Thrombosis Research 132 (2013) 263–270

Contents lists available at ScienceDirect

Thrombosis Research

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

Regular Article

Studies of a microchip flow-chamber system to characterize whole blood thrombogenicity in healthy individuals

Yusuke Yamaguchi [a], Takanori Moriki [b,*], Atsuko Igari [a], Yumiko Matsubara [a], Tomoko Ohnishi [c], Kazuya Hosokawa [c], Mitsuru Murata [a]

[a] Department of Laboratory Medicine, Keio University School of Medicine, Tokyo, Japan
[b] Health Center, Keio University, Tokyo, Japan
[c] Research Institute, Fujimori Kogyo Co., Yokohama, Kanagawa, Japan

ARTICLE INFO

Article history:
Received 29 November 2012
Received in revised form 20 April 2013
Accepted 27 May 2013
Available online 16 June 2013

Keywords:
Thrombus formation
Platelet
Flow-chamber system
Thrombogenicity

ABSTRACT

Introduction: A whole blood flow-chamber system, the Total Thrombus-formation Analysis System (T-TAS), was developed for quantitative analysis of platelet thrombus formation (PTF) using microchips with thrombogenic surfaces (collagen, PL chip; collagen plus tissue thromboplastin, AR chip) under shear stress conditions. We evaluated the usefulness of the T-TAS for assessing individual thrombogenicity compared with other platelet function tests.

Materials and Methods: Blood samples from 31 healthy volunteers were applied to the T-TAS to measure PTF starting time (T_{10}: time to reach 10 kPa), occlusion time (T_{60} for PL chip; T_{80} for AR chip), and area under the curve (AUC_{10}, area under curve until10 min for PL chip; AUC_{30}, 30 min for AR chip) under various shear rates (1000, 1500, 2000 s^{-1} for PL chip; 300 s^{-1} for AR chip). Platelet functions were also tested using platelet aggregometry, the PFA-100 (collagen and epinephrine [C/EPI], collagen and adenosine diphosphate [C/ADP]), and the VerifyNow P2Y12 assay.

Results: Individual pressure waveforms, including PTF starting and ending points, varied among healthy subjects. In the PL chip, T_{10} and AUC_{10} showed a shear-dependent correlation with C/EPI or C/ADP. VerifyNow P2Y12 values were not significantly associated with the parameters of the T-TAS. Platelet counts were correlated with all AR measurements, and mostly with PL measurements.

Conclusion: The results of the T-TAS were associated with those of the PFA-100 in many respects, indicating that its characteristics are related to shear-induced PTF. The T-TAS showed few correlations with platelet aggregometry and the VerifyNow P2Y12 assay. The T-TAS may allow for the measurement of comprehensive parameters of individual thrombogenicity under whole blood flow conditions.

Introduction

The initial step of thrombus formation at the site of vascular injury in an artery is the adhesion of platelets to the exposed collagen-bound von Willebrand Factor (VWF), followed by platelet activation to bind stably to the collagen on the subendothelium under high-shear blood flow. Activated platelets undergo morphologic changes and aggregate together by bridging with VWF and fibrinogen, and release soluble agonists such as adenosine diphosphate, thromboxane A_2, and thrombin, which further enhance platelet activation. The coagulation pathway on the platelet plasma membrane is accelerated to generate localized thrombin, leading to enhanced platelet activation and fibrin formation, and resulting in the growth of platelet thrombus formation (PTF) [1,2]. This process, hemostasis, is essential for maintaining blood circulation *in vivo* to prevent blood loss due to bleeding.

Arterial thrombosis, however, is attributed to pathologic PTF at sites of atherosclerotic plaque rupture, which leads to ischemia or necrosis of the organs downstream, such as acute coronary syndrome, myocardial infarction, and ischemic stroke [3,4]. Arterial thrombotic diseases are a major cause of morbidity and mortality, particularly in industrialized countries, and the numbers continue to grow worldwide, increasing healthcare costs. Prothrombotic conditions, e.g., high platelet reactivity, enhanced coagulation, and reduced fibrinolysis, have been identified in disorders that lead to atherosclerotic thrombosis [5], such as diabetes [6,7], hypertension [8], dyslipidemia [9], obesity [10], and metabolic syndrome [11,12], as well as in smokers [13] and the elderly [14].

