Thrombosis Research 154 (2017) 7–15

Contents lists available at ScienceDirect

Thrombosis Research

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

Full Length Article

Effect of platelet-derived β-thromboglobulins on coagulation

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ARTICLE INFO

Article history:
Received 19 December 2016
Received in revised form 18 February 2017
Accepted 28 March 2017
Available online 30 March 2017

Keywords:
Platelet-derived β-thromboglobulins
Coagulation
Thrombin generation
Factor X

ABSTRACT

Background: β-thromboglobulins are derived from the cleavage of the CXC chemokine platelet basic protein and are released in high concentrations by activated platelets. Platelet-derived β-thromboglobulins (βTG) share 70% homology with platelet factor 4 (PF4), another CXC chemokine released by activated platelets. PF4 modulates coagulation by inhibiting heparin-antithrombin interactions, promoting protein C activation, and attenuating the activity of activated protein C. In contrast, the effect of βTG on coagulation is unknown.
Aim/Methods: Clotting times, thrombin generation, chromogenic clotting factor assays, and surface plasmon resonance (SPR) were used to assess the effect of purified βTG on coagulation.
Results: In normal pooled plasma, βTG shortened the lagtime and time to peak thrombin generation of tissue factor (TF)-dependent and TF-independent thrombin generation. In factor VIII and factor IX-deficient plasmas, βTG induced thrombin generation in the absence of a TF stimulus and in the presence of anti-TF and factor VIIa inhibitory antibodies. The procoagulant effect was not observed when thrombin generation was independent of factor X activation (supplementation of factor X-deficient plasma with factor Xa). Cleavage of a factor Xa-specific chromogenic substrate was observed when βTG was incubated with factor X, suggesting a direct interaction between βTG and factor X. Using SPR, βTG were found to bind to immobilised factor X in a dose dependent manner.
Conclusion: βTG modulate coagulation in vitro via an interaction with factor X.
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1. Introduction

The β-thromboglobulins are a group of immunologically cross-reactive proteins derived from the proteolytic cleavage of platelet basic protein (PBP). PBP is a 10.3 kDa, 94 amino acid protein of the CXC ELR+ chemokine family. CTAPIII (connective tissue activating peptide III) is an 8.3 kDa, 85 amino-acid long, amino-terminal truncation product of PBP. It was previously referred to as low-affinity platelet factor 4. CTAPIII activates connective tissue cells by increasing glucose consumption, mitogenesis, and the secretion of hyaluronic acid and glycosaminoglycans [1], inhibits megakaryopoesis [2], possesses heparanase activity [3], stimulates cellular sphingomyelin import into lymphocytes [4], and acts as a chemokine for endothelial cells [5]. β-thromboglobulin is an 8.8 kDa, 81 amino acid long amino-terminal truncation product of PBP. Its physiological effects are poorly characterized although it has been reported to possess mild chemotactic activity for fibroblasts and endothelial cells [5,6]. NAP-2 (Neutrophil Activating Peptide-2) is a 7.6 kDa, 71 amino acid long, amino-terminal truncation of PBP. It is also referred to as CXCL7 (CXC Ligand 7) and small inducible cytokine subfamily B7. It is a potent chemoattractant and activator of neutrophils, acting via the CXC receptor 1 (CXCR1) and CXC receptor 2 (CXCR2) [7,8]. In contrast to NAP-2, PBP, CTAPIII, and β-thromboglobulin do not possess neutrophil chemotactic activity, suggesting that full cleavage of PBP is required to reveal the ELR motif that interacts with CXCR1 and CXCR2 on neutrophils.

Platelets are the main cellular source of β-thromboglobulins. These platelet derived β-thromboglobulins (βTG) are stored in platelet α-granules and released in high concentrations during platelet activation.

