Original Article

Flow Cytometric Analysis of Platelet Function in Micro- and Macroangiopathy

Durchflußzytometrische Analytik der Plättchenfunktion bei Mikro- und Makroangiopathie

M. Koksch[1,2], F. Zeiger[1], Kathrin Wittig[1], Annelie Siegemund[1], D. Pfeiffer[1] und Chr. Rühlmann[1]

Abstract: Aim of this study was the development of a flow cytometric assay to detect platelet activation using in vitro stimulation with physiological agonists. Therefore, in healthy subjects and insulin-dependent diabetics with and without microangiopathy (retinopathy and/or nephropathy) the surface expression density of fibrinogen receptor complex gpIIb/IIIa (CD41), von Willebrand factor complex gpIb/IX (CD42b), α-granule protein GMP-140 (CD62P), and of lysosomal protein gp53 (CD63) were measured ex vivo and after in vitro stimulation using ADP and thrombin receptor-activator peptide 6 (TRAP-6). Best discrimination ($p<0.05$) between diabetics and controls were observed in the surface expression density of GMP-140 after maximum stimulation with the weak agonist ADP (20 µmol/l) and sub-maximal stimulation with the strong agonist TRAP-6 (5 µmol/l). Induced platelet aggregometry using collagen, ristocetin, ADP, and TRAP-6 and plasma concentrations of platelet factor 4 and ß-thromboglobulin failed to show any differences between the groups. GMP-140 levels were analyzed in a dual-color whole blood assay (CD41-PE and CD62P-FITC) in 50 healthy controls and 60 patients suffering from peripheral arterial occlusive disease. Highly significant differences ($p<0.005$) were found between the two groups in the ex vivo as well as in the in vitro stimulated samples (mean fluorescence ±SEM ex vivo: 0.43 ±0.02 vs. 0.50 ±0.02; 5 µmol/l ADP 0.93 ±0.03 vs. 1.30 ±0.04; 10 µmol/l TRAP-6 3.30 ±0.17 vs. 3.91 ±0.16; all $p<0.005$). In conclusion, the in vitro platelet stimulation assay is an easy, fast, and sensitive tool for the detection of platelet hyperreactivity.

Keywords: Flow Cytometry; Platelet Activation; Diabetic Angiopathies/blood.

Zusammenfassung: Ziel der vorliegenden Studie war der Aufbau eines durchflußzytometrischen Assays zur Untersuchung der Thrombozytenfunktion. Neben der Analyse von Aktivierungsmarkern ex vivo sollte der Stellenwert der in vitro Stimulation mit physiologischen Agonisten evaluiert werden. Es wurde die Expressionsdichte des Fibrinogenrezeptors (GpIIb/IIIa; CD41), des von Willebrand Faktor-Rezeptors (GpIb/IX; CD42b), des α-Granulaproteins GMP-140 (CD62P) und des lysosomalen Proteins Gp53 (CD63) auf der Thrombozytenoberfläche bei Gesunden und bei insulinpflichtigen Diabetikern mit Mikroangiopathie (Retinopathie und / oder Nephropathie) und ohne Mikroangiopathie ex vivo und nach in vitro Stimulation mit ADP und TRAP-6 untersucht. Mit einer maximalen Konzentration des schwachen Agonisten ADP (20 µmol/l) und einer submaximalen Konzentration des starken Agonisten TRAP-6 (5 µmol/l) wurden signifikante Unterschiede ($p < 0,05$) in der GMP-Expressionsdichte zwischen den Gruppen gemessen. Die Thrombozytenaggregation nach Induktion durch Kollagen, Ristocetin, ADP und TRAP-6 und die Plasmakonzentrationen von Plättchenfaktor 4 und ß-Thromboglobulin zeigten keine Unterschiede zwischen Gesunden und Diabetikern. Mit einem durchflußzytometrischen 2-Farb-Vollblutassay (CD41-PE und CD62P-FITC) wurden die Plättchen von 50 Gesunden und 60 Patienten mit peripherer arterieller Verschlußkrankheit untersucht. Hochsignifikante Unterschiede zwischen beiden Gruppen wurden dabei ex vivo und nach in vitro Stimulation gefunden (mittlere Fluoreszenz ±SEM für Gesunde und Patienten ex vivo: 0,43 ±0,02 vs. 0,50 ±0,02; 5 µmol/l ADP 0,93 ±0,03 vs. 1,30 ±0,04; 10 µmol/l TRAP-6 3,30 ±0,17 vs. 3,91 ±0,16; alle $p<0,005$). Die vorliegende Studie zeigt, daß der in vitro Stimulationsassay ein einfacher, schneller und sensitiver Test zur Analyse thrombozytärer Hyperreagibilität ist.

