneth G. Mann, Saulius Butenas *

Department of Biochemistry, University of Vermont College of Medicine, Burlington, VT, USA

ARTICLE INFO

Article history:
Received 23 July 2013
Received in revised form 11 November 2013
Accepted 25 November 2013
Available online 19 December 2013

Keywords:
Hemostasis
Factor VIIa
Platelet
Tissue factor
Flow cytometry

ABSTRACT

Background: Expression of tissue factor (TF) antigen and activity in platelets is controversial and dependent upon the laboratory and reagents used. Two forms of TF were described: an oxidized functional form and a reduced nonfunctional form that is converted to the active form through the formation of an allosteric disulfide. This study tests the hypothesis that the discrepancies regarding platelet TF expression are due to differential expression of the two forms.

Methods: Specific reagents that recognize both oxidized and reduced TF were used in flow cytometry of unactivated and activated platelets and western blotting of whole platelet lysates. TF-dependent activity measurements were used to confirm the results.

Results: Western blotting analyses of placental TF demonstrated that, in contrast to anti-TF#5, which is directed against the oxidized form of TF, a sheep anti-human TF polyclonal antibody recognizes both the reduced and oxidized forms. Flow cytometric analyses demonstrated that the sheep antibody did not react with the surface of unactivated platelets or platelets activated with thrombin receptor agonist peptide, PAR-1. This observation was confirmed using biotinylated active site-blocked factor (F)VIIa: no binding was observed. Likewise, neither form of TF was detected by western blotting of whole platelet lysates with sheep anti-hTF. Consistent with these observations, no FXa or FIXa generation by FVIIa was detected at the surface of these platelets. Similarly, no TF-related activity was observed in whole blood using thromboelastography.

Conclusion and significance: Platelets from healthy donors do not express either oxidized (functional) or reduced (nonfunctional) forms of TF.

1. Introduction

Blood coagulation is initiated at sites of vascular injury by formation of the tissue factor (TF)/factor (F)VIIa complex which activates FIX and FX. FIXa assembles into the intrinsic FXase complex on the surface of activated platelets to generate additional FXa, while FXa assembles into platelet-bound prothrombinase to generate thrombin [1]. Thrombin amplifies, propagates and sustains the coagulant response through the recruitment of additional activated platelets to the site of injury, and activation of plasma coagulation factors [2]. Sequestration of TF from plasma under normal, physiological conditions by limiting its constitutive expression to subendothelial cells restricts thrombin generation to sites of vascular injury and prevents inappropriate clotting [3].

This paradigm has been challenged by studies suggesting the expression of TF activity and antigen by platelets [4–13]. In contrast, the data from our laboratory [14–16] have clearly demonstrated using well-characterized reagents and employing immuno- and functional assays that platelets do not express TF. The recent identification of a role for an allosteric disulfide in regulation of its coagulant function [17–21] led to the hypothesis that these discrepant data could be a result of differential expression of oxidized/reduced TF in platelets. Our previous studies utilized a monoclonal antibody directed against the active, oxidized form of TF. In the current study, these observations were extended by assessing platelet TF expression using a specific polyclonal antibody and active site-blocked recombinant FVIIa that both recognize the oxidized and reduced forms of TF.

2. Methods

2.1. Subjects

Healthy volunteer blood donors with normal coagulation histories were recruited and advised according to a protocol approved by the Institutional Review Board of the University of Vermont Human Studies Committee. Informed written consent was obtained from all subjects prior to blood collection.

Abbreviations: TF, tissue factor; F, factor; CTI, corn trypsin inhibitor; hTF, human TF; PE, phycoerythrin; FPRck, D-Phe-Pro-Arg-CH_2Cl; rFVIIa, recombinant FVIIa; PAR, protease activated receptor; LPS, lipopolysaccharide; RGDS, Arg-Gly-Asp-Ser; HRP, horseradish peroxidase; PCPS, 80% phosphatidylcholine/20% phosphatidylserine containing vesicles; TEG, thromboelastography; PRP, platelet-rich plasma; TAT, thrombin-antithrombin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 5× SPB, 312.5 mM Tris, pH 6.8, 10% sodium dodecyl sulfate, 50% glycerol, 0.05% bromophenol blue; Mr_{app}, apparent molecular weight; kDa, kilodalton

* Corresponding author at: Department of Biochemistry, University of Vermont College of Medicine, Colchester, VT 05446, USA. Tel.: +1 802 656 0350; fax: +1 802 656 2256.
E-mail address: sbutenas@uvm.edu (S. Butenas).

