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

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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

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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

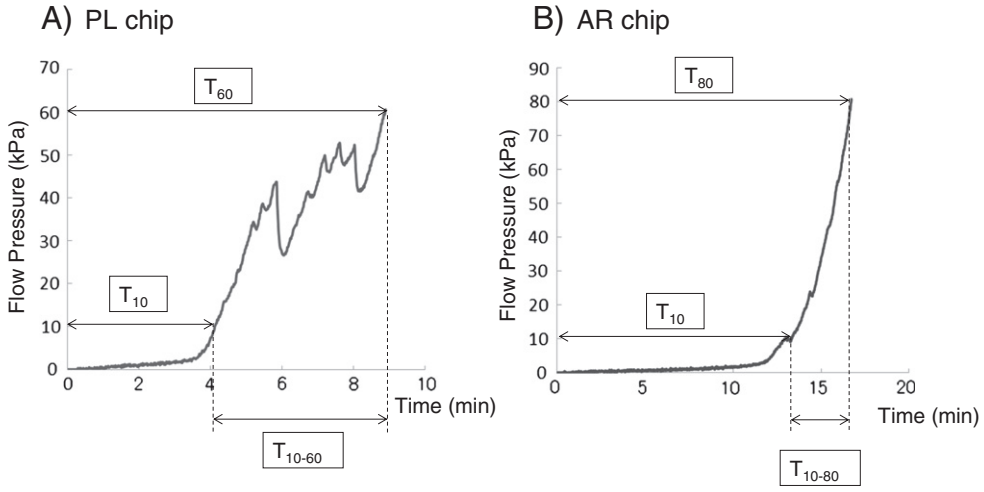

Fig. 1. T-TAS flow pressure curves and parameters. A) PL chip. T_{10}: time from baseline pressure to reach 10 kPa (PTF starting time). T_{60}: time to reach 60 kPa (occlusion time). $T_{10\text{-}60}$: T_{60} minus T_{10} (PTF growth rates). AUC_{10}: area under the flow pressure curve for 10 min. B) AR chip. T_{10}: time from baseline pressure to reach 10 kPa. T_{80}: time to reach 80 kPa. $T_{10\text{-}80}$: T_{80} minus T_{10}. AUC_{30}: area under the flow pressure curve for 30 min.

22 nmol/l of prostaglandin E_1, the other contained iso-thrombin receptor agonist peptide. The results are expressed as P2Y12 reaction units (PRU).

Platelet Function Analyzer-100

The platelet function analyzer (PFA)-100 (Dade-Behring, Marburg, Germany) is a device used to assess platelet function under high-shear rates (5000–6000 s^{-1}) using citrated whole blood. We used 3.8% sodium citrate in this study [23,24]. The sample is subjected to constant flow through a capillary to a microscopic aperture cut into the membrane coated with collagen and epinephrine (C/EPI) or collagen and ADP (C/ADP). These agonists and high-shear rates induce platelet attachment, activation, and aggregation, resulting in occlusion of the aperture. The time required for full occlusion is reported as the closure time (CT). In this study, CT values greater than 300 s were regarded as 300 s for statistical analysis.

Complete Blood Count Tests and Biomarkers

Complete blood count tests were performed using the Sysmex KX-21, an automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Plasma levels of biomarkers were measured using commercially available kits as follows; VWF:CB (VWF-CBA enzyme-linked immunosorbent assay [ELISA]; PROGEN Biotechnik GmbH, Germany), VWF:Ag (IMUBIND® VWF Activity ELISA; American Diagnostica, Stamford, CT), ADAMTS13:Ag (IMUBIND® ADAMTS13 ELISA; American Diagnostica), ADAMTS13:Ac (FRETS-VWF73, Peptide Institute, Inc., Osaka, Japan), Fibrinogen:Ag (Human Fibrinogen ELISA Kit; Innovative Research, Novi, MI), plasminogen activator inhibitor (PAI)-1:Ag (IMUBIND Plasma PAI-1 ELISA; American Diagnostica), PAI-1:Ac (PAI-1, Active, Human, ELISA kit; Innovative Research), glycocalicin (Glycocalicin EIA Kit; Takara Bio Inc, Shiga, Japan), high sensitive C-reactive protein (hs-CRP; CircuLex High-Sensitivity CRP ELISA Kit; CycLex Co., Ltd., Nagano, Japan).

