CLINICAL-DIAGNOSTIC STUDIES

ASSESSMENT OF PLATELET FUNCTIONAL ACTIVITY IN HEALTHY INDIVIDUALS AND PATIENTS RECEIVING ANTIPLATELET THERAPY. POSSIBLE INCONSISTENCIES BETWEEN AGGREGATION AND FLOW CYTOMETRY TESTS

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Platelet functional activity was assessed in healthy volunteers (HV, n=92), patients with stable angina pectoris (SA, n=42) and acute coronary syndrome (ACS, n=73), treated with acetylsalicylic acid (ASA) + clopidogrel and ASA + ticagrelor, respectively. In all HV and patients we have compared parameters of platelet aggregation (maximum light transmission and velocity, T_{max} and V_{max}) and parameters, characterizing exposure of platelet activation markers, evaluated by flow cytometry. HV platelets were activated by 10 μM, 1 μM TRAP, and 20 μM, 5 μM, 2.5 μM ADP; patient platelets were activated by 10 μM TRAP and by 20 μM and 5 μM ADP. Strong and significant correlations between the aggregation and flow cytometry parameters (the r correlation coefficient from 0.4 up to >0.6) most frequently were registered in HV platelet during activation by 1 μM TRAP and in SA patients during platelet activation by 20 μM and 5 μM ADP. However, in many other cases these correlations were rather weak (r <0.3) and sometimes statistically insignificant. In HV the differences in PAC-1 binding parameters between platelets activated by 10 μM TRAP (the strongest agonist) and all ADP concentrations were negligible (≤10%), while CD62P binding (at all ADP concentrations) and LTA parameters for (5 μM and 2.5 μM ADP) were significantly lower (by 40–60%). Antiplatelet therapy in patients decreased all parameters as compared to HV, but to varying extents. For 10 μM TRAP the MFI index for PAC-1 binding (40–50% decrease) and for both ADP concentrations the T_{max} values (60–85% decrease) appeared to be the most sensitive in comparison with the other parameters that decreased to a lesser extent. The data obtained indicate a possibility of inconsistency between different LTA and flow cytometry parameters in assessing platelet activity and efficacy of antiplatelet drugs.

Key words: platelets; platelet aggregation; flow cytometry; glycoprotein IIb-IIIa; P-selectin; antiplatelet drugs

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INTRODUCTION

Assessment of the functional activity of platelets and the effectiveness of antiplatelet drugs, both in clinical and experimental studies, are most often carried out using light transmission aggregometry (LTA) and flow cytometry tests [1, 2]. To measure aggregation, platelets are activated in constantly stirred platelet-rich plasma (PRP) with various agonists (ADP, TRAP (thrombin receptor activating peptide), collagen, arachidonic acid, etc.) and the light transmittance (T%) of the suspension increases during aggregate formation. In most instruments the aggregation curves are characterized by two parameters: the maximum level of aggregation, calculated as the maximum increase in light transmission (T_{max}), and the maximum aggregation rate (slope of the curve), calculated as the maximum change in light transmission per minute (V_{max}) [3, 4]. In contrast to the aggregation test, platelet activity measurements using flow cytometry can be performed in whole blood, in small volumes (less than 1 ml) and when platelet counts are low. During the test, whole blood platelets are pre-identified (gated) by their size and binding to platelet-specific antibodies (CD42b or CD41). To assess platelet activity by flow cytometry, agonists (the same as in the aggregation assay) are added to the platelets, and the level of activation is measured by the binding of fluorescently labeled antibodies against specific activation markers displayed on the platelet surface. Most often for these purposes the PAC-1 antibody and various variants of CD62P antibodies are used. The PAC-1 antibody recognizes the activated conformation of glycoprotein (GP) IIb-IIIa (CD41/CD61), while CD62P antibodies interact with the P-selectin protein, which is located in resting platelets in the membranes of α-granules, and appears on the surface of activated platelets due to exocytosis of granules. The level of binding of fluorescently labeled antibodies is usually characterized by two parameters: the mean fluorescence intensity of the peak (MFI, mean fluorescence intensity, arbitrary units, a.u.) or the percentage of positively labeled platelets [5, 6]. Despite the widespread use of both aggregometry and flow cytometry for measurement of the platelet activity, it is unclear how consistent the results obtained by these methods are with each other.

Abbreviations used: ACS – acute coronary syndrome; ADP – adenosine diphosphate, ASA – acetylsalicylic acid; GP – glycoprotein; HV – healthy volunteers; LTA – light transmission aggregometry; MFI – mean fluorescence intensity; PRP – platelet-rich plasma; SA – stable angina; TRAP – thrombin receptor activating peptide.

In this study, we have investigated the functional activity of platelets in healthy volunteers (HV) and patients with cardiovascular diseases receiving antiplatelet therapy by comparing the results obtained using light transmission aggregometry (LTA; T_{max} and V_{max}) and flow cytometry (MFI parameters and the percentage of PAC-1 positive platelets and CD62P-positive platelets).