0304-4165/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2013.11.024

2.2. Materials

Corn trypsin inhibitor (CTI) was isolated as previously described [22]. The monoclonal antibodies anti-FXI-2, anti-FIX-40, anti-FX-1D and anti-TF#5 [23] were obtained from the Biochemistry Antibody Core Laboratory (University of Vermont). Sheep anti-human TF (hTF) antibody was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). The control mouse and sheep IgGs were bought from Jackson ImmunoResearch (West Grove, PA), respectively. Mouse IgG, sheep IgG, anti-TF#5, and sheep anti-hTF were conjugated to AlexaFluor488 (Invitrogen, Carlsbad, CA). Anti-CD62-phycoerythrin (PE) was purchased from BD Biosciences (Franklin Lakes, NJ). Streptavidin-AlexaFluor488 was purchased from Invitrogen. Human Fc was purchased from EMD Millipore (Billerica, MA). Placental TF was purified and subsequently reduced and alkylated as described previously [17]. D-Phe-Pro-Arg-CH_2Cl (FPRck) was produced in house. Human FX and FIX were isolated from fresh frozen plasma using anti-FX and anti-FIX mAb-coupled Sepharose [24]. FXa was a gift from Dr. R. Jenny (Haematologic Technologies, Essex, VT) and recombinant TF_{1-242} a gift from Dr. R. Lundblad (Baxter Healthcare Corp., Duarte, CA). Recombinant FVIIa (rFVIIa), a gift from Dr. U. Hedner (Novo Nordisk, Denmark), was active site blocked and biotinylated in house. Streptavidin conjugated to horseradish peroxidase (HRP), prostaglandin E_1, protease activated receptor (PAR) 1 agonist peptide (SFLLRN-NH_2), lipopolysaccharide (LPS) and Arg-Gly-Asp-Ser (RGDS) were bought from Sigma (St. Louis, MO). Chemiluminescence reagent was purchased from Perkin Elmer (Waltham, MA). Goat anti-mouse IgG (H + L)-horseradish peroxidase (HRP) and rabbit anti-sheep IgG (H + L)-HRP were purchased from SouthernBiotech (Birmingham, AL) and Affinity Biologicals (Ancaster, ON, Canada), respectively. Human monocytic cells (THP-1) were from ATCC (Rockville, MD).

2.3. Blotting analyses

Reduced and alkylated or non-reduced and nonalkylated placental TF, THP-1 lysates, or platelet lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 4–20% gradient gels under non-reducing conditions [25]. Following transfer to nitrocellulose [26], the resolved proteins were probed with mouse anti-TF#5 (5 μg/ml) or sheep anti-hTF (5 μg/ml). Primary antibody reactivity was detected using goat anti-mouse IgG (H + L)-HRP or rabbit anti-sheep IgG (H + L)-HRP (1:10,000 dilutions), respectively, followed by chemiluminesence. A similar protocol was used for western blotting of FIX and FX activation samples with anti-FIX-40 and anti-FX-1D monoclonal antibodies, respectively.

2.4. Preparation of THP-1 cells and platelets

Human monocytic cells (THP-1) were cultured per the manufacturer's instructions. Cells (2.5×10^6 cells/mL) were stimulated with 250 ng/ml *Escherichia coli* LPS (4 h, 37 °C) to induce expression of TF [27]. For western blotting, cells (5×10^6 cells/mL) were lysed by multiple freeze/thaw cycles followed by dilution with 312.5 mM Tris, pH 6.8, 10% sodium dodecyl sulfate, 50% glycerol, 0.05% bromophenol blue (5× SPB) (one part 5× SPB plus four parts cell lysate).