Statistical Analysis

Data are shown as the mean ± SD unless indicated otherwise. The Shapiro-Wilk test was performed to assess for the normal distribution of all measured parameters. Correlations of parametric or non-parametric data were analyzed with Pearson's correlation coefficient or Spearman's correlation coefficient, respectively. Statistical analysis was performed using SPSS version 16.0 (SPSS Inc, Chicago, IL). Two-tailed values were reported, and P values of less than 0.05 were considered statistically significant. The reference intervals of the measurements were defined as the range within 95% confidence intervals. Figures were prepared using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA).

Results

Measurements by T-TAS

The individual time-dependent continuous inner pressure increase in the microchips was represented as a waveform (Fig. 2). The starting and ending points of PTF using the PL or AR chip varied among healthy subjects, possibly characterizing the individual potential for thrombus formation under blood flow conditions.

The results (mean ± SD, range) of the PTF starting time (T_{10}), occlusion time (T_{60} or T_{80}), growth rates ($T_{10\text{-}60}$ or $T_{10\text{-}80}$), and AUC (AUC_{10} or AUC_{30}) using the PL or AR chip are summarized in Table 1. Intra-assay coefficients of variation (CV) in PL chip measurements (1500 s^{-1}) were as follows: T_{10} 6.0%, T_{60} 4.2%, $T_{10\text{-}60}$ 6.2%, AUC_{10} 2.5% ($n = 5$), and in AR chip measurements: T_{10} 7.9%, T_{80} 6.0%, $T_{10\text{-}80}$ 20.2%, AUC_{30} 5.0% ($n = 4$). In the PL chip, T_{10} and T_{60} both decreased in accordance with an increase in shear rates. Furthermore, $T_{10\text{-}60}$ was also decreased, indicating that the shear rates accelerated the growth rate of PTF. Consequently, a shear-dependent increase in AUC_{10} was observed, indicating a comprehensive parameter related to the platelet-mediated thrombogenicity under high shear stress. In addition, the standard deviation (SD) of T_{10} at 1000 s^{-1} was greater than at 1500/2000 s^{-1}, and SD of $T_{10\text{-}60}$ at 1000/1500 s^{-1} was greater than that at 2000 s^{-1}, whereas the SD of T_{60} and AUC_{10} was almost the same. Accordingly, the difference in individual thrombogenicity might be well expressed by the T_{10} and $T_{10\text{-}60}$ at low shear rates. The distribution of measurements illustrates the characteristics of these parameters (Fig. 3).

In this study, the reference intervals of the measurements were defined as the range within 95% confidence intervals. Among the 31 subjects, a 55-year-old woman showed decreased T_{10} in the PL chip at 2000s^{-1} (1:01), possibly related to enhanced thrombogenicity, although she exhibited no abnormalities in the LTA, VerifyNow P2Y12 assay, and PFA-100. Among the 31 subjects, 8 showed prolonged T_{10}, $T_{10\text{-}60}$, or T_{60}, or reduced AUC_{10}, possibly related to reduced thrombogenicity. Of

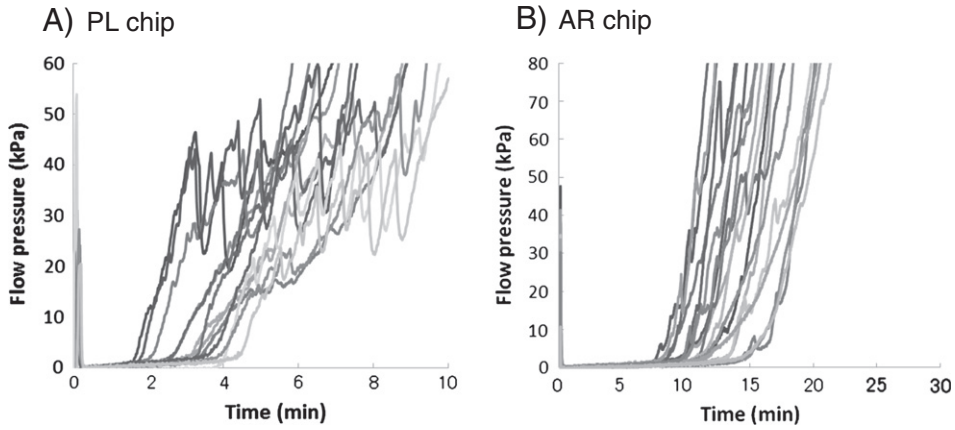

Fig. 2. Variations in individual time-dependent flow pressure curves depicted as superimposed waveforms. A) Measurements by PL chip from 13 subjects. B) Measurements by AR chip from 19 subjects.

