Comparison of two methods to assess variability of platelet response to anti-platelet therapies in patients with acute coronary syndrome undergoing angioplasty

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Summary
The study investigated the clinical usefulness of a new method to evaluate platelet activation and the variability of platelet response to anti-platelet therapy in patients undergoing percutaneous transluminal coronary angioplasty (PTCA). Platelet activation was assessed in parallel by a new method for platelet density measurements (MPC, Mean Platelet Component Concentration), on the automated ADVIA 120 Hematology System and by the classic measurement of P-selectin (CD62P) expression, on a fluorescence flow cytometer. Patients received a loading dose of clopidogrel (300 mg; n = 29) or a bolus of abciximab (0.25 mg/kg; n = 15). Blood samples were collected before (baseline) and at different times after PTCA and anti-platelet drugs administration. Our data showed a close inverse correlation between the change in MPC and the CD62P fluorescence surface marker expression (r = -0.776, P<0.0001). Individual platelet activation determinations in patients receiving either clopidogrel or abciximab showed a variation in platelet activation as assayed by MPC and CD62P expression. Patients were characterized as having either high platelet activity upon admission and positive response to treatment or no detectable platelet activation before or after treatment. This study demonstrates the heterogeneity of platelet activation states in ACS patients undergoing coronary angioplasty. The present work also illustrates the potential use of the MPC parameter, generated on an automated hematology system, to define high risk patients and to monitor the variability of platelet response to anti-platelet therapies.

Keywords
Platelets activation, anti-platelet drugs, ADVIA 120, CD62P

Introduction
Platelets play a major role in the development of thrombus formation in patients with arterial thromboembolic diseases. Abnormal platelet behavior is associated with several pathological conditions such as acute coronary syndromes (ACS), and in patients undergoing transluminal coronary angioplasty (1-4). Consequently, anticoagulant and anti-platelet therapies are widely used for cardiovascular disease patients. For example, treatment of ACS patients with aspirin, ADP receptor inhibitors, peptide and non-peptide glycoprotein IIb/IIIa (GPIIb/IIIa) receptor inhibitors, and other novel inhibitors of platelet function have been shown to significantly improve both short-term and long-term outcome by reducing the incidence of recurrent MI and death (5-13). However, clinical risk of thrombosis or bleeding is associated with these treatments (14). Inter-individual variation in response to abciximab has been reported both in terms of extent and duration of inhibition of platelet function...
(13, 15, 16). A high level of platelet function inhibition has been reported to be associated with a decrease in the incidence of major adverse cardiac events (17). Currently, decisions to use anti-platelet treatment are based on clinical grounds because of a lack of clinically applicable methods to monitor platelet function in individual patients. Artificial in vitro platelet activation represents the main problem in assessing platelet function. The ability to measure platelet activation in a highly specific and sensitive manner raises the possibility that such a method may be used in the early identification of patients who will benefit most from targeted anti-platelet regimens and to guide therapy. Several instruments have been developed to assess the global platelet response to such treatment. Within the last decade, a well-established method to assess platelet activation has been studied in whole blood using fluorescence flow cytometry (18). The technique is specific, and sensitive, since fluorescence measurements can detect as few as 0.8%-activated cells. However, it is expensive, labor intensive, and data analysis is imprecise. Furthermore, in some patients, activated platelets lose their marker receptors but continue to circulate and function, which leads to missed detection with activation-specific fluorescence-labeled antibodies (19).

Here we report the clinical usefulness of a new approach to assess platelet function and monitor anti-platelet drug therapy on the automated ADVIA 120 Hematology System. The method may provide an opportunity for rapid clinical measurements of platelet activation. The ADVIA 120 Hematology System’s technology offers two-dimensional platelet analysis on an automated analyser that in addition to measuring the conventional hematologic indices, also provides activation-related information about platelets. Platelet analysis by this system detects two light scatter-related indices, also provides activation-related information about platelets. Platelet density parameter, mean platelet component concentration (MPC) is reported in 30 seconds. A decrease in platelet density, measured by a reduction in MPC is indicative of platelet activation. MPC also shows, in vitro, excellent correlation with the well-defined fluorescence marker, CD62P-Phycoerythin (CD62P-PE) (20-22).

This study has evaluated the effects of clopidogrel and abciximab therapy on platelet activation during coronary angioplasty in ACS patients using two platelet activation measurement methods, the new ADVIA 120 Hematology System and the well established CD62P-PE flow cytometry system.