Schlüsselwörter: Thrombozytenaktivierung; Durchflußzytometrie; Diabetische Angiopathien/Blut.

Platelets play an essential role in primary hemostasis. After damage of the vascular endothelium platelets are activated by contact to subendothelial structures, e.g. collagen fibrils, von Willebrand factor, fibronectin. A whole cascade of changes in platelet morphology and function follows the primary adherence of platelets via specific receptors. Circulating dis-

[1] Abt. Kardiologie/Angiologie, Medizinische Klinik und Poliklinik I, Universität Leipzig (Direktor: Prof. Dr. J. Schauer)
[2] Author for correspondence: Dr. Mario Koksch, Universität Leipzig, Medizinische Klinik und Poliklinik I, Abt. Kardiologie / Angiologie, Johannisallee 32, D-04103 Leipzig. Fax +49-341-97-12659
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coid platelets become spherical and develop variously shaped projections from the surface turning into multiple pseudopodia. Receptor- and cytoskeletal-mediated outside-in-signaling processes accompany morphological changes. Platelets liberate compounds stored in α, dense, and lysosomal granules via transport through the surface-connected open canalicular system. Furthermore, inside-out-signaling leads to conformational changes of the heterodimer glycoprotein complex gpIIb/IIIa, known as the fibrinogen receptor complex. Due to specific changes in the conformation of the two polypeptide chains within the complex fibrinogen can now be bound and can serve as a bridging molecule leading to platelet aggregation.

While platelet activation processes maintain vascular integrity under physiological conditions, the same mechanisms play a crucial role in the pathogenesis of various diseases [1-5]. Flow cytometric assays has prove to be a sensitive and elegant technique for quantification of platelet activation. *Tschoepe* et al. demonstrated the importance of activated platelets in the pathogenesis of diabetes mellitus [6-8]. They detected a significantly larger fraction of circulating CD62- and CD63-positive platelets in long-time diabetics and also in newly diagnosed insulin-dependent diabetes mellitus patients. In coronary heart disease as one manifestation of degenerative atherosclerotic vessel disease activated platelets are a well established risk factor [9-11] and were found in myocardial infarction, instable angina pectoris, during cardiopulmonary bypass surgery, or after coronary angioplasty [12-18]. In patients suffering from peripheral arterial occlusive disease (PAOD) platelet hyperreactivity was demonstrated using stagnation point flow aggregometry [19, 20], but could not be confirmed in a study from *Galt* et al. using flow cytometry [21].

The aim of our study was the development of a flow cytometric assay for platelet function analysis by introducing in vitro platelet activation and testing its clinical relevance. First, insulin-dependent diabetics and healthy controls were used as reference groups for establishing the method. Then, due to the rare and controversial data concerning PAOD patients that population was examined with the improved protocol.

Material and methods

Patients

In the first part of the study 15 insulin-dependent diabetics without signs of microangiopathy (diabetes I: 9 men, 6 women, age 28 ±8 years), 15 patients with signs of diabetic nephropathy and/or retinopathy (diabetes II: 7 men, 8 women, age 53 ±12 years), and 15 healthy controls (9 men, 6 women, age 35 ±11 years) were included. None of them had taken anti-platelet medication during the 2 weeks prior to blood collection.

60 consecutive PAOD patients (age 63 ±11 years, 48 men, 12 women) hospitalized for angiological assessment and treatment were included in the second part of the study. Patients were classified according to the FONTAINE stages (stage IIa n=27; IIb n=9; III n=11; IV n=13). Distribution of atherosclerotic risk factors among the study population were as follows: smoking 45/60, hyperlipoproteinemia 40/60, hypertension 34/60, diabetes mellitus 17/60, and hyperuricemia 12/60. 20 patients were on 100 mg acetylsalicylic acid (ASA) medication, none of them had taken ticlopidine or non-steroidal anti-inflammatory drugs. Platelet function tests were run before starting infusion therapy or invasive diagnostics. 50 healthy probands (36 men, 14 women, age 31 ± 11 years) served as controls (smoking: 7, HLP: 6, hypertension: no, diabetes: no, hyperuricemia: 3, ASA: no).