these 8, 3 exhibited abnormalities in other tests. A 31-year-old woman showed prolonged T_{10} in the PL at 1000 s^{-1} (5:53) and in the AR (16:20) with reduced ristocetin-induced MPA (49.0%) and prolonged C/ADP (185 s). A 52-year-old man exhibited a prolonged T_{60} (6:28) with reduced epinephrine-induced MPA (35.7%) and reduced PRU (175). A 28-year-old man showed only reduced AUC_{10} in the PL chip at 2000s^{-1} (262.7), although he had reduced epinephrine-induced MPA (26.7%) and AUC (6679.9), ADP-induced MPA (53.6%) and AUC (13979.6), collagen-induced MPA (49.2%) and AUC (7444.9), and prolonged C/EPI (>300 s). The remaining 5 subjects exhibited no abnormalities in other tests.

Other Platelet Function Tests

Platelet function tests of the healthy subjects were performed using the LTA, VerifyNow P2Y12 assay, and PFA-100 (Fig. 4), and the results were compared with the T-TAS measurements. In LTA, 4 of 31 subjects showed abnormalities; 3 of these 4 had abnormalities in T-TAS. Among the remaining subjects, a 57 year-old man exhibited reduced ADP-induced MPA (55.4%) with prolonged C/ADP (155 s), but no abnormalities in the T-TAS. In the VerifyNow P2Y12 assay, only one subject showed reduced PRU (175) with normal T-TAS measurements. In the PFA-100, two subjects showed prolonged C/EPI (>300). Of these, one showed reduced epinephrine-induced MPA (26.7%) with slight abnormality in the T-TAS (reduced AUC_{10} at 2000 s^{-1}), and the other showed normal epinephrine-induced MPA (83.4%) with no abnormality in the T-TAS. And two subjects showed prolonged C/ADP. Of these, one exhibited prolonged T_{10} in the PL at 1000 s^{-1} and the other had no abnormality in the T-TAS.

Correlation between T-TAS and other Platelet Function Tests

In the PL chip, T_{10} was correlated with C/EPI and C/ADP (Fig. 5), and AUC_{10} was correlated with C/EPI (data not shown) under all of the shear rate conditions. Furthermore, the relationship was enhanced in accordance with an increase in the shear rates. Thus, the findings from the PL chip were associated with those of the PFA-100 in many respects, indicating that its characteristics were related to high shear-induced PTF. In addition, T_{60} and AUC_{10} correlated with the AUC of collagen-induced platelet aggregation curve under all shear rate conditions ($|r| = 0.404 \sim 0.531$). In the AR chip, $T_{10\text{-}80}$, reflecting the rate of PTF, was significantly correlated with C/ADP ($r = -0.366$, $p = 0.043$), although few AR measurements were associated with other platelet function tests. The VerifyNow P2Y12 assay values were not significantly associated with the parameters of the T-TAS.

T-TAS and CBC/biomarkers

To identify the blood constituents that would affect this system, we investigated the correlations between CBC, hematocrit, VWF, fibrinogen, ADAMTS13, PAI-1, hs-CRP, and glycocalicin. The results of CBC, VWF, and fibrinogen tests are shown in Table 2. Interestingly, platelet counts were correlated with all parameters in the AR chip (Fig. 6), and mostly with those in the PL chip. The VWF results were not associated with any of the T-TAS measurements. Fibrinogen was associated with T_{10} in the PL at 1000 s^{-1}. Other biomarkers tested were irrelevant. For reference, as previously reported [38], C/EPI and C/ADP of the PFA-100 were correlated with VWF ($r = -0.501, -0.382$). The VerifyNow P2Y12 assay values were associated with red blood cells, hemoglobin, and hematocrit ($r = -0.725, -0.788, -0.732$, respectively).

Table 1
Mean ± SD, range of T-TAS measurements (min:s or min · kPa).

	PL chip			AR chip	
	1000 s^{-1}	1500 s^{-1}	2000 s^{-1}	300 s^{-1}	
T_{10}	3:44 ± 1:02 (1:57–6:01)	2:43 ± 0:51 (1:10–4:30)	2:32 ± 0:41 (1:00–3:50)	**T_{10}**	11:39 ± 2:15 (8:39–16:44)
T_{60}	8:00 ± 1:14 (5:51–10:10)	5:47 ± 1:36 (3:03–9:26)	4:55 ± 1:12 (3:13–7:57)	**T_{80}**	15:48 ± 2:56 (11:42–21:21)
T_{10-60}	4:16 ± 1:04 (2:16–6:30)	3:03 ± 1:03 (1:26–5:07)	2:23 ± 0:48 (1:05–4:35)	**T_{10-80}**	4:08 ± 1:11 (2:12–6:42)
AUC_{10}	271.1 ± 58.2 (176.6–369.1)	369.1 ± 71.8 (196.0–487.4)	396.6 ± 55.8 (262.7–492.5)	**AUC_{30}**	1318.5 ± 203.2 (924.9–1576.1)