MATERIALS AND METHODS

Healthy Volunteers and Patients

The study included: (i) healthy volunteers (HV) (n=92, age 46±19 years, the male/female ratio 49/43) who had not taken any medications for 10 days before blood sampling; (ii) patients with stable angina pectoris (SA) (n=42, age 65±11 years, the male/female ratio 28/14) treated with acetylsalicylic acid (ASA) (100 mg per day) and clopidogrel (75 mg per day), (iii) patients with acute coronary syndrome (ACS) (n=73, age 62±10 years, the male/female ratio 55/18) treated with ASA (100 mg per day) and ticagrelor (90 mg ×2 times per day). The ACS group included patients with myocardial infarction (n=58) and unstable angina (n=15). All patients underwent percutaneous coronary intervention on the first day of hospitalization. Patients in both groups were expectedly older than HV (p<0.001), and the number of men in the ACS group was greater than in the HV group (p=0.003). Blood from ACS patients was collected 4–6 days after their hospitalization. All patients were treated at the Chazov National Medical Research Center for Cardiology (NMRC for Cardiology).

Platelet Aggregation

Platelet aggregation was measured as described previously [7]. Blood was collected in 3.8% sodium citrate (the blood/anticoagulant ratio, 9/1); PRP was obtained by centrifugation at 180 g for 10 min. The platelet count was determined using an Abacus Junior B hematology analyzer (Diatron Ltd., Austria). Aggregation in PRP was recorded in a BIOLA analyzer (BIOLA, Russia) at 37°C and a stirring speed of 800 rpm. In the HV group, platelet aggregation was induced by 10 μM and 1 μM TRAP (sequence SFLLRN, peptide provided by M.V. Ovchinnikov, NMRC for Cardiology) and 20 μM, 5 μM, and 2.5 μM ADP (AppliChem GmbH, Germany), and in patients platelet aggregation was induced by 10 μM TRAP and 20 μM and 5 μM ADP. Agonists were added 0.5 min after the start of recording, and aggregation was measured for 4.5 min. The maximum level (T_{max}, %T) and the maximum rate (V_{max}, %T/min) of aggregation were determined.

Platelet Flow Cytometry

Platelet activity was assessed using flow cytometry by measuring surface exposure of activated GP IIb-IIIa and P-selectin, as described in detail previously [8]. Activated GP IIb-IIIa was detected by PAC-1-FITC binding (BD Biosciences, USA), and P-selectin by CD62P-FITC binding (IMTEK, Russia). Control mouse IgG-FITC (IMTEK, Russia) was used to measure nonspecific binding. Blood was collected in 3.8% sodium citrate (the blood/anticoagulant ratio 9/1) and diluted 1/6 with Tyrode buffer without $CaCl_2$ (137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 0.1% dextrose, 5 mM HEPES, 1 mM $MgCl_2$, pH 7.35) containing 0.35% bovine serum albumin. CD42b-APC (3 μl) and PAC-1-FITC (10 μl), or CD62P-FITC (5 μl), or mouse IgG-FITC (5 μl; control samples) were added to 60 μl of diluted blood. Platelets were left unactivated or activated with TRAP or ADP using the same concentrations as in the case of the LTA assay. Samples were incubated for 15 min at 37°C in the dark without stirring, fixed with an equal volume of 2% paraformaldehyde in phosphate-buffered saline (140 mM NaCl, 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.35) for 40 min at room temperature in the dark, and then analyzed in a BD FACS CantoTM II flow cytometer using BD FACS DivaTM software (BD Biosciences). Platelets in whole blood were gated according to their size and CD42b-APC staining, and 10,000 platelet events were analyzed. Platelets in activated samples were considered PAC-1- and CD62P-positive (PAC-1+ and CD62P+) when their fluorescence exceeded that of 95% of the platelets in control samples (non-activated platelets with mouse IgG-FITC controls). The average peak fluorescence level (MFI, a.u.) and the percentages of PAC-1- and CD62P-positive platelets (PAC-1, % and CD62P, %) were calculated.

Statistics

Statistical analysis was performed using Statistica 12 software (StatSoft. Inc., USA). Most of the analyzed variables followed normal distribution (the Shapiro-Wilk test). Data were presented as means ± standard deviations (SD). The significance of differences was assessed using the t-test for means or the paired t-test (as indicated). Correlations were assessed using the Pearson test.