Evaluation of the prothrombotic status is expected to contribute to the primary prevention of arterial thrombosis by improving lifestyle habits, such as diet and exercise, or the consideration of antithrombotic medication, but the most appropriate test for evaluating thrombogenicity in an

* Corresponding author at: Health Center, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160–8582, Japan. Tel.: +81 3 5363 3973; fax: +81 3 3359 6963.
E-mail address: TakaMoriki@aol.com (T. Moriki).

0049-3848/$ – see front matter
http://dx.doi.org/10.1016/j.thromres.2013.05.026

artery is unclear. The platelet aggregation test using light transmission aggregometry (LTA) is currently the gold standard method of evaluating platelet function. Although this test is useful for detecting platelet function deficiencies, it is not adequate for estimating high platelet reactivity, has poor reproducibility, requires skillful manipulation, and is time consuming [15–17].

A new flow-chamber system was recently developed to quantitatively evaluate the growth of PTF using whole blood samples under flow conditions [18,19]. This device, the Total Thrombus-formation Analysis System (T-TAS, Fujimori Kogyo Co., Yokohama, Kanagawa), analyzes the process of PTF by monitoring the continuous pressure increase in the capillary channels of two types of microchips with thrombogenic surfaces. The first chip, the PL chip, contains 25 capillary channels coated with collagen. A whole blood sample anticoagulated with the thrombin inhibitor hirudin is applied to the chip under constant flow speed until occlusion. The continuous increase in the inner pressure is measured, reflecting the specific thrombogenicity mainly mediated by platelets. The second chip, the AR chip, contains a single capillary channel coated with collagen and tissue thromboplastin. Collected citrated whole blood is recalcified immediately before testing, and then tested in a similar way, allowing for the assessment of parameters related to the formation of a platelet thrombus rich in fibrin fibers, representing whole blood thrombogenicity under flow conditions [18]. This system is reported to be useful for evaluating the effects of some clinical anticoagulants (e.g., heparin, argatroban, abciximab, aspirin, $P2Y_{12}$ antagonist) [18,19], human blood products (prothrombin complex concentrates, fresh frozen plasma) [20], and factor VIII or IXa deficiency [21].

In the present study, we aimed to characterize the effectiveness of this system for evaluating the thrombogenicity of whole blood under flow conditions in healthy individuals by comparing the measurements with those of other standard platelet function tests. In addition, we investigated some blood constituents that could affect the measurements obtained using this system.

Materials and Methods

Subjects

Healthy volunteers (11 men, 20 women), between 25 and 62 (mean ± SD, 39 ± 11) years of age, were recruited from Keio University School of Medicine. Individuals taking medication or dietary supplements within the previous 2 weeks that could affect platelet function or coagulation were excluded. All individuals were apparently healthy based on a medical questionnaire. Among them, 26 subjects received regular physical checkups within 6 months in the facility, and the following data were recorded. Mean ± SD body mass index (21 ±2.7 kg/m^2), blood pressure (117 ± 17/69 ± 11 mmHg), glucose (96 ± 9 mg/dl), triglyceride (79 ± 40 mg/dl), HDL-cholesterol (67 ± 18 mg/dl), and LDL-cholesterol (110 ± 31 mg/dl). The study was conducted according to the guidelines of the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of Keio University. Written informed consent was obtained from all subjects before beginning the study.

Blood Samples

Blood samples were collected from the antecubital vein with a 21-gauge butterfly needle into the following tubes: a Hirudin Blood Tube (MP0600: Dynabyte GmbH, Munich, Germany, final concentration of hirudin: 25 μg/ml), a vacuette tube (31–114: Nipro neotube, Nipro, Japan) and blood collection tubes (VP-CW050K, VP-C050K, VP-P052K: Venoject II, Terumo, Japan). Plasma or serum samples to measure biomarkers were prepared by centrifuging whole blood at 2500 rpm for 10 min at room temperature. All platelet function tests were performed from 1 to 4 hours after blood sample collection.