Materials and methods

Patients
A total of 44 patients who underwent elective percutaneous coronary angioplasty and stenting were evaluated. Patients were enrolled in the study after informed consent had been obtained. Their baseline variables including age, gender, smoking history and medication were documented. The treatments were assigned based on the angiographic characteristics. Patients with presence of thrombus and high risk vessel disease were assigned to abciximab. Two thirds (n=29) of patients received a loading dose of clopidogrel (300 mg) before the beginning of the PTCA procedure followed by 75 mg daily for 1 month. One third (n=15) patients received intravenously a 0.25 mg/kg, bolus of abciximab before the beginning of the PTCA procedure followed by a 0.125 mg/kg/min infusion for 12 hours. These patients also received clopidogrel a few hours after PTCA.

Reagents
Platelet control, mouse IgG1 PE; Platelet control, mouse IgG1 FITC; monoclonal antibodies, CD61a (GPIIIa) FITC-conjugate; CD62P-PE-conjugate; and CaliBRITE beads were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Dulbecco’s PBS (PBS), catalog # 14040, was obtained from Gibco/BRL (Grand Island, NY). Formaldehyde, 10% Ultra-pure Methanol-free, Catalog # 04018 was obtained from Polyscience, (Warrington, PA). ADVIA 120 Hematology OPTIpoint, SETpoint calibrator, TESTpoint controls, Low, Normal, and High were obtained from Bayer HealthCare, LLC (Tarrytown, NY).

Other materials
VACUTAINER K$_3$-EDTA tubes were obtained from Becton Dickinson VACUTAINER Systems (Franklin Lakes, NJ). Instrumentation: FACScan: PowerPC Macintosh G3/300MHZ, equipped with an argon laser, and Lysis II analysis software from Becton Dickinson Immunocytometry Systems (San Jose, CA). Bayer’s ADVIA 120 Hematology System and Bayer’s Customized Proprietary Refractive Index Series Software were acquired from Bayer HealthCare, LLC, Diagnostics Division (Tarrytown, NY and Dublin, Ireland).

Collection of blood
Blood was drawn with 21 gauge needle into EDTA (5 mM) tubes, mixed immediately with the anticoagulant avoiding frothing during the mixing procedure. The first 1-2 ml of blood were discarded to avoid the effects of traces of thrombin generated during venipuncture. Universal precautions were taken at all times during phlebotomy.

Whole blood specimens for measurement of the ADVIA 120 System platelet parameters and CD62P-PE flow cytometry analysis were taken prior to (baseline), 2 hours, 5 hours, and 24 hours after administration of a loading charge of clopidogrel and PTCA, and prior to, 10 minutes, 2 hours and 24 hours after administration of the abciximab bolus and PTCA. All specimens were processed within 1 hour after blood collection.

Fluorescence flow cytometry analysis
Whole blood samples were fixed with methanol-free paraformaldehyde (final concentration 0.5%). Fixed blood
samples (5 μl) were incubated at room temperature for 15 min in the dark with monoclonal antibody CD61a-FITC (10 μl) to identify the platelets and CD62P-PE (10 μl) to measure the expression of p-selectin. Platelets labeled with FITC- or PE-conjugated isotype control antibody were used as control for nonspecific staining. Samples were diluted to 1 ml with PBS. FACScan calibration and compensation were performed as recommended by the manufacturer. Five thousand (5,000) CD61a positive events were acquired on the FACScan flow cytometer equipped with Lysis II software. Data analysis was performed using a threshold based on the FITC -fluorescence correlating to the platelet specific binding of CD61a-FITC antibodies. A gate was drawn around the fluorescent platelet population. The CD61a-FITC labeled platelets that were also positive for PE-conjugated activation marker antibody, (CD62P) were quantitated and presented expressed as percentage of positive events.

**ADVIA 120 automated hematology system platelet density measurement**

The two-dimensional platelet method is based on the analysis of platelets from whole blood by flow cytometry using the ADVIA 120 (21). The system is linear for MPC values where the reference normal mean is 27.2 ± 1.3 g/dL (23). The system was standardized and calibrated as per manufacturer’s instructions. The ADVIA 120 Hematology System was quality controlled using TEST point Hematology control reagents (Bayer HealthCare, LLC, Tarrytown, NY). The whole blood specimens anticoagulated with EDTA were processed within 1 hour after specimen collection. The ADVIA 120 platelet density parameter, MPC, was acquired as a measure of baseline platelet activation and 10 minutes, 2 hours, 5 hours, and 24 hours after antiplatelet drug therapy.

**Statistical analysis**

Platelet CD62P expression and MPC were compared using the paired t-test to test for significant differences for repeated measures. To control for potential errors introduced by deviations from normal distribution, we validated the results of the t-test by the Friedman’s test, followed by the Wilcoxon’s matched pairs signed ranks test. The nonparametric tests always confirmed the results of parametric tests. P values < 0.05 were considered statistically significant.