Platelets were isolated from human venous blood as described previously [28]. Platelets (1×10^9 platelets/mL) were lysed with 1% triton X-100 and diluted with 5× SPB prior to SDS-PAGE and western blotting. For flow cytometric analyses, platelets were activated with PAR1 peptide (100 μM) (1×10^8 platelets/mL) for 15 or 120 min at 37 °C in the presence of RGDS to prevent platelet aggregation. Prior to flow cytometric analyses, platelets were either subjected to fixation with 2% paraformaldehyde (TF immunostaining) or activation was stopped by the addition of prostaglandin E_1 (5 μM) (rFVIIa-biotin binding).

2.5. Flow cytometric analyses

LPS-stimulated THP-1 cells (1×10^6/mL) or unactivated and activated platelets (1×10^7/mL) were incubated with 0.1 μM sheep anti-hTF-AlexaFluor488 or a control sheep IgG-AlexaFluor488 (45 min, ambient temperature) in the presence of 10 μg/mL human Fc. In other experiments, LPS-stimulated THP-1 cells or platelets were incubated with 0 or 10 nM biotinylated active site-blocked rFVIIa (20 min, ambient temperature). Following centrifugation, the dry cell pellets were incubated with streptavidin-AlexaFluor488 (10 μg/mL, 45 min, ambient temperature).

Following extensive washing the cells were subjected to fixation with 2% paraformaldehyde and stored at 4 °C until flow cytometric analyses. Cells (10,000) were analyzed on a BD LSRII flow cytometer. Platelet activation was confirmed by immunostaining with anti-CD62-PE. The data were analyzed using FlowJo (version 7.6.5) software.

2.6. TF activity measurements

TF-dependent FXa and FIXa generation were determined as described previously [16,29]. Briefly, platelets (2×10^8/mL) were incubated with 10 nM or 100 nM rFVIIa and 100 μM PAR1 peptide for 15 or 120 min at 37 °C prior to the addition of FX (170 nM) or FIX (90 nM). The rate of FXa generation was determined by chromogenic assay and western blotting. FIXa generation was also assessed by western blotting. Control reactions used TF_{1-242} relipidated into 80% phosphatidylcholine/20% phosphatidylserine containing vesicles (PCPS) [30,31] (20 pM TF/100 μM PCPS) as a TF source.

2.7. Thromboelastography (TEG)

Fresh whole blood was added to a TEG cup containing CTI (100 μg/mL) and anti-FXI-2 (667 nM) to block the contact pathway of blood coagulation, in the presence or absence of PAR1 peptide (100 μM) and anti-TF#5 (667 nM). Analysis was carried out on each sample using a TEG Haemoscope 5000 (Haemonetics, Braintree, MA) at 37 °C. TEG parameters were extracted using TEG V4 software (Haemonetics). Reactions were quenched after 70 min with an inhibitor cocktail (50 mM EDTA, 20 mM benzamidine, 100 μM FPR-ck). The samples were subjected to centrifugation, and the soluble material was frozen at −80 °C until analysis of thrombin–antithrombin (TAT) complex [32].

3. Results

Previous observations have clearly demonstrated that unactivated platelets and platelets activated under different conditions do not express TF when analyzed by highly sensitive activity assays and flow cytometry using a well-characterized anti-TF antibody, anti-TF#5 [14–16]. Western blotting analyses of reduced, alkylated (reduced) and non-reduced, nonalkylated (oxidized) placental TF demonstrated that this antibody recognizes only the oxidized (active) form of TF (Fig. 1; mouse anti-TF#5). In contrast, a sheep polyclonal antibody directed against human TF (sheep anti-hTF) recognizes both oxidized and reduced forms of the protein (Fig. 1). As platelets (unactivated and activated) do not express active (oxidized) TF as demonstrated by functional assays and flow cytometry using anti-TF#5 [14–16], the expression of the inactive (reduced) form of TF by platelets was examined. Reactivity of sheep anti-hTF with cell surface expressed TF was confirmed using LPS-stimulated THP-1 cells. Flow cytometric analyses demonstrated substantial immunostaining of these cells with sheep anti-hTF conjugated to AlexaFluor488 as compared to cells stained with the control sheep IgG-AlexaFluor488 used at the same concentration and same dye to protein ratio (Fig. 2A). In contrast, no reactivity with unstimulated THP-1 cells was observed consistent with a lack of cell surface expressed TF (data not shown). Similarly, no expression