Microchip Flow-chamber System

The PTF process was monitored quantitatively using the T-TAS as previously reported [18,19]. Briefly, this system analyzes PTF generation by monitoring the continuous pressure increase in the capillary channels of each of two microchips with thrombogenic surfaces.

One chip, the PL chip, contains 25 capillary channels 40 μm wide × 40 μm deep coated with type I collagen [19]. A whole blood sample (320 μl) anticoagulated with the thrombin inhibitor hirudin is applied to the chip under constant flow until occlusion. The continuous increase in the inner pressure is measured, reflecting the specific thrombogenicity mainly mediated by platelets in the absence of coagulation and fibrinolysis pathways [19,22]. Another chip, the AR chip, contains a single capillary channel 300 μm wide × 120 μm deep coated with type I collagen plus tissue thromboplastin [18]. Whole blood taken with 3.13% sodium citrate is mixed with $CaCl_2$ and corn trypsin inhibitor (CTI) immediately before testing to restore the coagulation system except for Factor XII, and the mixture of 450 μl is tested similarly under constant shear rate (300 s^{-1}), allowing for the measurement of parameters related to the formation of the fibrin-rich platelet thrombus, representing whole blood thrombogenicity under flow. Activity of the tissue thromboplastin coated on the AR chip and the effect of CTI on intrinsic coagulation pathway were assessed elsewhere (Figs. S1 and S2).

The typical flow pressure patterns and measured parameters using the PL or AR chips are depicted in Fig. 1. The PTF starting time was defined as T_{10} (min: s), the time in minutes for pressure to reach 10 kPa from baseline. Based on the pressure increase, the time required to reach 60 kPa, T_{60}, was defined as the occlusion time for the PL chip [22], and similarly, the time required to reach 80 kPa (T_{80}) was defined as the occlusion time for the AR chip. The growth rates of PTF were calculated as T_{60} minus T_{10} ($T_{10\text{-}60}$) or T_{80} minus T_{10} ($T_{10\text{-}80}$). AUC refers to the area under the flow pressure curve; AUC_{10}, until 10 min for the PL chip, and AUC_{30}, until 30 min for the AR chip. All of the measurements were performed under a constant flow speed of 12, 18, and 24 μl/min, corresponding to shear rates of 1000, 1500, and 2000 s^{-1} for the PL chip, and 10 μl/min, corresponding to 300 s^{-1} for the AR chip.

Platelet Aggregometry

The light transmission aggregometry (LTA) test was performed using 3.8% citrated whole blood samples. Platelet-rich plasma was prepared by centrifuging at 700 rpm for 15 min and platelet-poor plasma was prepared by centrifuging at 2500 rpm for 10 min at room temperature. Platelet-rich plasma was adjusted to 300,000 platelet counts/μl using platelet-poor plasma. Thereafter, 22 μl of each agonist was added to 200 μl of the adjusted platelet-rich plasma. Platelet aggregation was induced by a final concentration of 5 μmol/l adenosine diphosphate (ADP; Trinity Biotech, Co., Wicklow, Ireland), 2 μg/ml collagen (Takeda Pharmaceutical International GmbH, Zurich, Switzerland), 10 μmol/l epinephrine (Daiichi Sankyo Co., Ltd., Tokyo, Japan) or 1.2 mg/ml ristocetin (Nacalai Tesque, Kyoto, Japan). The rate of maximum platelet aggregation (MPA) and area under the aggregation curve (AUC) were measured using Easy Tracer ET-800 (Tokyo Koden, Tokyo, Japan).

VerifyNow P2Y12 Assay

The VerifyNow P2Y12 system (Accumetrics, San Diego, CA) is a whole-blood, light transmission-based optical detection assay that measures ADP-induced platelet aggregation in a cartridge containing fibrinogen-coated beads. The blood sample in a 3.13% sodium citrate tube was applied to the cartridge with two channels according to the manufacture's instruction; one contained 20 μmol/l of ADP and