**Results**

**Baseline characteristics of patients**

Clinical and demographic features of study patients are shown in Table 1. All patients had symptomatic coronary artery disease, unstable angina and/or myocardial infarction. The majority of patients had received aspirin before hospitalization.

| Table 1: Clinical and demographic characteristics of the ACS patients. |
|-----------------------------|-----------------------------|
|                            | Clopidogrel n = 29          | Abciximab n = 15 |
| Sex                        | M: 21 ; F: 8                | M: 13 ; F: 2    |
| Age                        | 60 ± 13                     | 59 ± 10         |
| Diagnosis                  |                             |                 |
| UA                         | 23 (79)                     | 8 (53)          |
| MI                         | 6 (21)                      | 6 (40)          |
| SCAD                       | 0                           | 1 (7)           |
| Medication received before |                             |                 |
| Aspirin or en tropken      | 26 (90)                     | 13 (86)         |
| Oral anticoagulant         | 16 (55)                     | 10 (66)         |
| History of Hypertension    | 13 (45)                     | 8 (53)          |
| History of Diabetes        | 3 (10)                      | 1 (6)           |
| Current Smoking            | 11 (38)                     | 5 (33)          |
| Previous Smoking           | 2 (7)                       | 0               |
| Prior PTCA                 | 8 (28)                      | 4 (27)          |
| Prior CABG                 | 1 (3)                       | 3 (20)          |

SCAD : stable coronary artery disease; (%)

**Figure 1: Inhibition of circulating activated platelets by clopidogrel (A) or by abciximab (B) treatment. Results are reported as MPC values (expressed in g/dL) and percent positive (%) CD62P-PE events. Values are expressed as the mean (SEM; error bars), n = 29 for clopidogrel treatment and n = 15 for abciximab treatment. Statistical significance between baseline and time point: * p<0.05, ** p<0.01.
Platelet activation status in patients receiving clopidogrel and abciximab

In the population of clopidogrel patients (n = 29), mean CD62P expression levels decreased significantly after treatment (means ± SE, 9.5 ± 3.3%, 3.1 ± 1.8%, 1.2 ± 0.7% and 1.8 ± 1.1%, for baseline, 2hrs, 5hrs and 24hrs, respectively). Correspondingly, mean MPC levels increased (means ± SE, 27.8 ± 0.4 g/dL, 28.4 ± 0.3 g/dL, 28.9 ± 0.2 g/dL and 28.7 ± 0.2 g/dL, n = 29, for baseline, 2hrs, 5hrs and 24hrs, respectively) (Fig. 1A).

In the population of abciximab patients (n = 15), similar results were seen. CD62P expressions were (mean ± SE) 9.3 ± 4.0%, 7.9 ± 5.4% and 0.7 ± 0.3%, for baseline, 2hrs and 24hrs, respectively, and MPC levels were (mean ± SE) 28.0 ± 0.3 g/dL, 27.9 ± 0.3 g/dL, 28.0 ± 0.4 g/dL and 28.4 ± 0.2 g/dL, for baseline, 10 min, 2hrs and 24hrs, respectively (Fig. 1B).

Individual platelet activation determinations in patients receiving either clopidogrel or abciximab demonstrated heterogeneity of platelet activation. The patients were characterized based on the combination of the baseline platelet activation parameters, MPC and CD62 expression, and the response to the anti-platelet therapy. For our population, we have already reported a normal MPC value, 27.9 ± 0.9 (22). In a larger set of data (n = 51), the MPC in normal healthy donors was 28.1 ± 1.0 g/dL (data not published). The CD62P-PE % positive events was < 2% for normal healthy donors (22).

The majority of patients could be characterized by high platelet activity upon admission and positive response to treatment or by no detectable platelet activation before or after treatment. Several patients (n = 7) demonstrated platelet activation levels that were normal upon admission, and became activated after treatment and surgical intervention. Patients who exhibit-
ed high baseline platelet activation responded to clopidogrel treatment as indicated by a decrease in CD62P percent positive expression at time points after treatment ((mean ± SE) 23.4 ± 6.6%, 2.0 ± 1.0%, 0.3 ± 0.1% and 1.5 ± 0.5%, p<0.01, for baseline, 2hrs, 5hrs, and 24hrs, respectively). A concomitant increase in MPC values (25.7 ± 0.8 g/dL, 28.0 ± 0.5 g/dL, 28.8 ± 0.3 g/dL and 28.4 ± 0.3 g/dL, p<0.01, for baseline, 2hrs, 5hrs, and 24hrs, respectively) was seen in those patient samples. Individual changes in MPC and CD62P values of patients with and without baseline activated platelets are presented in figure 2. Similar patterns to those found in clopidogrel patients were observed in abciximab patients.