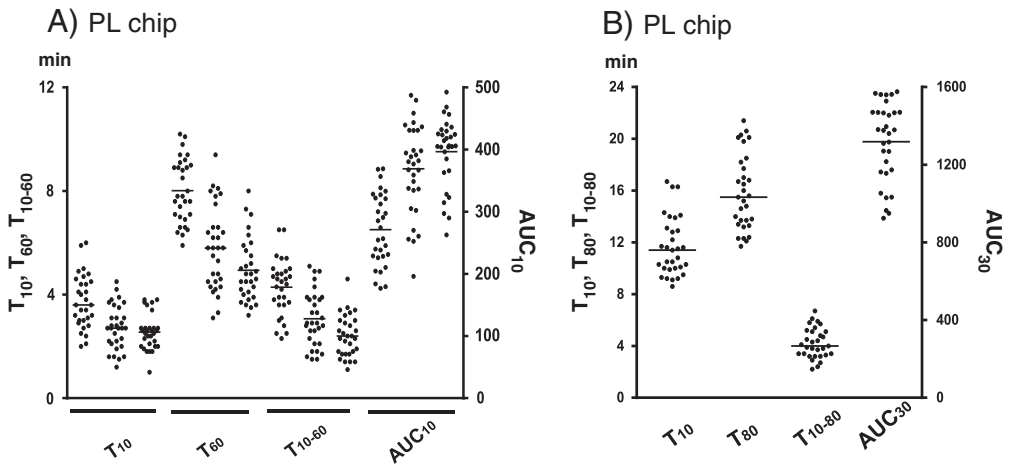

Fig. 3. Distribution of the T-TAS measurements from 31 healthy subjects. A) PL chip. In each measurement, shear rates were 1000, 1500, and 2000 s^{-1} from left, middle, and right, respectively. B) AR chip. Shear rate: 300 s^{-1}. T_{10}: PTF starting time. T_{60}, T_{80}: occlusion time. $T_{10\text{-}60}$, $T_{10\text{-}80}$: PTF growth rates. AUC_{10}, AUC_{30}: area under the flow pressure curve until 10 or 30 min.

Discussion

The process of thrombus formation is a complex pathway in which blood constituents, vessel wall function, and blood flow are integrated (Virchow's triad) [25,26]. Many blood tests have been developed to assess factors related to hemorrhagic or thrombotic diathesis, such as platelets, coagulation, fibrinolysis, and endothelial cell function, although it remains unclear which clinical test is most appropriate for evaluating total thrombogenicity.

The T-TAS, a flow chamber system, uses whole blood samples, and flow on thrombogenic surfaces (collagen: PL chip; collagen plus thromboplastin: AR chip) is measured under various shear rate conditions. Accordingly, this system allows for measurements of at least two factors of the triad, blood constituents and flow, and the blood volume required is practical for clinical application. The starting and ending points of occlusion by clotting are monitored as the pressure is increased (Fig. 1), allowing for measurement of the parameters possibly linked to individual thrombogenicity. The pressure increase is represented as a waveform specific to each individual (Fig. 2). The distribution of the occlusion starting time (T_{10}), ending time (T_{60} or T_{80}), growth rate ($T_{10\text{-}60}$ or $T_{10\text{-}80}$), and AUC (AUC_{10} or AUC_{30}) varied among healthy subjects (Fig. 3). These findings suggested that the measurement

