Original Article

Platelet Function Measured Using a Whole Blood Aggregometer Can Predict Bleeding Events

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Aim: We hypothesized that excessive suppression of platelet function due to antiplatelet therapy can increase the incidence of bleeding complications. The aim of the present study was to find whether we could predict bleeding events by measuring platelet function.

Methods: We enrolled 743 subjects whose platelet function was measured using a whole blood aggregometer based on a screen filtration pressure method. Of these subjects, 551 (74.2%) were treated with some type of antiplatelet agent. The endpoints were bleeding or ischemic events requiring hospitalization or extension of hospital stay. We prospectively compared the platelet function of subjects with and without bleeding or ischemic events.

Results: During 556 ± 207 days of follow-up, 52 (7.0%) bleeding events and 20 (2.7%) ischemic events were observed. Kaplan-Meier analysis using the log-rank test revealed that an aggregation rate of $<20\%$ induced by $8 \mu\text{M}$ adenosine diphosphate (ADP) was significantly associated with a greater number of bleeding events (11.9% vs. 5.2%; $p=0.0007$). Cox proportional hazards model showed that age >75 years (hazard ratio [HR], 1.78; 95% confidence interval [CI], 1.03-3.10; $p=0.039$), estimated glomerular filtration rate $<60 \text{ mL/min/1.73 m}^2$ (HR, 1.82; 95% CI, 1.06-3.18; $p=0.031$) and aggregation rate $<20\%$ induced by $8 \mu\text{M}$ ADP (HR, 2.18; 95% CI, 1.24-3.80; $p=0.0071$) were independent predictors of bleeding events.

Conclusions: Low platelet function demonstrated using a whole blood aggregometer was an independent predictor of bleeding complications.

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Key words; Platelet function assay, Screen filtration pressure method, Aggregation rate, Antiplatelet agents, Bleeding event

Introduction

Aspirin has been considered essential for the secondary prevention of atherosclerotic disease¹⁾, and dual antiplatelet therapy with aspirin and thienopyridine or clopidogrel is the cornerstone for the management of patients undergoing percutaneous coronary intervention; however, antiplatelet therapy occasionally

induces bleeding complications. Since Asian populations have a relatively high incidence of bleeding compared with other ethnic groups²⁻⁵⁾, it is prudent to carefully monitor Japanese patients treated with antiplatelet agents to prevent severe bleeding.

Recently, it was reported that residual platelet reactivity or resistance to antiplatelet agents, as measured with a platelet function assay, is strongly associated with ischemic events⁶⁻¹⁰⁾, and the efficacy of the platelet function assay for patients treated with antiplatelet agents has been validated; however, these studies did not focus on bleeding events but rather focused on ischemic events, including coronary artery stent thrombosis. Furthermore, there have been few studies

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on the association between the results of ex vivo platelet function tests and the frequency of bleeding events. We therefore examined whether we could predict bleeding events in patients treated with antiplatelet agents using data derived from platelet function assays.

Methods

Study Population and Clinical Endpoints

This was a single-center, prospective observational study. Subjects were eligible for inclusion in the present study if their platelet aggregation function had been measured at Chugoku Workers Compensation Hospital. Subjects were excluded if they had been previously diagnosed with serious hematological disease, significant anemia with a hemoglobin level <10 g/dL, severe liver damage (Child-Pugh class B or C), and severely impaired renal function, defined as the estimated glomerular filtration rate (eGFR)¹¹⁾ <30 ml/min/1.73m². Additional exclusion criteria were uncontrolled hypertension and regular use of nonsteroidal antiinflammatory drugs or selective serotonin reuptake inhibitors. After enrollment, subjects were scheduled to be followed up for 18 months to ascertain the occurrence of clinical events. Follow-up was by medical visits or telephone calls.

The primary endpoint was a bleeding event requiring hospitalization or extension of hospital stay. The secondary endpoint was an ischemic event defined as an ischemic stroke, myocardial infarction (including coronary artery stent thrombosis), or peripheral artery occlusion. Patients were censored at clinical endpoints, loss to follow-up, discontinuation of prescribed antiplatelet or anticoagulant agents, and death.

Written informed consent was obtained from all patients, and the study protocol was approved by the research committee of our institution.

Platelet Function Measurement

Platelet function was measured using a whole blood aggregometer based on a screen filtration pressure method (WBA analyzer; LMS Co., Ltd., Tokyo, Japan). Study procedures were conducted according to the method described by Ozeki *et al.*¹²⁾ and Sudo *et al.*¹³⁾. Briefly, whole blood aggregation was induced by adding 8 μ M adenosine diphosphate (ADP) to 200 μ L whole blood. Five minutes later, the absorbing pressure of the aggregated whole blood was measured through the screen of a microsieve (300 μ m² pore size). The results are expressed as aggregation rates. A negative pressure of -130 mm Hg was defined as 100% and -6 mm Hg as 0%.