Platelet preparation

Blood was taken through a 21-gauge needle with low tourniquet and collected in trisodium citrate. Samples were immediately stored at 37 °C and processed between 15 and 45 minutes after blood drawing. In preliminary studies the lowest rate of in vitro activation had been demonstrated during this period of time interval. Citrated whole blood was diluted in Hank's Balanced Salt Solution (HBSS, Sigma, Deisenhofen, Germany) containing 1mg/ml bovine serum albumin (BSA, Sigma, Deisenhofen, Germany) and platelet count was adjusted to approximately 20,000 platelets / µl. Aliquots of the platelet suspension were activated 10 min at 37 °C using 5 µmol/l ADP (final concentration, Sigma, Deisenhofen, Germany) and 10 µmol/l thrombin-receptor-activating peptide 6 (final concentration, TRAP-6, Bachem, Bubendorf, Switzerland), respectively. Samples were then incubated for 5 min at 37 °C with saturating concentrations of a PE-labeled anti-gpIIb/IIIa monoclonal antibody (clone P2, CD41, Coulter-Immunotech Diagnostics, Hamburg, Germany) and saturating concentrations of a FITC-labeled anti-GMP 140 monoclonal antibody (clone CLB-Thromb/6, CD62P, Coulter-Immunotech Diagnostics, Hamburg, Germany). Platelet activation and staining were stopped using 2 ml of 4 °C HBSS buffer.

Some experiments were carried out in platelet rich plasma (PRP). PRP was obtained after centrifugation of citrated whole blood (15 min at 150 *g*). After dilution in HBSS to 20,000 platelets/µl samples were activated using 20 µmol/l ADP, 5 µmol/l, or 100 µmol/l TRAP-6 and stained with saturating concentrations of the FITC-labeled anti-GMP 140 (clone CLB-Thromb/6, CD62P), anti-gp53 (cloneCLB-Gran/12, CD63), anti-gpIb (clone SZ2, CD42b), and anti-

Non standard abbreviations: ASA, acetylsalicylic acid; BSA, bovine serum albumin; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; GMP-140, granulate membrane protein 140; HBSS, Hank's balanced salt solution; PAOD, peripheral arterial occlusive disease; PF4, platelet factor 4; PE, phycoerythrin; PRP, platelet rich plasma; SEM, standard error of mean; ß-TG, ß-Thromboglobulin; TRAP-6, thrombin receptor activator peptide 6.

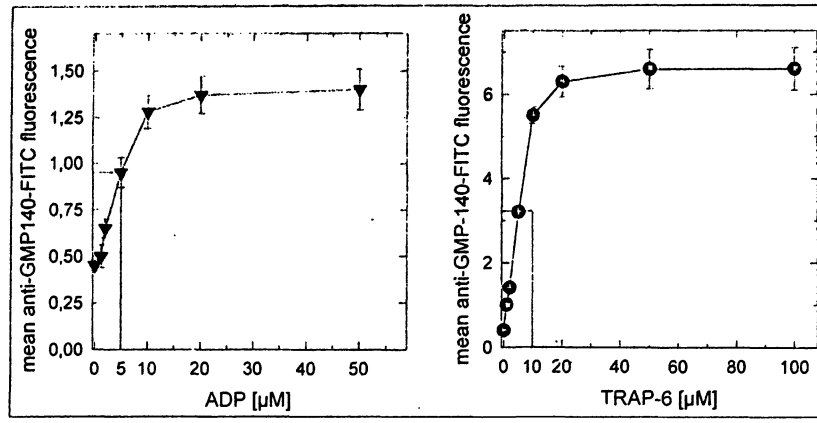

Figure 1 Mean fluorescence of ADP (1-50 µmol/l) or TRAP-6 (1-100 µmol/l) stimulated platelets stained with saturating concentrations of anti-GMP-140-FITC monoclonal antibodies in platelet rich plasma. Values are means ±SEM of three independent experiments.

gpIIb/IIIa monoclonal antibodies (clone P2, CD41, all from Coulter-Immunotech Diagnostics, Hamburg, Germany).