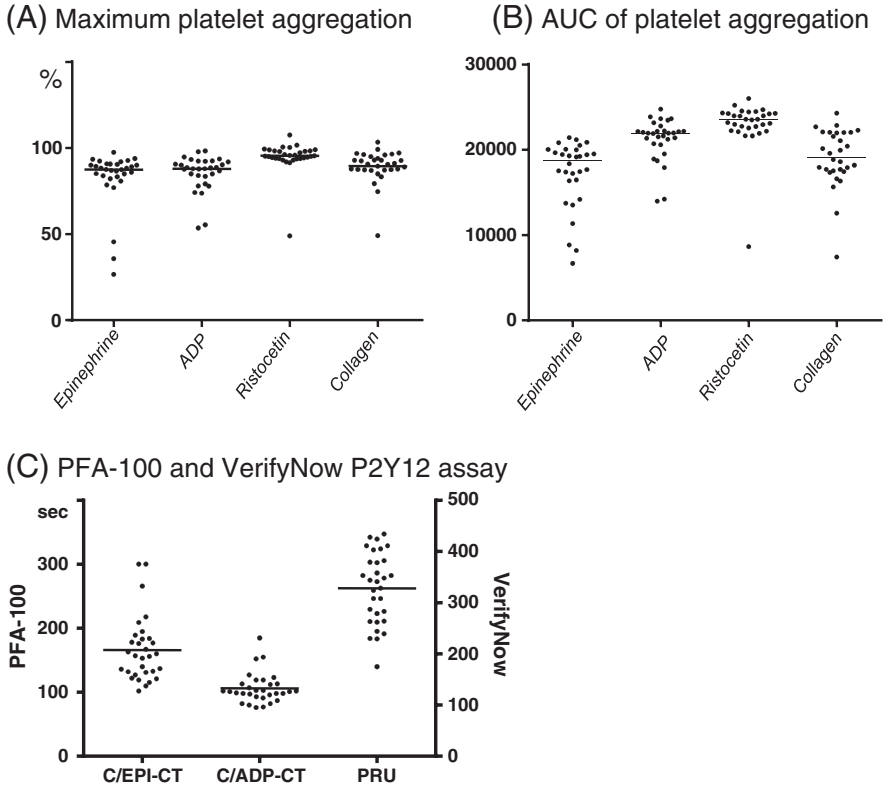

Fig. 4. Distribution of measurements by platelet function tests from 31 healthy subjects. A) LTA maximum platelet aggregation by agonists. B) AUC (area under curve) of platelet aggregation curve induced by agonists. C) Closure time of the C/EPI and C/ADP cartridge by PFA-100 (left), and PRU of the P2Y12 assay by VerifyNow (right).

