Influence of Platelet Aggregate Formation in Blood Samples on Light Transmission Aggregometry Results

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Background: Light transmission aggregometry is a standard method used to evaluate platelet function. However, in clinical settings, light transmission aggregometry results sometimes fail to reflect actual platelet hyperactivity. In patients with suspected platelet hyperactivity such as thrombosis, platelet aggregates are frequently detected in citrated blood samples using a scattergram of a hematology analyzer. This study aimed to evaluate the effects of platelet aggregate formation on light transmission aggregometry results. *Methods*: We used 19 citrated blood samples in which platelet aggregate formation was intentionally induced by a hematology analysis process. Employing fully automated light transmission aggregometry and agonists including adenosine diphosphate or collagen, light transmission aggregometry maximum aggregation percentage, platelet count, and mean platelet volume of platelet-rich plasma before and after platelet aggregate formation were evaluated. *Results*: Light transmission aggregometry maximum aggregation percentage with adenosine diphosphate or collagen was significantly lower in the samples after than before platelet aggregate formation. Platelet count and mean platelet volume were both decreased by platelet aggregate formation ($P < .01$), suggesting that maximum aggregation percentage reduction was caused by the decrease in activated large platelets in the platelet-rich plasma. *Conclusion*: This study clarified that platelet aggregate formation in blood samples interfered with an accurate assessment of platelet hyperactivity. To ensure reliability of light transmission aggregometry results, we must confirm that platelet aggregates have not formed in the sample, especially in those of patients with platelet hyperactivity.

Key Words: Platelet aggregate—light transmission aggregometry—hematology analyzer—platelet count—mean platelet volume—platelet-rich plasma

Introduction

Light transmission aggregometry (LTA), the standard method used to evaluate platelet aggregation ability,[1] has been widely used to measure the effect of antiplatelet drugs.[2-5] However, no studies have established the benefit of LTA in the assessment and management of thrombosis risk.[6,7] In contrast to the LTA-based assessment of platelet hypoactivity, that of platelet hyperactivity is less reliable in the clinical setting. The precise reason for this uncertainty remains unclear.

When platelet activity is increased, platelet aggregates (PAs) are likely to appear in citrated blood samples. In

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fact, we previously established a new method to detect increased PA formation in citrated blood samples on a scattergram of a hematology analyzer.[8-10] Such PA formation may produce misleading LTA results, especially in patients with platelet hyperactivity. This study aimed to evaluate the effects of PA formation and verify the association between PA formation in citrated blood samples and LTA results.

Materials and Methods

Subjects

In this study, we examined 19 citrated blood samples in which PA was detected on a scattergram of a hematology analyzer during a hematology analysis. Samples were obtained after written informed consent was provided by 6 healthy volunteers and 13 patients (cerebral infarction in 8, transient ischemic attack in 2, carotid artery stenosis in 2, and cerebral hemorrhage in 1). Eight patients received antiplatelets (aspirin in 4, aspirin/cilostazol in 2, clopidogrel/aspirin or clopidogrel/cilostazol in 1 each), and 2 patients received anticoagulant (apixaban or dabigatran in each). Statins were administered to 4 patients. Healthy persons took no medicines that affected platelet function. This study followed the principles of the Declaration of Helsinki and was approved by our institutional ethics committee.

Sample Preparation

Blood (approximately 13 mL) was drawn via an antecubital vein using a 21G needle. The first 2 mL was collected into an ethylenediaminetetraacetic acid (EDTA)-2K–containing tube, while the remaining blood was mixed in a 109-mM sodium citrate–containing tube (whole blood:sodium citrate = 9:1). The EDTA-2K and citrated blood samples were analyzed by an automated hematology analyzer (CELL-DYN Sapphire Hematology System; Abbott Diagnosis, Abbott Park, IL). PA formation was defined as a PA fraction detected on the scattergram of the hematology analyzer (Fig 1B),[8-10] and all 19 citrated blood samples in this study fulfilled this criterion. The EDTA-2K blood samples were negative for PA formation (Fig 1A). Actual PA formation was confirmed in a smear of citrated blood (Fig 1C). PA is thought to form gradually through the automated stirring process of the hematology analysis. In this study, the analysis was repeated up to 4 times until PA appeared; blood samples containing PA in the first analysis were excluded because this study focused on the effect of PA formation during the blood test procedure on the LTA result.

Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) for LTA analysis were prepared from citrated blood samples before and after hematology analysis, i.e., before and after PA formation. The PRP was separated from the citrated whole blood samples by a first centrifugation (85 × *g* for 15 minutes at room temperature), while a subsequent centrifugation (150 × *g* for 5 minutes at room temperature) separated out the PPP.