Flow cytometric analysis

A Coulter XL flow cytometer (Coulter Corporation, Hilaleah, FL, USA) equipped with standard filters for FITC and PE fluorescence analysis, and XL System II ™ software R.1.0 or a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Josè, CA, USA) equipped with standard filters for FITC and PE fluorescence analysis, and LYSIS II ™ were used for measurements. In the whole blood assay platelets were gated in the forward scatter vs. FL2 dotplot based on the characteristic FSC signal and the high FL2 emission signal. Threshold or discriminator were set in FL2 to exclude all CD41-negative events. After backgating to the forward vs. side scatter dotplot for separation of platelets from debris, aggregates or leukocytes 10,000 platelets were acquired in list mode at a flow rate of < 500 particles/sec and analyzed. In the PRP studies platelets were gated in the forward scatter vs. side scatter dotplot based on the typical light scatter characteristics with threshold setting in FSC to exclude debris.

Induced platelet aggregometry

Freshly drawn venous blood was collected into 0.1 vol 3.8% trisodium citrate. Platelet-rich plasma (PRP) was obtained after centrifugation of anticoagulated whole blood at 150 *g* for 15 min at 22 °C. The aggregation studies were performed using a PAP-4C aggregometer (Bio/Data, Horsham, PA, USA) in 250 µl PRP (200.000 platelets/µl) with 10 µg/ml collagen, 1.5 mg/ml ristocetin, 10 µmol/l ADP, 100 µmol/l TRAP-6, or 5 µmol/l TRAP-6 as final agonist concentrations.

PF4 and ß-TG ELISA

Test kits for PF4 and ß-TG analysis (Asserachrom-PF4 and Asserachrom ß-TG, Boehringer Mannheim, FRG) were used. Blood collection, sample preparation and measurement at 492 nm were carried out according to the manufacturer's instructions.

Statistics

Software package SPSS for Windows was used for statistical analysis. According to results of Kolmogorov-Smirnov-test for normal distribution of values independent-samples T-test (all PAOD patients vs. controls) or Mann-Whitney U-test (controls vs. diabetes I or II group) were used. Data are presented as mean ±SEM.

Results

Concentration-dependent increase of mean fluorescence of anti-GMP-140-FITC labeled platelets in PRP after in vitro activation using 1-50 µmol/l ADP and 1-100 µmol/l TRAP-6 is shown in Fig. 1. Both agonists continuously increased the release of GMP-140 from the α-granule. TRAP-6 mimicking the thrombin stimulation and known as a very strong platelet agonist led to 5-fold higher maximal fluorescence signal of anti-GMP-140-FITC stained platelets compared to maximum ADP stimulation. 5 µmol/l ADP and 10 µmol/l TRAP-6 were identified as those concentrations inducing half-maximum activation. For the first part of the study 20 µmol/l ADP were chosen as a maximum ADP concentration and 5 µmol/l as well as 100 µmol/l TRAP-6 as a submaximum and maximum TRAP-6 concentration.

Fig. 2 A-D shows the agonist-induced increase in surface expression of gpIIb/IIIa (A), GMP-140 (C), and gp53 (D) and the activation-induced decrease of gpIb molecules (B) on the platelet surface. A good discrimination of study populations was achieved by use of 5 µmol/l TRAP-6 and 20 µmol/l ADP in the GMP-140 expression density, but not by 100 µmol/l TRAP-6 (Fig. 2 C). The activation markers in diabetics with microangiopathy were higher composed to those without. A similar trend was also observed in gpIIb/IIIa and gp53 surface expression densities (Fig. 2 A and D). The strong activator TRAP-6 at maximum concentration was less efficient in discriminating the three study populations than at submaximum TRAP-6 con-