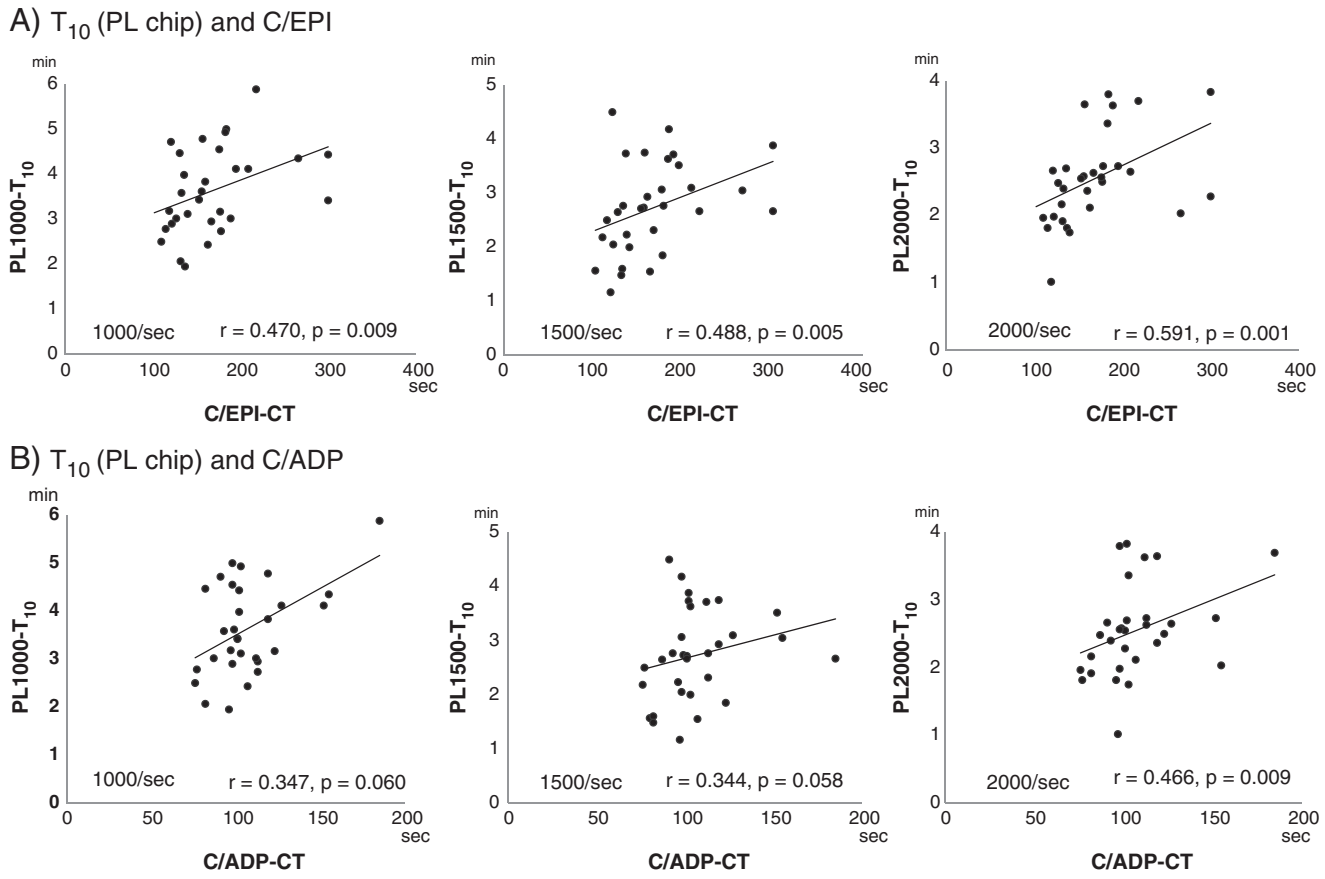

Fig. 5. Correlation of PTF starting time (T_{10}) by T-TAS/PL-chip and the closure time of C/EPI (top) and C/ADP (bottom) by PFA-100. Shear rates were 1000, 1500, and 2000 s^{-1} from left, middle, and right, respectively. r, correlation coefficient. p, probability value.

parameters of the T-TAS reflect individual characteristics of thrombus formation.

In the PL chip, the thrombus is formed under high shear rates without the generation of thrombin, thus the measurements reflect the parameters mainly mediated by platelets in the absence of coagulation and fibrinolysis pathways. At high-shear rates, the interaction between the platelet membrane glycoprotein GPIb-IX-V complex and collagen-bound VWF plays a key role in the first step of platelet adhesion [27,28]. As the shear rates increased, T_{10}, T_{60}, $T_{10\text{-}60}$ were decreased and AUC_{10} was increased (Table 1, Fig. 3), supporting the involvement of the GPIb-IX-V/VWF interaction in these measurements. Previous reports showed contribution of the interaction during the thrombus formation in the T-TAS using GPIb inhibitor [19] and anti-VWF monoclonal antibodies [32].

The standard deviation of T_{10}, T_{60}, and $T_{10\text{-}60}$ in the PL chip diminished in accordance with an increase in the shear rate, thus individual differences might be poorly expressed under higher shear rate conditions. It is noteworthy that T_{10} and AUC_{10} showed a shear-dependent correlation with C/EPI or C/ADP. Because the PFA-100 uses citrated whole blood under high-shear rates (5000–6000 s^{-1}), it is reasonable that the results of the PL chip were associated with the results of the PFA-100 in many respects, based on its characteristics related to high shear-induced PTF [29]. We speculate that the PL chip might detect individual differences more sensitively than the PFA-100, because of the lower shear rates and the use of only collagen as a physiologic agonist. In contrast, however, the results of the LTA and VerifyNow P2Y12 assay rarely correlated with those of the PL chip. In the LTA, only the AUC of the collagen-induced platelet aggregation curve correlated with T_{60} and AUC_{10} under all shear rate conditions ($|r| = 0.404 \sim 0.531$). For stable platelet adhesion on the collagen in subendothelium after GPIb-IX-V/VWF interaction, subsequent activation of integrin GPIIb-IIIa and collagen receptors is involved in the binding to fibrinogen, VWF, and collagen [1,2]. The collagen-induced AUC might be associated with the platelet reactivity to collagen under flow conditions in a certain way in the PL chip. Interestingly, fibrinogen levels were associated with a measurement in the PL chip (Table 2), suggesting the contribution of fibrinogen as a bridging factor among platelets, and some clinical reports identified the fibrinogen level as a possible risk factor for cardiovascular disease [30,31]. It is not clear why VWF levels were not associated with any of T-TAS measurements (Table 2). Patients with VWD were reported to exhibit delayed thrombus formation in the T-TAS measurements [32,33]. In healthy subjects, however, thrombus formation should be the consequence of comprehensive reactions in addition to the GPIb-IX-V/VWF interaction, thus the VWF levels alone were not supposed to correlate with the T-TAS measurements. Within normal range of VWF level, higher shear rates might be necessary to detect the effect of VWF levels on PTF in this system. Consequently, the parameters measured in the PL chip are thought to reflect platelet reactivity to adhesion on the collagen surface under whole blood flow conditions with high shear rates between 1000 and 2000s^{-1}.