LTA Reagents

Agonists used for LTA including Revohem adenosine diphosphate (ADP) (final concentrations: .25, 1.0, 2.0, 5.0, and 10.0 μM) and Revohem Collagen (collagen) (final concentrations: .05, .5, 2.0, and 5.0 μg/mL) were purchased from Sysmex Corporation (Kobe, Japan).

Assays

Using a fully automated LTA machine (CS-2000i; Sysmex Corporation, Kobe, Japan), LTA maximum aggregation percentage (MA%) between samples before and after the PA-forming hematology analysis (PA formation) was compared. Light transmission values of 0% and 100%

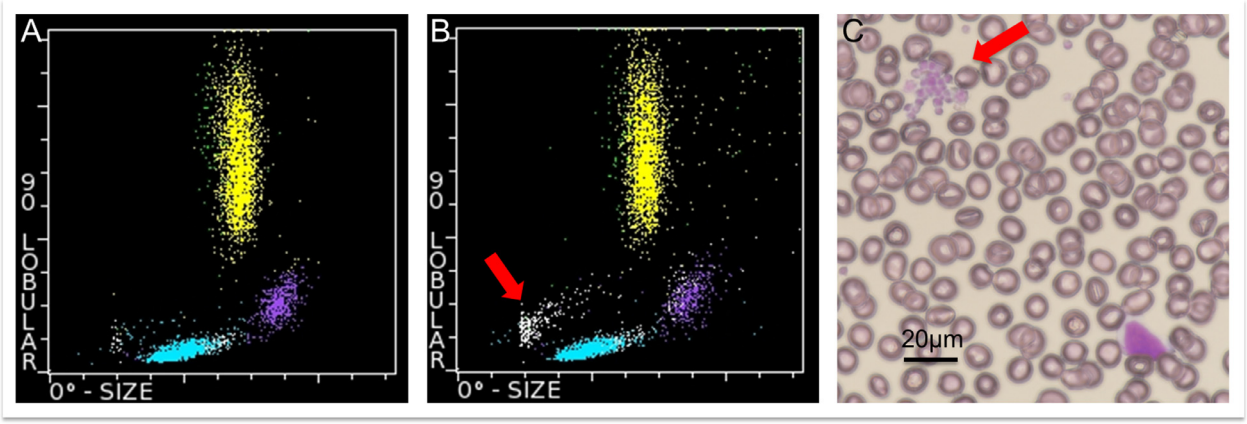

Figure 1. *Platelet aggregates (PAs) in the hematology analyzer. (A) Scattergram of EDTA-2K containing whole blood from a healthy person is shown. Lymphocytes (blue), monocytes (pink), and granulocytes (yellow) are expressed in the scatter plot area of white blood cells. The vertical axis represents side scatter, while the horizontal axis represents forward scatter. (B) Scattergram of citrated blood from the same person is shown. Platelet PA appears as a white streamed fraction (red arrow). (C) Citrated blood smear from the same person is shown (May-Giemsa stain, × 400). Red arrow indicates the PA. (Color version of figure is available online.)*

were automatically set by PRP and PPP samples, respectively, prior to the addition of agonists. Each agonist (20 μL at each concentration per assay) was automatically added to the PRP (140 μL per assay). Reaction time was set to 300 seconds, at which point the aggregation was measured and evaluated as the MA%. LTA studies were performed within 4 hours after the blood collection. In addition to LTA, platelet counts and mean platelet volume (MPV) of citrated whole blood and PRP before and after PA formation were also measured.

Statistical Analysis

Intergroup differences in age and the number of cerebrovascular risk factors (hypertension, dyslipidemia, diabetes mellitus, and atrial flutter) were analyzed using the Mann−Whitney U test. Sex ratios were analyzed by Fisher's exact test. Differences between patients and healthy persons in the number of hematology analysis tests in which PA first appeared in the scattergram of the citrated blood samples were also analyzed using the Mann−Whitney U test. Platelet count and MPV differences were analyzed by Student's *t* test and the Mann−Whitney U test, respectively. Differences in platelet count ratio of PRP/whole blood and LTA MA% were analyzed by the Wilcoxon signed-rank test. JMP 10.0.2 (SAS Institute Inc., Cary, NC) was used for the statistical analyses, and values of $P < .05$ were considered significant.

Results

Background

The healthy persons were significantly younger than the patients (mean age ± SD, 43.2 ± 8.7 versus 74.5 ± 11.3) ($P = .0013$). Sex ratios were not significantly different between patients and healthy subjects. Healthy persons did not have any cerebrovascular risks. In contrast, the patients had a mean risk of 1.6 ± .9 ($P = .0020$) (Table 1).