**Correlation between MPC and CD62P**

The correlation between fluorescence surface marker expression of CD62P-PE and the activation state determined by the change in MPC is shown in Figure 3. The correlation was performed separately using platelet activation values for baseline and for the response to anti-platelet therapy. Increased percentage CD62P-positive platelets are strongly correlated with decreased MPC values ($r = -0.776$, $p<0.0001$, $n = 42$ and $r = -0.775$, $p<0.0001$, $n = 102$, for baseline values and for values on treatment, respectively). An unexpected high CD62P-PE value (75%) was observed in one patient at 2 hours after abciximab and angioplasty. The corresponding MPC value was very low (22.8 g/dL) indicating a high level of platelet activation. The baseline CD62 and MPC were 4.2% and 28.3 g/dL respectively. This patient had some complications of pacemaker installation and did not finish the 24-hour sampling.

**Discussion**

Early detection of platelet activation status might be useful to identify ACS patients who would benefit from platelet antagonist therapy. Inter-individual variation in response to anti-platelet therapy and clinical risk of thrombosis or bleeding associated with these treatments have been reported by several studies (14-16). Currently, decisions for anti-platelet treatment are based on clinical data but not on platelet activation studies in individual patients. There is a well-recognized need for clinically applicable methods to monitor platelet function in the treatment and prevention of ACS.

The ADVIA 120 Hematology System’s platelet analysis is an automated method that uses both volume and density measurements to derive an accurate platelet count, as well as a platelet density value, MPC. In this study and others, MPC has been shown to be increased in inactivated platelets and decreased in activated platelets (20, 22). The measurement of MPC does not require specimen preparation, platelet activation specific receptors, or activation-specific receptor labels. The MPC values showed very good inverse correlation with the percentage of platelets expressing CD62P antigen as detected by fluorescent flow cytometry after activation by thrombin (22). The present study was designed to establish the correlation and to evaluate the clinical usefulness of these parameters to measure the magnitude of baseline platelet activation, as well as to follow anti-platelet therapy in patients with acute coronary syndrome undergoing coronary angioplasty.

Our data showed a close correlation between CD62P-PE expression and MPC measured on the ADVIA 120 Hematology System. Individual platelet activation determinations in patients receiving either clopidogrel or abciximab showed het-
ergogeneity of platelet activation as assayed by MPC and CD62P-PE expression. Patients were characterized as having either high platelet activity upon admission and positive response to treatment or no detectable platelet activation before or after treatment. In the group of patients with the greatest baseline platelet activation, response to clopidogrel exhibited a significant reduction in platelet activation as determined by both CD62P decrease and platelet density change expressed by increasing MPC. It has been shown that clopidogrel suppresses expression of platelet activation markers CD62, CD63, and PAC-1 after stimulation with ADP or thrombin in healthy subjects (24) and in patients after MI (25).

In our study, the majority of patients, including patients with increased baseline platelet activation, received aspirin therapy. The platelet inhibition effect of aspirin was not demonstrated by the classic or new platelet activation measurements reported by this study. The apparent lack of aspirin effect may be in part due to the aspirin resistance phenomena. Several clinical trials suggest that there are individual differences in the response to aspirin dose used in clinical trials (26) and influence of genetic polymorphisms (27).

Other investigators, using other methods to measure platelet activation, have reported similar variability of platelet activation in patients with coronary artery diseases (28, 29). These observations raise the question of whether or not clinicians should uniformly use aggressive and costly anti-platelet strategies, including platelet GP Ib/IIa inhibitors without individual assessment of platelet status in patients with ACS. In theory, patients who exhibit activated platelets would be ideal candidates for aggressive anti-platelet strategies, whereas patients with diminished or absent platelet activity would be expected to derive less benefit and could be at greater risk for bleeding complications.

The use of the MPC parameter for anti-platelet therapy monitoring may provide rapid access to platelet activation test results and may be helpful in detecting the heterogeneity of platelet activation among ACS patients (and perhaps other patient groups), which may account for the variability of patient response to treatment. More extensive and widespread studies are needed to confirm our results and to evaluate new and varied anti-platelet drugs of the future.

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References

18. Michelson AD. Platelet activation by thrombin can be directly measured in whole blood through the use of the peptide GPRP and flow cytometry: Methods and clinical applications. Blood Coagul Fibrinolysis 1994; 5: 121-31.