In the AR chip, citrated whole blood was recalcified and subjected to flow on the surface coated with collagen and tissue thromboplastin under shear rates of 300 s^{-1}, corresponding to those in large-sized arteries [34]. Thus, the extrinsic coagulation pathway and platelet adhesion/activation on the collagen surface under flow were totally involved in the process of fibrin-rich PTF in this microchip. The starting and ending points of individual waveforms clearly varied among healthy subjects, although the rates of thrombus formation

Table 2
Correlation of T-TAS measurements with blood constituents.

	WBC	HCT	PLT	VWF:CB	VWF:Ag	Fibrinogen
PL-1000						
T_{10}	−0.219	−0.035	−0.304	−0.101	−0.201	0.375
T_{60}	−0.060	0.373	−0.398	−0.026	−0.077	0.24
$T_{10\text{-}60}$	0.143	0.503	−0.162	0.069	0.096	-0.087
AUC_{10}	0.189	−0.128	0.412	0.090	0.195	-0.315
PL-1500						
T_{10}	0.021	−0.010	−0.359	−0.203	−0.221	0.137
T_{60}	−0.223	0.213	−0.502	−0.016	−0.023	0.146
$T_{10\text{-}60}$	−0.355	0.336	−0.471	0.140	0.103	0.111
AUC_{10}	0.150	−0.138	0.484	0.090	0.086	−0.146
PL-2000						
T_{10}	0.079	0.083	−0.164	−0.201	−0.181	0.197
T_{60}	−0.223	0.193	−0.355	−0.093	−0.037	0.203
$T_{10\text{-}60}$	−0.403	0.152	−0.395	0.031	−0.012	0.138
AUC_{10}	0.095	−0.145	0.305	0.115	0.024	−0.266
AR						
T_{10}	−0.103	0.136	−0.411	−0.019	−0.164	0.135
T_{80}	−0.091	0.193	−0.520	−0.004	−0.095	0.025
$T_{10\text{-}80}$	−0.030	0.245	−0.503	0.026	0.041	−0.179
AUC_{30}	0.115	−0.169	0.509	0.009	0.087	−0.047

Values shown are correlation coefficients. Enclosed figures are statistically significant ($p < 0.05$). WBC: white blood cell counts. HCT: hematocrit. PLT: platelet counts. VWF CB: von Willebrand factor collagen binding activity. VWF Ag: von Willebrand factor antigen. Mean ± SD (range) of each measurement was as follows: WBC ($x10^2/\mu l$), 50 ± 12 (24–78); HCT (%), 36.9 ± 3.3 (30.6–44.6); PLT ($x10^3/\mu l$), 224 ± 44 (155–342); VWF:CB (%), 95.2 ± 22.2 (54.3–142.9); VWF:Ag (%), 112.8 ± 39.9 (48.3–210.5); Fibrinogen (mg/dl), 275.9 ± 55.9 (157.8–402.2).

seemed almost constant (Fig. 1B). Accordingly, T_{10} or T_{80} could be a useful parameter for evaluating individual differences. Unlike the PL chip, T_{10}, T_{80}, and AUC_{30} were not associated with other platelet function tests, but only $T_{10\text{-}80}$ was likely associated with C/ADP, suggesting the involvement of ADP as a key agonist for thrombus formation under physiologic conditions. These findings suggest that the parameters measured in the AR chip reflect unique characteristics of individual PTF, different from those in other platelet function tests, including the PL chip. Further studies are necessary, however, to characterize the parameters in the AR chip under different conditions such as higher shear rates.

Which blood constituents might contribute to the measurements in T-TAS? With regard to CBC, high platelet counts were strongly associated with a shortened PTF time, especially in the AR chip (Table 2, Fig. 6). A previous report suggested platelet counts and aggregation responsiveness induced by ADP were related to increased coronary heart disease mortality in healthy middle-aged men [35], although other large-scale prospective studies did not detect an association [36,37]. We speculated that higher platelet counts might provide a larger platform with phospholipids for thrombin generation enhanced by agonists such as ADP released from activated platelets, resulting in the efficient and stable fibrin-rich PTF, although the clinical significance remains to be elucidated. In the PL chip, higher platelet counts are likely correlated with a lower PTF time at lower shear rates (Table 2), suggesting that platelet counts contribute to platelet aggregation in the capillary channels even in the absence of thrombin generation, especially under relatively lower shear rate conditions. It is unclear, however, how platelet counts affect the process of PTF *in vivo* under various shear rate conditions. Studies on patients with thrombocytopenia or thrombocytosis might be useful to identify the contribution of platelet counts on the T-TAS measurements. We investigated the correlation between VWF, fibrinogen, ADAMTS13, PAI-1, hs-CRP, and glycocalicin with the T-TAS measurements, but detected no significant association, except fibrinogen level, as described before. As previously reported [38], the PFA-100 results correlated with VWF activity. The VerifyNow P2Y12 assay values correlated with red blood cells, hemoglobin, and hematocrit [39] in the present study. Further studies are needed to define the blood constituents related to the parameters in the T-TAS.