Platelets

The ordinal number of the hematology analysis in which PA first appeared in the scattergram of the citrated blood samples was the 2nd, 3rd, and 4th analysis in each 2 healthy persons and the 2nd assay in 11 patients and the 3rd assay in 2 patients. Thus, PAs were more easily detected in patients' blood with a lower number of analyses (2.2 ± .4) than in healthy subjects' blood (3.0 ± .9) ($P = .0213$) despite the use of antiplatelets or anticoagulants in most patients.

Platelet counts (mean ± SD) of all samples before and after PA formation in whole citrated blood were 172 ± 44 × 10^9/L and 114 ± 33 × 10^9/L, respectively. Platelet counts in whole citrated blood significantly decreased after PA formation ($P < .0001$). Likewise, platelet counts in PRP after PA formation (182 ± 53 × 10^9/L) were significantly lower than those in PRP before PA formation (360 ± 79 × 10^9/L) ($P < .0001$) (Fig 2). In addition, platelet count ratios of PRP/whole blood (median, interquartile range [IQR]) after PA formation (1.67, 1.31-1.85) were significantly lower than those before PA formation (2.06, 1.93-2.26) ($P = .0033$). Platelet counts of whole citrated blood and PRP were not significantly different between healthy persons and patients regardless of PA formation (Table 1).

MPVs (median, IQR) of whole citrated blood after PA formation (7.91, 7.53-8.41) were significantly higher than those of whole citrated blood samples before PA formation (6.65, 6.07-7.32) ($P < .0001$). In contrast, the MPV of PRP from samples after PA formation (5.59, 5.34-5.86) was significantly lower than that of PRP from samples before PA formation (6.43, 6.05-6.93) ($P < .0001$) (Fig 2).

Light Transmission Aggregometry

Because PRP samples after PA formation were in short supply, not all 19 samples were tested under all agonist conditions after PA formation. In ADP, .25, 1.0, 2.0, 5.0, and 10.0 μM reagents were used in 14, 15, 14, 8, and 4

Table 1. *Characteristics and platelet counts of healthy subjects and patients*

Characteristics		Healthy subjects (n = 6)	Patients (n = 13)	*P value*
Age		43.2 ± 8.7	74.5 ± 1.3	.0013*
Women (%)		50	30.8	.6169
Number of cerebrovascular risks		0	1.6 ± .9	.0020*
Number of hematology analysis until PA appearance		3.0 ± .9	2.2 ± .4	.0213*
Platelet counts (× 10^9/L)	Citrated blood before PA formation	174 ± 18	171 ± 52	.9260
	Citrated blood after PA formation	125 ± 14	109 ± 38	.3252
	PRP before PA formation	394 ± 51	344 ± 86	.2063
	PRP after PA formation	180 ± 28	182 ± 64	.9338

PA, platelet aggregates; PRP, platelet-rich plasma

Age, number of cerebrovascular risks, number of hematology analyses until PA appearance, and platelet counts are expressed as mean ± SD.

*$P < .05$.