RESULTS

The functional activity of platelets in HV who did not take pharmacological agents, SA patients treated with ASA + clopidiogrel, and ACS patients treated with ASA + ticagrelor was assessed using aggregometry and flow cytometry. In the HV group, platelets were activated by 10 μM and 1 μM TRAP as well as by 20 μM, 5 μM, and 2.5 μM ADP; and in the studied groups of patients platelets were activated by groups — 10 μM TRAP, and 20 μM and 5 μM ADP. 1 μM TRAP and 2.5 μM ADP were not used to stimulate patient platelets, since at such low concentrations these agonists stimulated weak platelet activation responses under conditions

of dual anitlatelet therapy. In the case of LTA assay, T_{max} and V_{max} were recorded, and the exposure of activation markers in a flow cytometer was assessed by MFI and the percentage of positive platelets for binding of PAC-1 and CD62P antibodies. The results of platelet activity measurements by LTA and flow cytometry are summarized in Table 1. In the HV group, the highest platelet activation rates were found for 10 μM TRAP, while PAC-1 binding rates were comparable to those for 20 μM and 5 μM ADP. In SA patients and ACS patients on dual antiplatelet therapy, all activation parameters were expectedly reduced as compared to HV. A more pronounced reduction was observed in ACS patients as compared with SA patients receiving ticagrelor, a stronger P2Y12 ADP receptor antagonist than clopidogrel. Significant differences between the SA and ACS groups were found for all parameters when platelets were activated with ADP (20 μM and 5 μM), while in the case of activation by 10 μM TRAP these differences were found only for PAC-1 binding parameters but not aggregation and CD62P binding.

In many cases the correlations between LTA and flow cytometry parameters for all agonists in all groups were weak (correlation coefficient (r) <0.3) and even statistically insignificant (p>0.05) (Tables 2 and 3). In the HV group, low and mostly nonsignificant correlations were found for platelet activation by 10 μM TRAP and 20 μM ADP, presumably due to their saturating effect on all platelet activation parameters except CD62P binding for ADP 20 μM. More pronounced correlations were often observed at low concentrations of ADP (5 μM and 2.5 μM), and the highest ones with platelet activation with 1 μM TRAP (r >0.4 and >0.5 for some correlations) (Table 2, examples of histograms for high and low correlations in HV, see Supplementary Materials, Fig. S1). In patients, antiplatelet therapy significantly reduced platelet activity, and in this situation, significant correlations between LTA and flow cytometry parameters were also registered when platelets were activated by 10 μM TRAP. High correlations were observed for platelet activation by 20 μM and 5 μM ADP (r >0.5 and >0.6 for some correlations). However, such correlations were more often detected in SA patients than in ACS patients, possibly due to greatly reduced aggregation/activation parameters in ACS patients treated with ticagrelor instead of clopidogrel, a more powerful blocker of P2Y12 ADP receptors (Table 3, examples of histograms for high and low correlations in patients — see Supplementary Materials, Fig. S2).

Stronger correlations were obtained for the two flow cytometry tests (Tables 2 and 3; examples of histograms for high and low correlations are given in Supplementary Materials, Fig. S3). In the HV group, correlations between PAC-1 and CD62P binding parameters (MFI and the percentage of positive platelets) were significant in the vast majority of cases for all TRAP and ADP concentrations. The highest correlation coefficients (r up to 0.8) were found for 1 μM TRAP. In SA patients, all correlations between PAC-1 and CD62P binding parameters were quite strong (r >0.5 for most relationships), but in ACS patients these same correlations were predominantly low and nonsignificant. At least with platelet activation by ADP, this could be explained by lower platelet activation responses due to more potent antiplatelet therapy (ticagrelor instead of clopidogrel).

TRAP (10 μM) is the most potent agonist used in our study. In the group of HV, various parameters of platelet activation were reduced to varying degrees by platelet activation by ADP as compared with 10 μM TRAP (defined as 100%) (Table 1, Fig. 1). For 1 μM TRAP, the reduction was almost the same for all LTA and flow cytometry parameters, about 50–60% (Table 1, Fig. 1). However, in the case of platelet activation by all concentrations of ADP, this difference was minimal for PAC-1 binding parameters (<10%), and for 20 μM ADP, PAC-1 and MFI were even significantly higher than for 10 μM TRAP. In contrast to PAC-1 binding, CD62P binding parameters were significantly lower with ADP as compared to 10 μM TRAP (40–60% reduction), and for LTA parameters these differences were highly dependent on ADP concentration (from 5–20% reduction to approximately 50% for 20 μM and 2.5 μM ADP, respectively) (Table 1, Fig. 1).

Antiplatelet therapy in SA and ACS patients leads to a statistically significant decrease in all parameters of platelet activation (Table 1, Fig. 2). However, this reduction was highly variable and depended not only on the strength of the agonist (expectedly less pronounced for 10 μM TRAP as compared with 20 μM and 5 μM ADP), but also on the particular parameter considered. In the case of platelet activation by 10 μM TRAP, antiplatelet drugs significantly reduced PAC-1, MFI (by 40–50%), while other parameters were reduced less effectively: <20% for LTA parameters, from 35% to 20% for PAC-1, the percentage and CD62P MFI, and <10% for CD62P, the percentage (Table 1, Fig. 2). In the case of platelet activation by ADP, the maximum decrease was observed for the T_{max} aggregation parameter: a decrease from 60% to 85% for 20 μM ADP in SA patients (ASA + clopidogrel) and for 5 μM ADP in ACS patients (ASA + ticagrelor), respectively (Table 1, Fig. 2). Other parameters, including aggregation V_{max} and all activity parameters measured by flow cytometry, were less sensitive to the therapy: reductions from 20% to 50% and from 40% to 60% in SA and ACS patients, respectively. The smallest decrease was observed for CD62P and MFI in patients with SA: only about 20% for both ADP concentrations used (Table 1, Fig. 2).