Abbreviations: CTAPIII, Connective Tissue Activating Peptide III; ETP, Endogenous thrombin potential; CTAPIII, Neutrophil Activating Peptide III; PBP, Platelet Basic Protein; βTG, Platelet-derived β-thromboglobulin; PF4, Platelet Factor 4; tpeak, Time to peak; TF, Tissue Factor.

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http://dx.doi.org/10.1016/j.thromres.2017.03.023
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CTAPIII is the main platelet isoform, representing >95% of the platelet intracellular stores [7]. Commercial preparations of βTG, including the preparation used in this study, consist of 95–100% CTAPIII [3]. It is believed that PBP is synthesised by megakaryocytes and then undergoes limited proteolysis to CTAPIII during megakaryocyte maturation and platelet formation [9,10]. Serum samples contain concentrations of βTG in the range of ~60–170 μg/ml (1.6–4.8 μM) [7], suggesting that substantial levels of βTG would be found at the site of a platelet-rich thrombus. In spite of their high abundance, the physiological role of βTG in haemostasis and thrombosis are unknown [11]. Interestingly, βTG share significant amino acid sequence homology and overlapping inflammatory properties with platelet factor 4 (PF4), another CXC chemokine released in high concentrations by activated platelets. PF4 is known to modulate the coagulation cascade, the interaction between heparin and antithrombin via its heparin binding properties [12–14], promoting protein C activation [15], and attenuating the anticoagulant of activated protein C [16]. The aim of this study was to determine a possible role of β-thromboglobulins in the coagulation process, in particular on the generation of thrombin.

2. Methods

2.1. Reagents

βTG, PF4, as well as factor V, factor Va, factor X, factor Xa, factor IXa, prothrombin (factor II) and α-thrombin (factor IIa) were purchased from Haematologic Technologies (Essex Junction, VT, USA). Normal pooled plasma, and plasmas deficient in factor VIII, protein C, Protein S, factor IX, factor V, and factor X, as well as factor Xa chromogenic substrate (CS-01-(65)) and factor IIa/thrombin chromogenic substrate (CS-01-(38)) were purchased from Hyphen Biomed (Neuville-sur-Oise, France). TFPI deficient plasma was purchased from Sekisui diagnostics (Japan). Calibrated automated thrombography reagents were purchased from Thrombinoscope BV (Maastricht, The Netherlands). HemosIL RecombiPlasTin 2G reagent and HemosIL SynthASil were purchased from Instrumentation Laboratory (Bedford, MA, USA). Recombinant NAP-2 was purchased from R & D Systems (Abingdon, UK). Mouse monoclonal anti-human NAP-2 antibody, mouse monoclonal isotype control antibody, rabbit polyclonal isotype control antibody were purchased from R & D systems (Abingdon, UK). Rabbit polyclonal anti-human NAP-2 antibody was purchased from US Biologicals (Salem, MA, USA).

2.2. Blood collection

Blood was collected from healthy volunteers free from anti-platelet and anticoagulant medication for at least 2 weeks. Written informed consent was obtained in accordance with the Declaration of Helsinki. Studies were approved by the host institutes ethics committee. Blood was collected in 1:10 (v/v) 3.2% trisodium citrate as anticoagulant. Platelet rich plasma was prepared by centrifugation of whole blood at 170*g* for 10 min at room temperature.

2.3. Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT)

PT and aPTT were measured using the fully automated ACL TOP 500 coagulometer (Instrumentation Laboratory) using HemosIL RecombiPlasTin 2G reagent and HemosIL SynthASil, respectively.