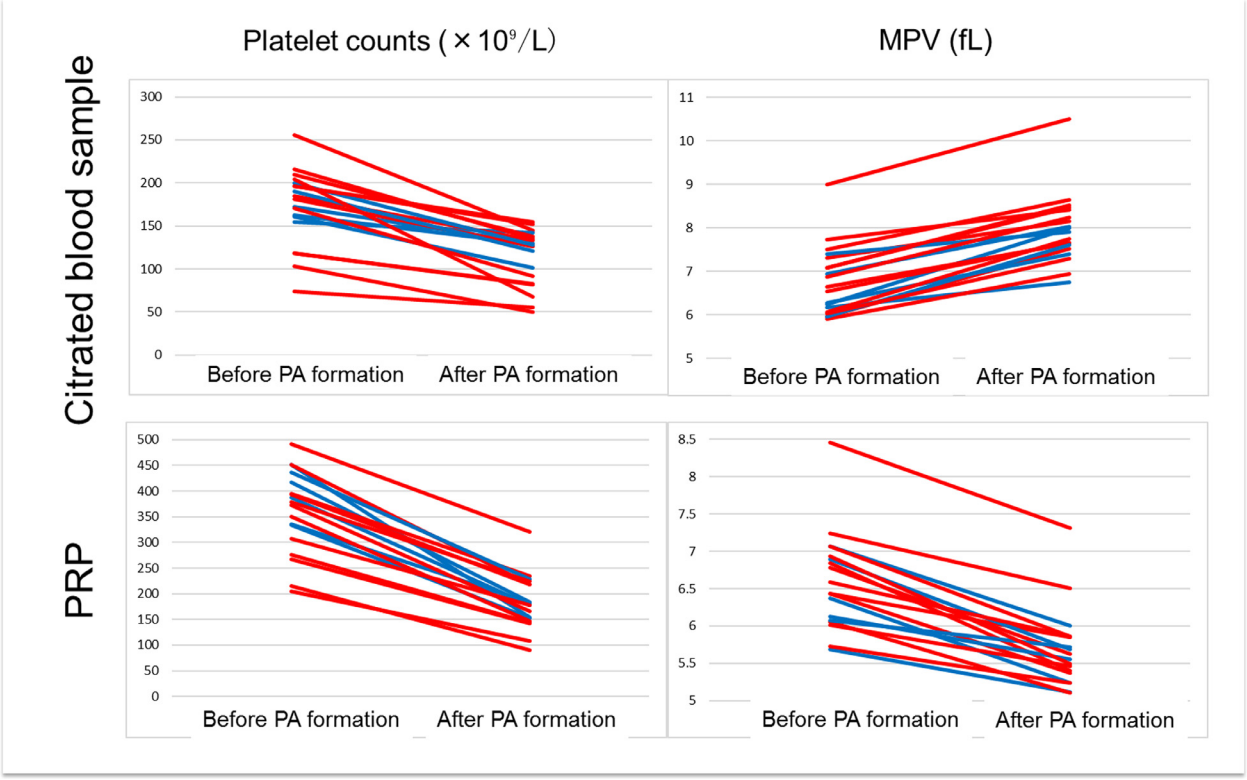

Figure 2. *Changes in platelet counts and MPV after PA formation. After PA formation, platelet counts significantly decreased (upper left panel) in citrated blood (P < .0001). MPV increased after PA formation (upper right panel) (P < .0001), but this increase was due to PAs being counted as giant platelets. In PRP, both platelet counts and MPV decreased after PA formation (lower panels) (P < .0001); PA being counted as giant platelets, as well as large, activated platelets, seemed to be removed by centrifugation (see text). MPV, mean plasma volume; PA, platelet aggregates. Red lines express patients' data and blue lines express healthy subjects' data. (Color version of figure is available online.)*

samples, respectively. Collagen reagents with .05, .5, 2.0, and 5.0 μg/mL concentrations were used in 6, 6, 4, and 4 samples, respectively.

MA% values with ADP in PRP after PA formation were significantly lower than those before PA formation at all concentrations (Figs 3, 4); the median MA% values (IQR) of PRP before and after PA formation respectively were 14.2 (11.9-22.0) and 6.1 (1.3-11.8) at .25 μM ADP ($P = .0002$), 70.0 (46.1-82.0) and 21.5 (14.9-36.9) at 1.0 μM ADP ($P < .0001$), 81.1 (71.6-86.8), and 41.2 (22.7-57.8) at 2.0 μM ADP ($P < .0001$), 84.3 (76.0-85.5), and 58.4 (26.6-68.5) at 5.0 μM ADP ($P = .0075$), and 84.5 (81.8-85.1) and 74.1 (66.9-74.3) at 10.0 μM ADP ($P = .0275$). MA% values of patients' PRP before PA formation at 1.0 and 2.0 μM