Table 1. Platelet activation parameters in LTA (T_{max}, V_{max}) and flow cytometry (PAC-1, MFI, PAC-1, %, and CD62P, MFI, CD62P, %) tests in healthy volunteers (HV), SA patients (treated with ASA + clopidogrel), and ACS patients (treated with ASA + ticagrelor)

Agonist	Parameter	HV (n – 83-92)	SA ASA + clopidogrel (n – 38-42) p(HV)≤0,001	ACS ASA + ticagrelor (n – 70-73) p(HV)<0,001
Aggregation				
10 μM TRAP	T_{max}, %T	65±10	55±10	54±11 p(SA)=0.467
	V_{max}, %T/min	87±20	75±17	77±19 p(SA)=0.576
1 μM TRAP	T_{max}, %T	32±29	—	—
	V_{max}, %T/min	42±31	—	—
20 μM ADP	T_{max}, %T	62±11	26±12	14±8 p(SA)<0.001
	V_{max}, %T/min	70±14	47±14	35±12 p(SA)<0.001
5 μM ADP	T_{max}, %T	51±16	15±9	8±6 p(SA)<0.001
	V_{max}, %T/min	57±15	35±12	25±11 p(SA)<0,001
2.5 μM ADP	T_{max}, %T	34±23	—	—
	V_{max}, %T/min	39±16	—	—
Flow cytometry, PAC-1 binding				
10 μM TRAP	PAC-1, MFI, a.u.	3258±986	1984±685	1559±473 p(SA)<0.001
	PAC-1, %	89±7	67±16	59±12 p(SA)=0.001
1 μM TRAP	PAC-1, MFI, a.u.	1788±882	—	—
	PAC-1, %	43±26	—	—
20 μM ADP	PAC-1, MFI, a.u.	3578±1064	2144±723	1586±527 p(SA)<0,001
	PAC-1, %	90±5	63±20	48±12 p(SA)<0,001
5 μM ADP	PAC-1, MFI, a.u.	3242±961	1903±637	1379±461 p(SA)<0,001
	PAC-1, %	86±7	57±20	38±12 p(SA)<0,001
2.5 μM ADP	PAC-1, MFI, a.u.	2917±957	—	—
	PAC-1, %	82±9	—	—
Flow cytometry, CD62P binding				
10 μM TRAP	CD62P MFI, a.u.	1680±407	1348±341	1243±301 p(SA)=0.095
	CD62P, %	94±4	88±9	88±7 p(SA)=0.903
1 μM TRAP	CD62P MFI, a.u.	555±262	—	—
	CD62P, %	37±22	—	—
20 μM ADP	CD62P MFI, a.u.	745±228	566±188	426±105 p(SA)<0.001
	CD62P, %	59±13	34±16	22±10 p(SA)<0.001
5 μM ADP	CD62P MFI, a.u.	654±197	523±160	391±87 p(SA)<0.001
	CD62P, %	53±14	30±15	19±10 p(SA)<0.001
2.5 μM ADP	CD62P MFI, a.u..	595±175	—	—
	CD62P, %	47±14	—	—

Data represent means ± SD; a.u. – arbitrary units; here and in other tables PAC-1, % and CD62P, % designate the percentage of PAC-1 and CD62P-positive platelets; n – the number of HV or patients; p(HV) ≤0.001 for the group of SA patients and p(HV) <0.001 for the group of ACS patients – significance of differences for all tested parameters from the group of HV; p(SA) – significance of differences for indicated parameters in the group of ACS in comparison with the group of SA patients (the *t*-test for means).

Table 2. Correlations of platelet activation parameters in LTA (T_{max}, V_{max}) and flow cytometry (PAC-1, MFI, PAC-1, %, and CD62, MFI, CD62, %) tests in the HV group

10 μM TRAP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.176	0.062	0.134	0.010
V_{max}	0.148	0.183	0.137	0.158
PAC-1, MFI	—	—	0.497***	0.312**
PAC-1, %	—	—	0.219	0.268*
1 μM TRAP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.256*	0.527***	0.446***	0.449***
V_{max}	0.247*	0.534***	0.452***	0.481***
PAC-1, MFI	—	—	0.593***	0.538***
PAC-1, %	—	—	0.794***	0.807***
20 μM ADP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.275**	0.287**	0.167	0.126
V_{max}	0.229*	0.140	0.130	0.074
PAC-1, MFI	—	—	0.319**	0.422***
PAC-1, %	—	—	0.173	0.190
5 μM ADP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.320**	0.245*	0.265*	0.323**
V_{max}	0.392***	0.249*	0.233*	0.202
PAC-1, MFI	—	—	0.343**	0.409***
PAC-1, %	—	—	0.250*	0.174
2.5 μM ADP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.274*	0.249*	0.349**	0.443***
V_{max}	0.249*	0.232*	0.262*	0.213
PAC-1, MFI	—	—	0.347**	0.392***
PAC-1, %	—	—	0.276*	0.179

Coefficients of correlation (r) are presented; *p<0.05, **p<0.01, ***p<0.001 – significance of correlation; n – 83-92.