2.4. Calibrated automated thrombography

Thrombin generation in platelet-poor plasma and platelet-rich plasma was assessed using a Fluoroskan Ascent plate reader (Thermo Lab System) in combination with Thrombinoscope software (Thrombinoscope BV). In platelet-poor plasma, 80 μl of plasma was incubated with 20 μl of MP reagent containing 4 μM phospholipids (60% phosphatidylcholine, 20% phosphatidylserine, and 20% phosphatidylethanolamine) or 20 μl of PPP low reagent containing 4 μM phospholipids and 1pM tissue factor (TF). In platelet-rich plasma, 80 μl of platelet rich plasma was incubated with 20 μl of PRP reagent containing 1 pM TF. Thrombin generation was initiated by automatic dispensation of a fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC) and 100 mM $CaCl_2$ into each well (final concentrations, Z-Gly-Gly-Arg-AMC·HCl, 0.42 mM and $CaCl_2$, 16.67 mM). Thrombin generation was quantified using a thrombin calibration standard, as described by Vanschoonbeek et al. [17]. Measurements were taken at 20-s intervals for 60 min, or until thrombin generation was complete. The lag time to start of thrombin generation, peak amount of thrombin generated (nM of IIa) time to peak thrombin (tpeak), and area under the thrombin generation curve, represented by the endogenous thrombin potential (ETP), were measured.

2.5. Chromogenic assays of factor X and prothrombin activation

The activation of factor X and factor II in a purified protein system was measured using factor Xa and factor IIa specific chromogenic substrates, respectively. For factor X activation, factor Xa (100 nM), factor X (2 μM), βTG (200 μg/ml), factor X plus βTG (2 μM + 200 μg/ml), or factor X plus factor IXa (2 μM + 200 μg/ml) were incubated for 1 h with shaking (450 rpm on an orbital shaker) in a 40 μl volume of a phosphate buffered saline solution supplemented with 3 mM $CaCl_2$. Following this, 200 μl of factor Xa chromogenic substrate (CS-01-(65), 5 mg/ml) was added and the absorbance at 405 nm was read after 20 min. For factor II activation, factor IIa (100 nM), factor II (2 μM), βTG (200 μg/ml), or factor II plus βTG (2 μM + 200 μg/ml) were incubated for 1 h with shaking (450 rpm on an orbital shaker) in a 40 μl volume of phosphate buffered saline supplemented with 3 mM $CaCl_2$. Following this, 200 μl of factor Xa chromogenic substrate (CS-01-(38), 5 mg/ml) was added and the absorbance at 405 nm was read after 20 min.

2.6. Surface plasmon resonance

A BIAcore 3000 system (GE Healthcare, UK) was used to evaluate βTG binding to immobilised factor X. Factor X was immobilised (25 μg/ml in 10 mM NaOAc, pH 4.5) onto the surface of a CM5 sensor chip at a flow rate of 10 μl/min for 10 min. The surface was capped with 1 M ethanolamine HCl (pH 8.5) and washed with 10 mM NaOH (flow rate 10 μl/min) to remove unbound or extraneous material. An uncoated flow cell was used to detect nonspecific binding. βTG (0–20 μg/ml in Hepes buffered saline (HBS-EP) with 0.005% (v/v) surfactant P20, pH 7.4) and factor IXa (0–4 μg/ml in HBS-EP with 0.05% (v/v) Tween-20, pH 7.4) was passed over the factor X surface at a flow rate of 30 μl/min. Association and dissociation phases were monitored for 3 and 10 min, respectively. Sensograms for each βTG and factor IXa concentration were fitted to a 1:1 interaction model using Biacore 3000 dedicated software. The surface was regenerated using 5 mM NaOH for βTG and 20 mM NaOH for factor IXa.

2.7. Statistics

Data were expressed as the mean plus the standard error of the mean. All data represent the mean of at least 3 independent experiments. Results were analysed using GraphPad Prism software (Horsham, Pa, USA). The Kolmogorov-Smirnov was used to determine whether data sets were parametric or non-parametric. Parametric data sets were analysed by paired *t*-test. Non-parametric data sets were analysed by Wilcoxon Signed Rank test. Paired tests were used to compare experimental conditions with and without βTG. A *p*-value < 0.05 was considered statistically significant. $^{*} = p < 0.05$. $^{**} = p < 0.01$. $^{***} = p < 0.001$.

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