The T-TAS is reportedly useful for assessment of the effects of some clinical anticoagulants, human blood products, and factor VIII

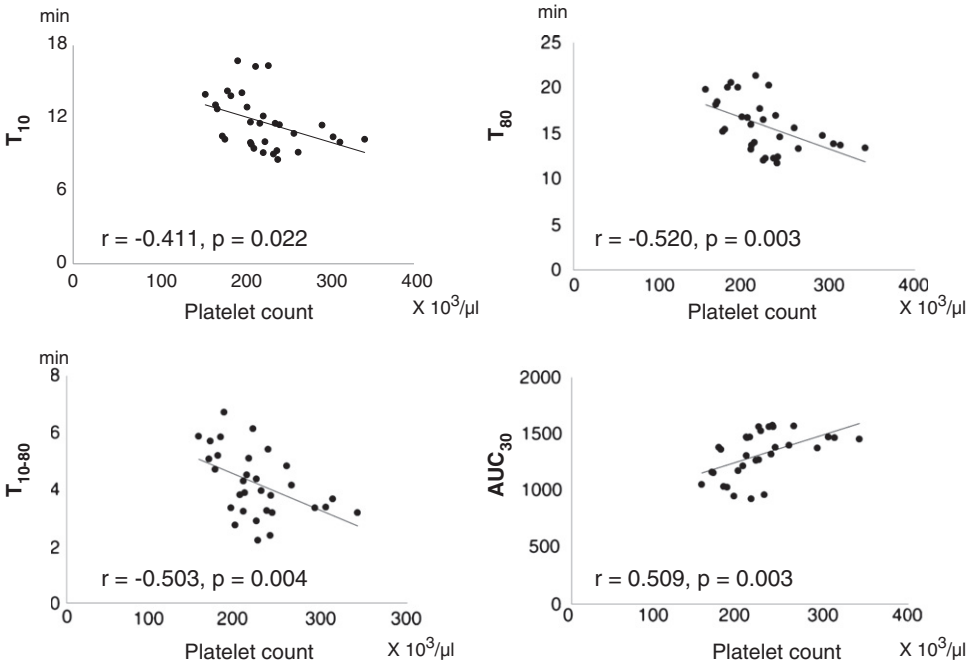

Fig. 6. Correlation of platelet counts and the T-TAS/AR-chip measurements, T_{10} (top left), T_{80} (top right), $T_{10\text{-}80}$ (bottom left), and AUC_{30} (bottom right). r, correlation coefficient. p, probability value.

or IXa deficiency [18–21]. Based on the findings of the present study, the parameters measured in the T-TAS could sensitively detect individual thrombogenicity. Therefore, this system might be a promising method of evaluating not only the bleeding tendency, but also the prothrombotic status in individuals having risk factors related to atherosclerotic thrombosis, such as metabolic syndrome, diabetes, hypertension, dyslipidemia, obesity, smoking and aging. Additional clinical studies are required for further characterization.

In conclusion, we identified that the measurements obtained using the T-TAS detected the characteristics of thrombus formation under shear rate conditions in healthy individuals. These parameters might be associated with the prothrombotic status of patients with atherosclerotic diseases, and thus T-TAS might be a useful monitoring device for primary or secondary prevention of thrombotic disease.

Conflict of Interest Statement

M. Murata: Hematology Consultant for Abbott, Advisory Committees of Daiichi-Sankyo, Advisory Committees of Sanofi-Aventis and Advisory Committees of Pfizer. T. Ohnishi and K. Hosokawa: Employees of the Fujimori Kogyo Co.

Acknowledgements

This study was supported in part by a grant from Keio Gijuku Academic Development Funds (T.M.) and a grant from the Ministry of Health, Labor, and Welfare of Japan (M.M), and a grant from Ministry of Education, Culture, Sports, Science and Technology of Japan (M.M).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2013.05.026.

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