International normalized ratio (INR) values were

also measured in subjects who were prescribed warfarin. The average aggregation rates and the INR values obtained on three different days were analyzed for each subject. All measurements were performed at least a week after the start of newly prescribed antiplatelet or anticoagulant agents for the enrolled subjects, except for the control subjects. (subjects not taking antiplatelet or anticoagulant agents)

Statistical Analysis

Continuous variables are presented as the mean $\pm$ SD and categorical variables as frequency (percentage). Continuous variables were assessed using Student's *t*-test, and categorical variables were compared using chi-square statistics. Aggregation rates induced by 8 μ M ADP were divided into quartiles ($<20\%$, 20% to $<40\%$, 40% to $<80\%$, $\geq 80\%$) to compare the frequency of bleeding events across quartiles, and were dichotomized according to the mean aggregation rate induced by 8 μ M ADP to determine whether there was a difference in the frequency of ischemic events between subjects with values below and above the mean aggregation rate. Kaplan-Meier analysis was used to assess the time taken for bleeding or ischemic events to occur. The log-rank test was used to compare the bleeding event-free survival between subjects within the first (lowest) quartile of aggregation rates induced by 8 μ M ADP and the remaining subjects, and to compare ischemic event-free survival between subjects with an aggregation rate below and above the mean aggregation rate induced by 8 μ M ADP. Univariate and multivariate Cox proportional hazards analyses were performed using clinical factors related to bleeding to determine predictors of a bleeding event. Variables with a *p* value of <0.1 in univariate analysis were included in multivariate models. A *p* value of <0.05 was considered significant. All analyses were performed using JMP software version 5.1 (SAS Institute Japan Co., Ltd., Tokyo, Japan).

Results

Patient Characteristics and Medications Used

We enrolled 743 subjects (mean age, 72 ± 11 years; 470 men and 273 women) between January 2007 and January 2008. The baseline characteristics of the entire group of study subjects are presented in **Table 1**. A little over half of all the subjects had ischemic heart disease and we found atrial fibrillation in slightly more than 10% of all the subjects. The mean platelet aggregation rate induced by 8 μ M ADP was $47.3 \pm 33.7\%$ (**Table 1**). A total of 117 (15.7%) subjects were treated with one type of antiplatelet agent

Table 1. Baseline characteristics of study subjects

<i>N</i>	743
Male	470 (63%)
Age (years)	72 ± 11
Underlying disease	
Hypertension	401 (54%)
Diabetes mellitus	253 (34%)
Ischemic heart disease	417 (56%)
Coronary artery bypass grafting	110 (15%)
Atrial fibrillation	89 (12%)
Previous stroke	49 (7%)
Arteriosclerosis obliterans	47 (6%)
Previous valve replacement	46 (6%)
Congestive heart failure	80 (11%)
Body mass index (kg/m ²)	23.8 ± 4.3
Estimated glomerular filtration rate (ml/min/1.73m ²)	65.2 ± 19.4
Hemoglobin level (g/dL)	12.8 ± 1.6
Platelet count (× 10 ⁴ /μL)	16.9 ± 5.4
Platelet aggregation rate induced by 8 μM ADP (%)	47.3 ± 33.7
Medication	
Antiplatelet agents	551 (74%)
Warfarin	241 (32%)
Proton pump inhibitor/H2 blocker	375 (50%)

alone, 246 (33.1%) with two or three types of antiplatelet agents, 53 (7.1%) with warfarin alone, 188 (25.3%) with a combination of antiplatelet agents and warfarin, and 139 (18.7%) with no antiplatelet or anticoagulation agents. Proton pump inhibitors or H2 blockers were prescribed in half of all the subjects (**Table 1**).

The types of antiplatelet agents prescribed and their usual daily doses were as follows: 81 mg aspirin, 200 mg ticlopidine, 75 mg clopidogrel, 100 mg cilostazol, and 120 μg prostaglandin. For subjects who received anticoagulant therapy, the dose of warfarin was based on INR.

Differences in Clinical Endpoints and Platelet Aggregation Rate Depending on Medications

The mean follow-up period was 556 ± 207 days. During this period, 52 (7.0%) bleeding events occurred; 25 (48.0%) gastrointestinal bleeds, 10 (19.2%) intracranial hemorrhages, 8 (15.4%) access site-related bleeds after percutaneous coronary intervention, and 9 (17.3%) bleeds due to other causes. We observed 20 (2.7%) ischemic events; 11 (55.0%) cerebral infarctions, 5 (25.0%) coronary artery stent thromboses, and 4 (20.0%) peripheral artery occlusions (**Table 2**).