DISCUSSION

To assess the functional state of platelets in healthy individuals, patients with hemostasis disorders, and patients treated with antiplatelet drugs, LTA and flow cytometry tests are routinely used. In our study, we have compared the parameters obtained using both tests for analysis of the platelet activity in HV and in SA and ACS patients on dual antiplatelet therapy (ASA + clopidogrel and ASA + ticagrelor, respectively). The results of this study were quite unexpected. The correlations between parameters characterizing platelet aggregation (T_{max} and V_{max}) and the expression of activated GP IIb-IIIa and P-selectin (MFI and the percentage of positive platelets for binding antibodies PAC-1 and CD62P, respectively) were not always high, but on the contrary, quite often low and even statistically insignificant. This is true even for the binding parameters of PAC-1, although the conformational changes of GP IIb-IIIa recorded by this antibody and the subsequent binding of fibrinogen by the activated receptor directly mediate platelet aggregation. In some cases, low correlations could be explained by: 1) saturation of aggregation/activation responses in the case of the use of potent agonists (for example, 10 μM TRAP for all parameters and 20 μM ADP for aggregation and PAC-1 binding parameters in the HV group), or 2) vice versa, very low responses when platelet activity was strongly suppressed by antiplatelet drugs (for example, when platelets were activated by 5 μM ADP in ACS patients treated with ASA and a powerful ADP receptor inhibitor, ticagrelor). From a more general point of view, the reported discrepancies between aggregation and flow cytometry could be explained by the different conditions used in these tests. Aggregation was carried out in undiluted, continuously stirred PRP at 37°C, while all flow cytometer activity the measurements were carried out in unstirred and highly diluted whole blood (partly

Table 3. Correlations of platelet activation parameters in LTA (T_{max}, V_{max}) and flow cytometry (PAC-1, MFI, PAC-1, %, and CD62, MFI, CD62, %) tests in SA patients (treated with ASA + clopidogrel), and ACS patients (treated with ASA + ticagrelor)

SA (ASA + clopidogrel)				
10 μM TRAP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.452**	0.578***	0.285	0.417**
V_{max}	0.482**	0.204	0.297	0.217
PAC-1, MFI	—	—	0.635***	0.410**
PAC-1, %	—	—	0.358*	0.540***
20 μM ADP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.562***	0.651***	0.496***	0.680***
V_{max}	0.422**	0.489**	0.269	0.407**
PAC-1, MFI	—	—	0.425**	0.551***
PAC-1, %	—	—	0.556***	0.677***
5 μM ADP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.524***	0.593***	0.426**	0.607***
V_{max}	0.456**	0.596***	0.288	0.560***
PAC-1, MFI	—	—	0.366*	0.502**
PAC-1, %	—	—	0.579***	0.655***
ACS (ASA + ticagrelor)				
10 μM TRAP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.364**	0.149	-0.001	-0.056
V_{max}	0.327**	-0.090	0.024	-0.056
PAC-1, MFI	—	—	0.417***	0.236
PAC-1, %	—	—	0.234	0.230
20 μM ADP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.507***	0.402***	0.294*	0.301*
V_{max}	0.484***	0.301*	0.160	0.132
PAC-1, MFI	—	—	0.233	0.231
PAC-1, %	—	—	0.417***	0.255*
5 μM ADP				
	PAC-1, MFI	PAC-1, %	CD62P, MFI	CD62P, %
T_{max}	0.315**	0.324**	0.322**	0.376**
V_{max}	0.239	0.263*	0.222	0.305*
PAC-1, MFI	—	—	0.171	0.186
PAC-1, %	—	—	0.308**	0.164

Coefficients of correlation (r) are presented; *p<0.05, **p<0.01, ***p<0.001 – significance of correlation; n – 38-41 for the group of SA patients and n – 78-82 for the group of ACS patients.

to prevent aggregation) and at room temperature. This assumption is supported by the fact that Frojmovic et al. [9] found a strong correlation between the parameters of aggregation and PAC-1 binding when both tests were performed in diluted PRP and under the same conditions (in this study, aggregation was recorded not by changes in light transmission, but by a decrease in the number of single (non-aggregated) platelets in the suspension). The most frequently high correlations between platelet aggregation and activation parameters measured by flow cytometry were found for intermediate (non-saturating and not very low) aggregation/activation responses, for example in the HV group, when platelets were activated by 1 μM TRAP, and in SA patients (treated with ASA + clopidogrel), with platelet activation by all agonists used (for 10 μM TRAP only for PAC-1 parameters).