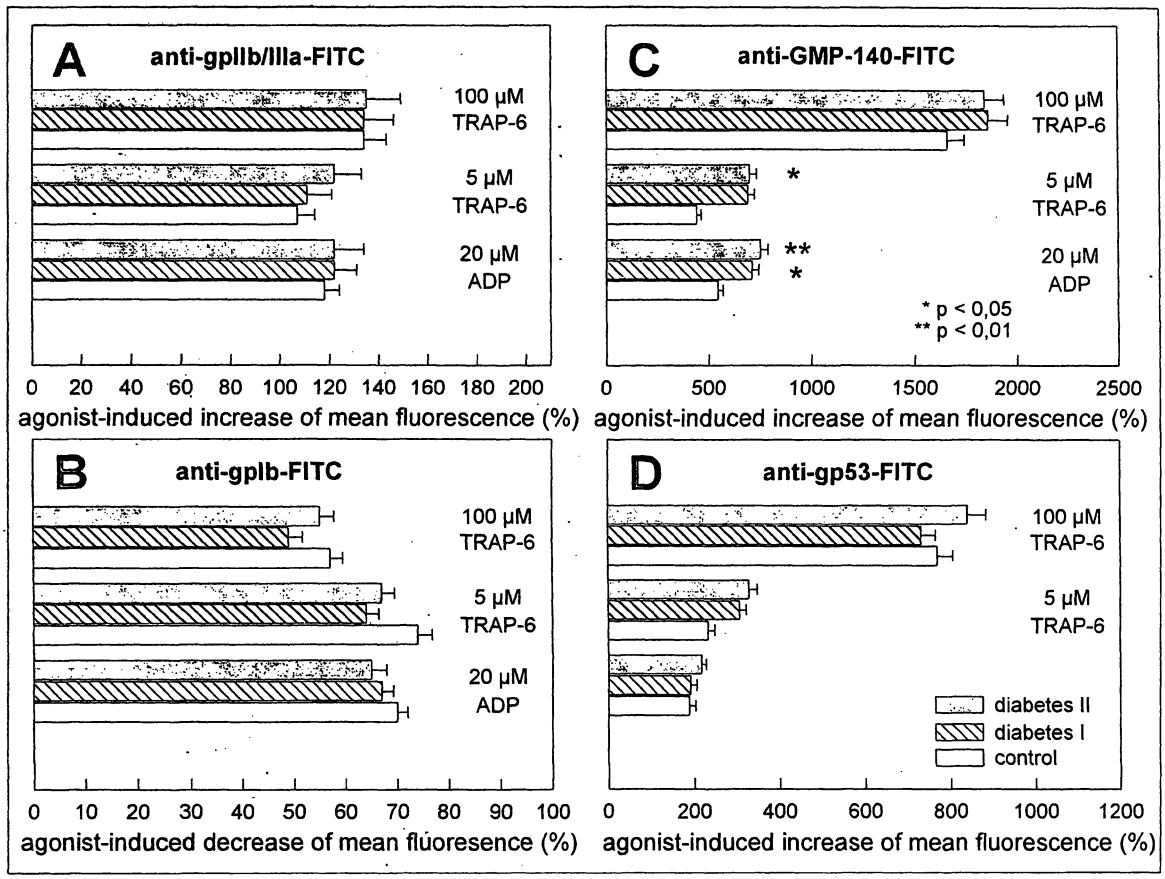

Figure 2 A-D Changes of mean fluorescence in percent of unstimulated samples for 20 µmol/l ADP, 5 µmol/l TRAP-6, and 100 µmol/l TRAP-6 stimulated platelet rich plasma samples of controls (n=15), diabetics without microangiopathy (diabetes I; n=15) or with microangiopathy (diabetes II; n=15). Mean fluorescence of unstimulated samples was set to 100%. After preparation of PRP (15 min centrifugation at 150 *g*) samples were diluted to 20,000 platelets/µl using HBSS buffer (containing 1 mg/ml BSA), activated 10 min by the indicated agonist cocncentrations, and stained 5 min with saturating concentrations of anti-gpIIb/IIIa-FITC (A), anti-gpIb-FITC (B), anti-GMP-140-FITC (C), or anti-gp53-FITC monoclonal antibodies (D). Values are means ±SEM.

centration and the weak agonist ADP. Both groups, the constitutionally expressed surface receptors gpIIb/IIIa and gpIb and the activation-dependent released granule proteins GMP-140 from α-granules and gp53 from dense granules, may serve as activation markers. However, due to a 5-15 fold elevation of mean anti-GMP-140 fluorescence of activated platelets this glycoprotein seems to have the widest diagnostic window.