The frequency of bleeding and ischemic events, the platelet aggregation rate induced by 8 μM ADP, and INR for each medication combination are shown

Table 2. Bleeding and ischemic events

Events	<i>n</i>
Bleeding events	52
Gastrointestinal bleeding	25 (48.0%)
Intracranial hemorrhage	10 (19.2%)
Access site-related bleeding during PCI	8 (15.4%)
Hemothorax	2 (3.8%)
Traumatic hemorrhage	2 (3.8%)
Severe hematuria	2 (3.8%)
Renal hemorrhage	2 (1.9%)
Intraperitoneal bleeding	2 (1.9%)
Bleeding from prostate	2 (1.9%)
Ischemic events	20
Ischemic stroke	11 (55.0%)
Coronary artery stent thrombosis	5 (25.0%)
Peripheral artery occlusion	4 (20.0%)

PCI, percutaneous coronary intervention

in **Table 3**. Subjects who took a combination of aspirin and warfarin and those who took other medication combinations (including various combinations of aspirin, cilostazol, clopidogrel, ticlopidine, prostaglandin, and warfarin) had a significantly greater number of bleeding events than those who took no antiplatelet or anticoagulant agents; however, there was no difference in the number of ischemic events between subjects taking and not taking any antiplatelet or anticoagulant agents. The aggregation rate was significantly lower in subjects taking antiplatelet agents than in those not taking these agents. However, the aggregation rate was similar in subjects taking cilostazol or warfarin and in those not taking any medication. INR was significantly lower in subjects taking a combination of aspirin, ticlopidine, and warfarin than in those taking warfarin alone.

Frequency of Bleeding or Ischemic Events According to Aggregation Rate Induced by 8 μM ADP and Kaplan-Meier Analysis

Significant differences in the frequency of bleeding events were observed between the first quartile of the aggregation rate induced by 8 μM ADP (<20%) and the third quartile (40% to <80%) (11.9% vs. 5.6%, *p* = 0.033) or fourth quartile (≥80%) (vs. 3.2%, *p* = 0.0014) (**Fig. 1**). The ischemic event rate was significantly higher in subjects with an aggregation rate ≥47% induced by 8 μM ADP compared with those with an aggregation rate <47% (4.1% vs. 1.6%, *p* = 0.038) (**Fig. 2**).

Kaplan-Meier analysis using the log-rank test revealed that aggregation rates of <20% were significantly associated with a greater number of bleeding

Table 3. Frequency of bleeding and ischemic events, platelet aggregation rate induced by 8 μ M ADP, and INR for each combination of medications

Medications	<i>n</i>	Bleeding events	<i>p</i> value*	Ischemic events	<i>p</i> value**	Aggregation rate (%)	<i>p</i> value***	INR	<i>p</i> value [§]
Aspirin and ticlopidine	208	12 (6%)	0.55	7 (3%)	0.27	28.9 $\pm$ 23.0	<0.001	-	-
Ticlopidine and warfarin	63	5 (8%)	0.29	1 (2%)	0.94	39.0 $\pm$ 31.1	<0.001	1.7 $\pm$ 0.5	0.55
Aspirin	61	3 (5%)	0.85	1 (2%)	0.91	48.5 $\pm$ 34.0	<0.001	-	-
Aspirin, ticlopidine, and warfarin	54	6 (11%)	0.08	1 (2%)	0.84	27.1 $\pm$ 21.0	<0.001	1.4 $\pm$ 0.4	0.002
Warfarin	53	3 (6%)	0.69	3 (6%)	0.1	76.2 $\pm$ 27.8	0.58	1.7 $\pm$ 0.7	-
Aspirin and warfarin	36	6 (17%)	0.009	1 (3%)	0.6	51.6 $\pm$ 30.0	<0.001	1.5 $\pm$ 0.3	0.1
Ticlopidine	28	1 (4%)	0.86	0 (0%)	0.52	39.7 $\pm$ 24.8	<0.001	-	-
Aspirin and clopidogrel	20	2 (10%)	0.27	1 (5%)	0.28	23.2 $\pm$ 13.3	<0.001	-	-
Cilostazol	12	0 (0%)	0.46	0 (0%)	0.68	60.2 $\pm$ 34.8	0.95	-	-
Cilostazol and warfarin	12	1 (8%)	0.4	1 (8%)	0.1	65.2 $\pm$ 42.0	0.86	1.9 $\pm$ 0.8	0.38
Other combinations [†]	57	7 (12%)	0.04	2 (4%)	0.35	23.2 $\pm$ 13.6	<0.001	-	-
None	139	6 (4%)	-	2 (1%)	-	75.2 $\pm$ 30.2	-	-	-
Total	743	52 (7%)	-	20 (3%)	-	47.3 $\pm$ 33.7	-	-	-

Aggregation rate, aggregation rate induced by 8 μ M ADP

* All differences between the frequency of bleeding events with each medication and without any medication.