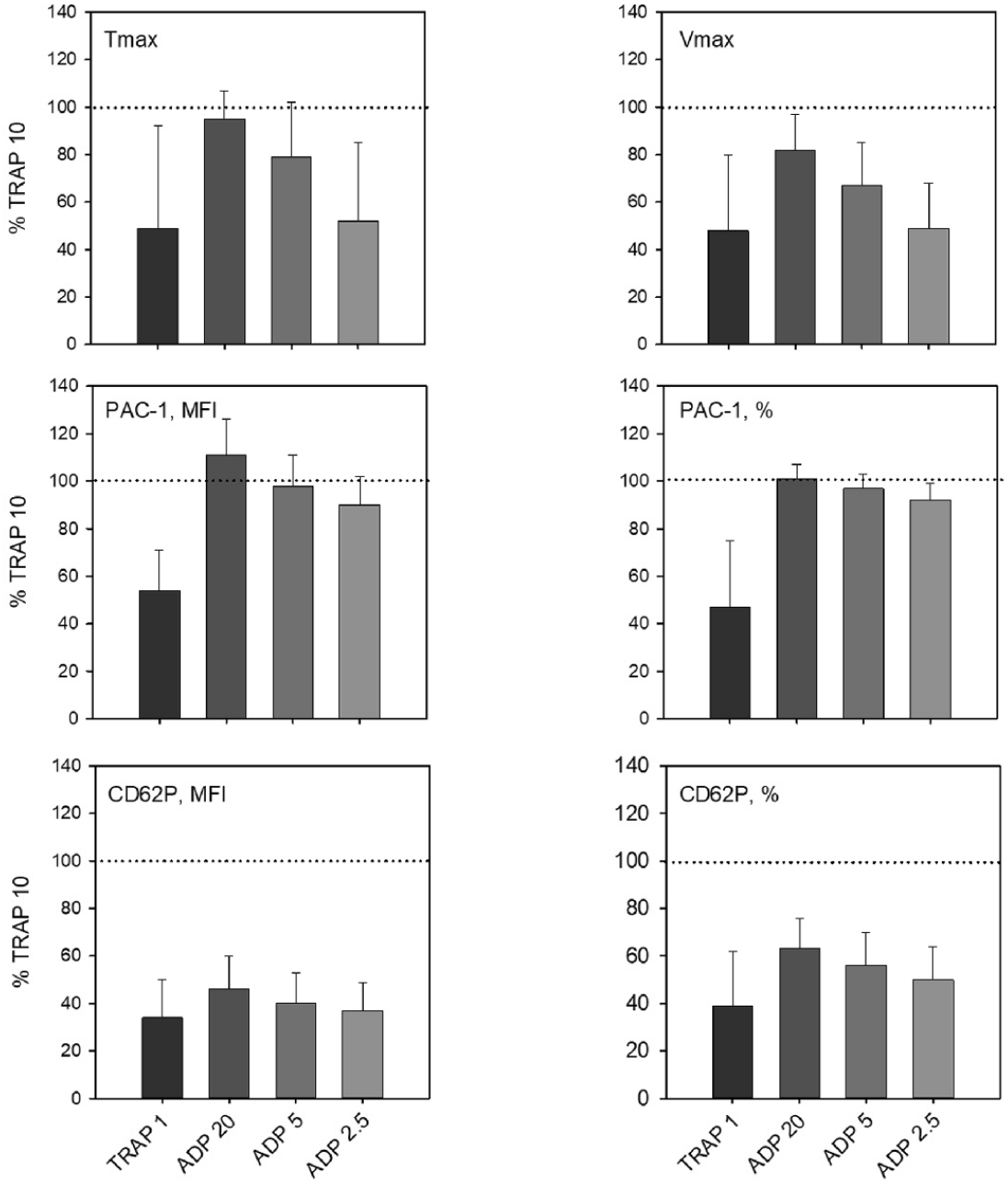

Figure 1. Comparison of the LTA (T_{max}, V_{max}) and flow cytometry (PAC-1, MFI, PAC-1, % and CD62P, MFI, CD62P, %) parameters of platelets activated by 10 μM TRAP (defined as 100%) and other agonists (1 μM TRAP, and 20 μM, 5 μM, 2.5 μM ADP) in the HV group. Data represent M ± SD. The decrease in all parameters from 10 μM TRAP was significant (p<0.001), except ADP 20 μM, MFI (a significant increase, p<0.001), 20 μM ADP, the percentage of PAC-1, (insignificant difference, p=0.102), and ADP 5 μM, PAC-1, MFI (insignificant difference, p=0.150) (paired t-test). Absolute values are given in Table 1.

Our results in patients with SA were comparable to those obtained by Gremmel et al. [10], who found almost the same correlations (r ≈0.5–0.6) between the level of platelet aggregation and the levels of exposure of activated GP IIb-IIIa and P-selectin during platelet activation by ADP in SA patients after percutaneous intervention, receiving the same antiplatelet therapy (ASA + clopidogrel).