Measurement of plasma concentrations of the released platelet granule products β-thromboglobulin and platelet factor 4 showed no significant differences between controls and diabetics (Tab. 1). Similar results were found in induced platelet aggregometry using the conventional high agonist concentrations of collagen, ristocetin, ADP, and TRAP-6 (Tab. 2). Differences between the groups were only observed in the low TRAP-6 stimulated aggregometry without reaching significance.

Based on the findings in the first part of the study, a population of 60 consecutive PAOD patients with clinical signs of macroangiopathy hospitalized for angiological assessment and therapy and 50 healthy controls were investigated. By incubation of platelets with half-maximum concentrations of ADP (5 µmol/l) and TRAP-6 (10 µmol/l) significantly higher GMP-140 expression densities ($p<0.005$) were found in PAOD patients both for unstimulated and for in vitro stimulated platelet samples (Fig. 3).

Discussion

Flow cytometric analysis of platelet activation is a sensitive tool for detection of platelet hyperreactivity in coronary heart disease [9-13, 15], diabetes mellitus

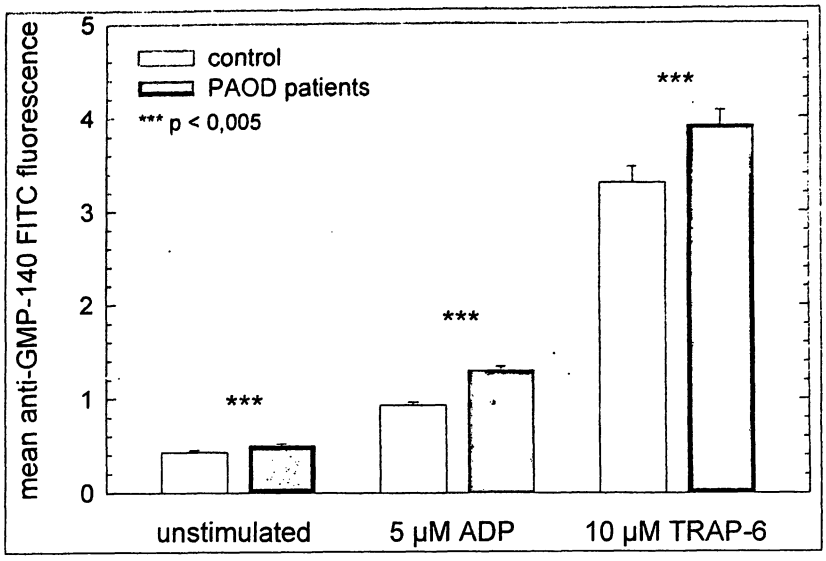

Figure 3 Mean anti-GMP-140-FITC fluorescence of unstimulated, 5 µmol/l ADP-, or 10 µmol/l TRAP-6-stimulated platelets of 60 PAOD patients and 50 controls, respectively. Activation was performed 10 min in HBSS buffer diluted whole blood containing approx. 20,000 platelets/µl. Platelets were stained using saturating concentrations of anti-gpIIb/IIIa-PE labelled and anti-GMP-140-FITC labelled monoclonal antibodies. Values are means ±SEM.

[6-8], chronic inflammatory bowel disease [22], transfusion medicine [23], sepsis [24], cardiopulmonary bypass surgery [17, 25], biocompatibility testing of artificial surfaces [14, 26], or hemostaseology [27]. In most of the studies an immediate fixation was performed after blood drawing and the fraction of activation marker-positive platelets among all platelets (e.g. CD62 or CD63) was measured. Discrimination cut-off for marker-positive platelets was set in platelet samples stained with an unspecific mouse IgG monoclonal antibody [6]. This assay protocol has the advantage of avoiding further platelet activation after blood drawing as well as during sample preparation and storage prior to analysis. However, fixatives (e.g. glyoxal, paraformaldehyde, or glutaraldehyde) per se may induce an artificial activation during the fixation process [28, 29]. Furthermore, after fixation a detailed investigation of the signal transduction mechanisms is impossible. In contrast, in vitro stimulation and investigation of signal transduction can be done using an assay protocol without fixation, but preparation- and storage-induced platelet activation may be critical.