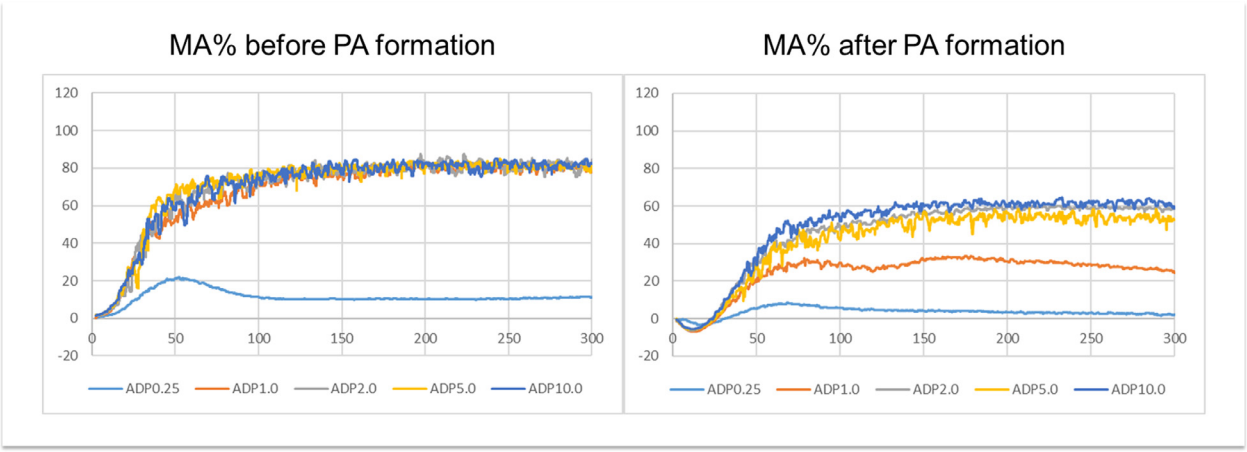

Figure 3. *LTA results with ADP before and after PA formation in an illustrative case. MA% values were decreased after PA formation (right panel), compared to those before PA formation (left panel). (Color version of figure is available online.)*

Figure 4. *Comparison of light transmission aggregometry MA% with ADP before and after PA formation. MA% values after PA formation were significantly lower than those before PA formation at all ADP concentrations. MA% in patients (represented by red lines) before PA formation were significantly lower than those in healthy subjects (represented by blue lines) at 1.0 μM and 2.0 μM ADP (P = .0169, P = .0196, respectively). MA%, maximum aggregation percentage; PA, platelet aggregation. (Color version of figure is available online.)*

ADP, respectively were 61.5 (45.0-72.9) and 78.3 (68.8-82.6), and those of healthy subjects' PRP were 82.0 (80.5-88.2) and 86.8 (85.8-90.0); MA% values of patients' PRP before PA formation were significantly lower than those of healthy subjects' PRP with 1.0 ($P = .0169$) and 2.0 μM ADP ($P = .0196$).

Although collagen-induced MA% showed similar tendencies to those of ADP-induced MA%, most differences did not reach statistical significance. The median MA% values (IQR) of PRP before and after PA formation respectively were 18.5 (14.4-24.2) and 14.4 (9.1-17.3) at .05 μg/mL collagen ($P = .11$), 42.9 (9.5-77.5) and 21.4 (5.8-40.8) at .5 μg/mL collagen ($P = .1314$), 80.7 (65.9-86.0) and 65.5 (46.9-81.0) at 2.0 μg/mL collagen ($P = .0422$), and 82.6 (75.2-84.2) and 70.8 (56.0-82.1) at 5.0 μg/mL collagen ($P = .0997$).

Discussion

PA formation frequently occurs during the blood testing process, especially in patients with platelet hyperactivity.[8-10] Here, we intentionally induced PA in citrated blood samples using a hematology analyzer to reproduce the PA formation in the LTA test process and evaluated the influence of PA formation on the LTA results. As a consequence, we demonstrated that LTA MA% was reduced by PA formation. This MA% reduction was associated with the decrease in platelet count and MPV in PRP after PA formation. Although both platelet count reduction (overall platelet decrease) and MPV reduction (selective large, activated platelet decrease) may contribute to the MA% reduction, our additional experiment proved the relative importance of a large activated platelet decrease (Supplement 1). In fact, the removal of large activated platelets was also suggested by the reduction of platelet count ratios of PRP/whole blood after PA formation during the PRP production process (centrifugation). Higher-speed centrifugation has been reported to reduce LTA MA% and MVP.[11] Although the PA activities of our samples were modified by antiplatelet drugs that were administered to the majority of patients, PAs formed more easily in patients than in healthy subjects during the hematology analysis process. The reason for the paradoxical lower MA% of initial PRP in patients might be the PA formation during centrifugation in the PRP preparation process rather than the effect of antiplatelet drugs.

Our study is limited in terms of study design in that we could not evaluate PRP after PA formation using all concentrations of agonists. In addition, we could not try several different centrifugation speeds in the PRP separation process, although we adopted rather slow centrifugation ($85 \times g$ for 15 minutes) to minimize the removal of active platelets from the PRP.[11]

In conclusion, we clearly demonstrated that PA formation in citrated blood samples led to a reduction of LTA MA%, probably because large activated platelets were removed during the LTA measurement process. Such PA formation may have been a cause of the lower reliability of the LTA-based assessment of platelet hyperactivity pointed out in the clinical setting. To ensure accurate LTA evaluation, it is necessary to confirm that PAs have not formed in the blood sample. Furthermore, when PAs are detected in the sample, it may be necessary to evaluate platelet function using another method.

Declaration of Interest

The authors declare no conflicts of interest.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jstrokecerebrovasdis.2018.12.014.

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