Levels of exposure of activated GP IIb-IIIa (evaluated by PAC-1 antibody binding) and α-granule specific protein, P-selectin (evaluated by CD62P antibody binding) registered by flow cytometry showed better correlation with each other than with aggregation parameters. Obviously, this is due to the fact that, unlike aggregation, these measurements are performed under identical conditions, although they record fundamentally different platelet reactions: activation of the fibrinogen receptor (GP IIb-IIIa) and exocytosis of α-granules. As in the case of aggregation, the highest correlations were gestered for 1 μM TRAP in the HV group and for all agonists in SA patients (ASA + clopidogrel).

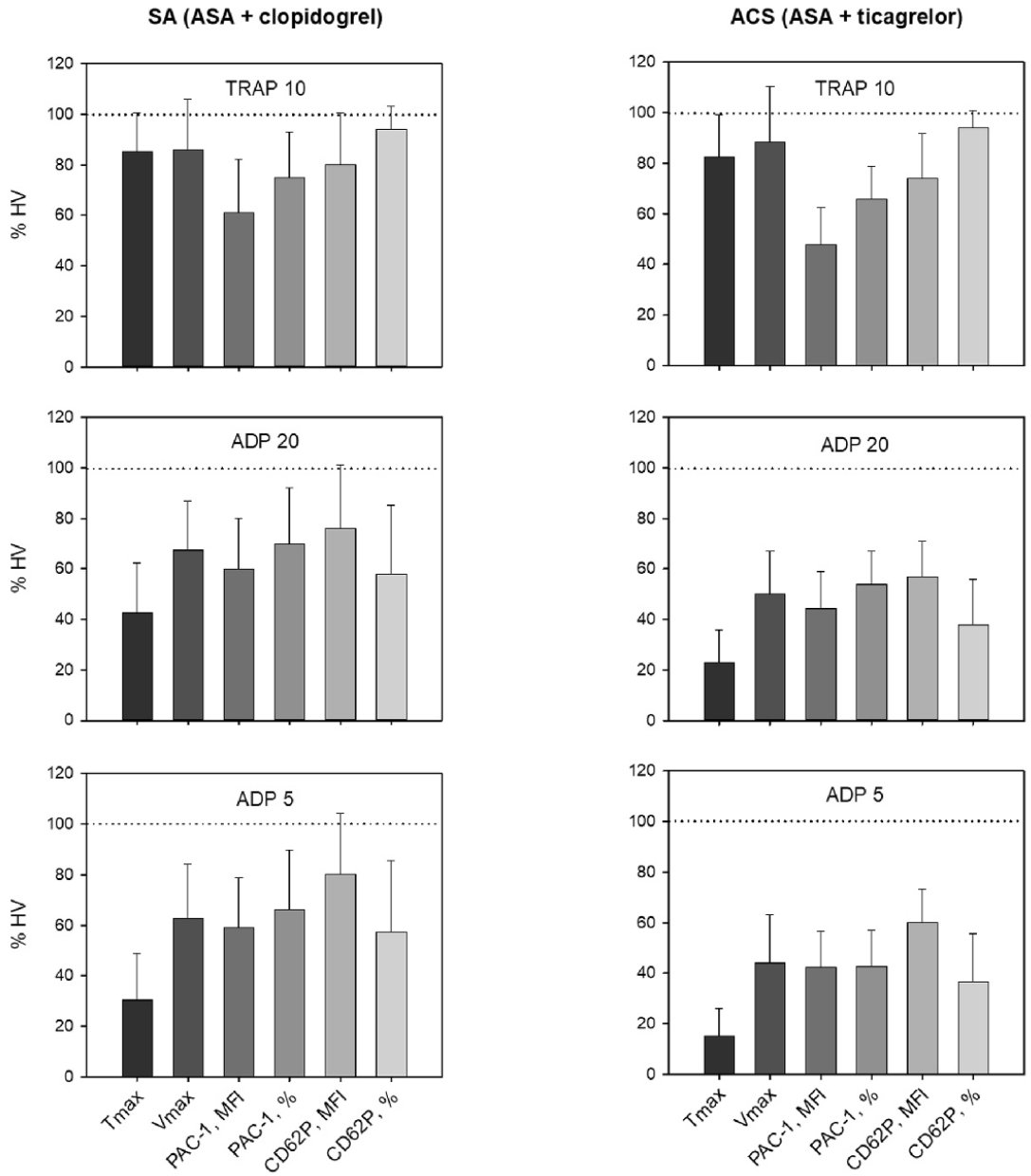

Figure 2. The decrease in LTA (T_{max}, V_{max}) and flow cytometry (PAC-1, MFI, the percentage of PAC-1, and CD62P, MFI, the percentage of CD62P) platelet activation parameters in SA (treated with ASA + clopidogrel) and ACS (treated with ASA + ticagrelor) patients in comparison with HV (defined as 100%). Data represent means ± SD. All differences form HV were statistically significant (p<0.001, t-test for means). Absolute values are given in Table 1.