In the present study we tested the hypothesis that a combination of activation marker analysis ex vivo and after in vitro stimulation using the agonists ADP and TRAP-6 may be used as a technique for measurement of platelet activation. In the first part of the study three populations were investigated: 1) healthy probands; 2) insulin-dependent diabetics without, and 3) insulin-dependent diabetics with microangiopathy. Similar to re-

Table 1 Plasma concentrations of platelet factor 4 (PF4; mean ±SEM) and ß-thromboglobulin (ß-TG; mean ±SEM) for controls (n=15), diabetics without (diabetes I, n=15), and diabetics with microangiopathy (diabetes II, n=15)

	α-granule proteins (IU/ml)	
	PF4 (mean ±SEM)	β-TG (mean ±SEM)
Controls	5.98 ± 1.87	5.92 ± 0.93
Diabetes I	3.20 ± 1.37	4.94 ± 1.37
Diabetes II	2.32 ± 0.53	5.35 ± 0.72

Table 2 Induced platelet aggregometry in percent ±SEM of controls (n=15), diabetics without microangiopathy (n=15), and with microangiopathy (n=15) using final concentrations of the following agonists: 10 µg/ml collagen, 1.5 mg/ml ristocetin, 10 µmol/l ADP, 100 µmol/l TRAP-6, and 5 µmol/l TRAP-6.

	Agonist-induced aggregation (percent ± SEM)				
	Collagen	Ristocetin	ADP	100 µmol/l TRAP-6	5 µmol/l TRAP-6
Controls	85±3.3	94±0.9	88±2.0	91±1.9	13±2.0
Diabetes I	89±1.1	91±1.7	87±2.2	93±1.8	15±2.5
Diabetes II	83±3.9	89±1.1	85±2.5	92±1.6	25±8.0

cent reports [6-8] a remarkable increase in platelet surface expression density of activation markers was found in diabetics compared to controls (Fig. 2 A-D). Best discrimination between the groups was seen with the activation marker GMP-140 and after stimulation with ADP and with low TRAP-6 concentration (Fig. 2 C). In contrast to PF4 and ß-TG measurements (Tab.1) and platelet aggregometry (Tab.2) flow cytometric data reached significant levels despite the small number of controls and patients in the three groups. A trend to higher values between controls and diabetics was only seen in 5 µmol/l TRAP-6-induced platelet aggregometry.

We concluded from this data that the assay conditions and activator concentrations presented may serve as an alternative protocol for platelet activation analysis. For this purpose half-maximum concentrations of both agonists were chosen for the second part of the study (Fig.1).

Patients suffering from peripheral arterial occlusive disease (PAOD) as one clinical manifestation of degenerative atherosclerotic disease are expected to have platelet hyperreactivity. This assumption refers to data from coronary heart disease patients and to the clinical benefit of anti-platelet medication [30,31]. However, the literature is controversial. In a group of 16 PAOD patients *Galt* et al. investigated unstimulated and ADP-stimulated GMP-140 levels using flow cytometry, plasma ß-thromboglobulin concentrations, and induced platelet aggregometry, but detected no differences to controls [21]. Using the stagnation point-adhesion aggregometer *Reininger* et al. demonstrated platelet hyperreactivity in PAOD patients in a flow model [19, 20]. In our study the surface expression density of GMP-140 ex vivo and after in vitro stimulation using 5 µmol/l ADP and 10 µmol/l TRAP-6 were examined in 60 PAOD patients and 50 controls. Highly significant differences ($p < 0.005$) were found in both the ex vivo and the in vitro stimulation measurements (Fig.3). The following factors could be responsible for the improved assay sensitivity: 1) changing the assay procedure from PRP in the diabetes study to whole blood analysis in the PAOD study with reduction of in vitro artefacts during sample preparation, 2) keeping a small time window between blood drawing and measurement, and 3) using very sensitive agonist concentrations for platelet stimulation. A difference in the mean age between patients and controls did not have considerable influence on platelet activation as seen in the diabetes part (controls: age 35 ±11, diabetics without microangiopathy 28 ±8 years, patients with signs of microangiopathy age 53 ±12 years). Furthermore, no statistically significant correlation was found between age and platelet activation (data not shown) within the PAOD patients or the control group.

In conclusion, a protocol for platelet activation measurements with marker analysis ex vivo and after in vitro stimulation by ADP and TRAP-6 was presented. This procedure without using fixation, allows a simple and fast detection of platelet hyperreactivity and could be used for further characterization of the signal transduction mechanisms in platelets.

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