In the HV group, platelet activation was registered when 10 μM and 1 μM TRAP and 20 μM, 5 μM, and 2.5 μM ADP were used. TRAP (10 μM) was the most powerful agonist and the highest rates of platelet aggregation and exposure of activation markers were expected recorded with its use. Reducing the TRAP concentration to 1 μM led to approximately the same decrease (50–60%) in all parameters; however, when platelets were activated by ADP (all concentrations), these changes widely varied. The aggregation parameters for 20 μM ADP were close to those for 10 μM TRAP and gradually decreased in the case of 5 μM and 2.5 μM ADP. At the same time, for all ADP concentrations, PAC-1 binding rates were almost identical, and CD62P binding rates were significantly lower as compared to 10 μM TRAP. These data indicate that comparisons of platelet activation stimulated by different agonists may yield widely varying results depending on the test used.

We have also found that sensitivity to dual antiplatelet therapy (ASA + clopidogrel in SA patients and ASA + ticagrelor in ACS patients) varied greatly when different methods of measuring platelet function were used. The degree ofinhibition of platelet activation in patients as compared with HV, when using the same agonist, could vary several times for different parameters. For example, in the groups of SA and ACS patients

platelet activation by 10 μM TRAP was accompanied by the 40–50% decrease in PAC-1 and MFI while the percentage of CD62P positive cells by less than 10%. When platelets were activated by 5 μM ADP, the T_{max} value of the aggregation test decreased by 70–85%, while CD62P and MFI only by 20–40%. Significant differences in inhibition levels were also found between PAC-1, MFI and aggregation parameters, when platelets were activated by 10 μM TRAP, and between the T_{max} parameter and all other parameters when platelets were activated by both doses of ADP.

CONCLUSIONS

The results obtained in this study indicate the possibility of a discrepancy between platelet aggregation and activation marker exposure (flow cytometry) during evaluation of platelet activity and the effectiveness of antiplatelet drugs.

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COMPLIANCE WITH ETHICAL STANDARDS

All volunteers and patients gave voluntary informed consent for the use of their blood samples for research purposes. The study was approved by the independent Ethics Committee of the NMRC for Cardiology (protocol No. 279 of April 25, 2022).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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ОЦЕНКА ФУНКЦИОНАЛЬНОЙ АКТИВНОСТИ ТРОМБОЦИТОВ У ЗДОРОВЫХ ЛИЦ И ПАЦИЕНТОВ, ПОЛУЧАЮЩИХ АНТИТРОМБОЦИТАРНУЮ ТЕРАПИЮ. ВОЗМОЖНЫЕ НЕСООТВЕТСТВИЯ ТЕСТОВ АГРЕГАЦИИ И ПРОТОЧНОЙ ЦИТОМЕТРИИ

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Функциональную активность тромбоцитов оценивали у здоровых добровольцев (ЗД, n=92), пациентов со стабильной стенокардией (СС, n=42) и острым коронарным синдромом (ОКС, n=73), получающих ацетилсалициловую кислоту (АСК) + клопидогрел и АСК + тикагрелор соответственно. У всех ЗД и пациентов сравнивали показатели агрегации тромбоцитов (максимальные светопропускание и скорость, T_{max} и V_{max}) и показатели, характеризующие экспонирование маркеров активации тромбоцитов, определяемые с помощью проточной цитометрии. В группе ЗД тромбоциты активировали, используя TRAP (thrombin receptor activating peptide, пептид активирующий рецептор тромбина) 10 мкМ и 1 мкМ и ADP 20 мкМ, 5 мкМ и 2,5 мкМ; а в группах пациентов TRAP 10 мкМ и ADP 20 мкМ и 5 мкМ. Достоверные и сильные взаимосвязи между показателями агрегации и проточной цитометрии (коэффициент корреляции, r от 0,4 до >0,6) чаще всего выявляли у ЗД при активации тромбоцитов TRAP 1 мкМ и пациентов со СС при активации тромбоцитов ADP 20 мкМ и 5 мкМ. В то же время во многих других случаях эти взаимосвязи были достаточно слабыми (r<0,3), а иногда статистически недостоверными. У ЗД различия между показателями связывания PAC-1 при активации тромбоцитов 10 мкМ TRAP и всеми концентрациями ADP были незначительными (≤10%), хотя показатели связывания CD62P (для всех концентраций ADP) и агрегометрии (5 мкМ и 2,5 мкМ ADP) были существенно снижены (на 40–60%). У пациентов антитромбоцитарная терапия достоверно снижала все показатели активации тромбоцитов в сравнении со ЗД, но в существенно разной степени. Для 10 мкМ TRAP показатель связывания PAC-1 MFI (снижение на 40–50%), а для ADP в обеих концентрациях показатель агрегации T_{max} (снижение на 60–85%) оказались наиболее чувствительными к действию антитромбоцитарных препаратов, по сравнению с другими показателями, снижавшимися в меньшей степени. Полученные данные указывают на возможность несоответствия между показателями агрегации тромбоцитов и экспонирования маркеров активации (проточная цитометрия) при оценке активности тромбоцитов и эффективности антитромбоцитарных препаратов.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: тромбоциты; агрегация тромбоцитов; проточная цитометрия; гликопротеин IIb-IIIa; Р-селектин; антиагрегантные препараты

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