** All differences between the frequency of ischemic events with each medication and without any medication.

*** All differences between the aggregation rates induced by 8 μ M ADP with each medication and without any medication.

[§] All differences between the INR values with each combination of antiplatelet agents and warfarin, and with warfarin alone.

[†] Other combinations include various combinations of medications chosen from aspirin, cilostazol, clopidogrel, ticlopidine, prostaglandin, and warfarin.

Fig. 1. Bleeding event rate according to quartiles of aggregation rate induced by 8 μ M ADP. **Fig. 2.** Ischemic event rate in subjects with a platelet aggregation rate above or below the median aggregation rate induced by 8 μ M ADP.

events (11.9% vs. 5.2%, $p=0.0007$; **Fig. 3**), and aggregation rates of $\geq 47\%$ were significantly associated with a greater number of ischemic events (4.1% vs. 1.6%, $p=0.042$; **Fig. 4**).

Independent Predictors of Bleeding Events

In univariate Cox proportional hazards analysis, age >75 years, eGFR <60 ml/min/1.73m², platelet count $<10 \times 10^4/\mu$ L, and an aggregation rate induced

by 8 μ M ADP $<20\%$ emerged as predictors of bleeding events. In multivariate models including these variables, age >75 years (hazard ratio [HR], 1.78; 95% confidence interval [CI], 1.03-3.10; $p=0.039$), eGFR <60 ml/min/1.73m² (HR, 1.82; 95% CI, 1.06-3.18; $p=0.031$), and an aggregation rate induced by 8 μ M ADP $<20\%$ (HR, 2.18; 95% CI, 1.24-3.80; $p=0.0071$) remained independent predictors of bleeding events (**Table 4**).

Fig. 3. Kaplan-Meier curves representing patients free of bleeding events.

Study subjects were divided into two groups based on the cut-off value for the aggregation rate induced by 8 μ M ADP for predicting ischemic events. Continuous line, aggregation rate $\geq 20\%$; broken line, aggregation rate $< 20\%$.

Fig. 4. Kaplan-Meier curves representing patients free of ischemic events.

Study subjects were divided into two groups based on the cut-off value for aggregation rates induced by 8 μ M of ADP for predicting ischemic events. Broken line, aggregation rate $\geq 47\%$; continuous line, aggregation rate $< 47\%$.

Table 4. Cox proportional hazards analysis for determining predictors of bleeding events

Variables	Univariate		Multivariate	
	HR (95% CI)	<i>p</i> value	HR (95% CI)	<i>p</i> value
Age > 75 years	1.88 (1.09-3.29)	0.023	1.78 (1.03-3.10)	0.039
Female	1.01 (0.56-1.76)	0.97		
Body mass index > 25 kg/m ²	0.74 (0.33-1.53)	0.43		
Hemoglobin level < 11 g/dL	1.36 (0.58-2.84)	0.45		
Estimated glomerular filtration rate < 60 ml/min/1.73m ²	1.99 (1.15-3.45)	0.014	1.82 (1.06-3.18)	0.031
Proton pump inhibitor/H ₂ blocker	0.98 (0.56-1.69)	0.94		
Platelet count $< 10 \times 10^4/\mu$ L	2.31 (1.01-4.64)	0.048	1.84 (0.80-3.76)	0.14
Aggregation rate induced by 8 μ M ADP $< 20\%$	2.50 (1.44-4.31)	0.0014	2.18 (1.24-3.80)	0.0071
Dual antiplatelet agents	1.03 (0.55-1.84)	0.92		
Single antiplatelet agent and warfarin	1.50 (0.75-2.77)	0.24		
Dual antiplatelet agents and warfarin	1.62 (0.67-3.36)	0.27		

HR, hazard ratio; CI, confidence interval

Discussion

To our knowledge, this is the first prospective study of the association between platelet function and the risk of bleeding. Our major finding was that a low platelet aggregation rate demonstrated with a whole blood aggregometer was a significant predictor of bleeding events. We identified bleeding events in 12% of 201 subjects with aggregation rates of $< 20\%$, which was comparable to the bleeding event rate for an INR of 3.0-4.4 in Italian subjects taking warfarin¹⁴. Chen and colleagues¹⁵ examined the relationship between platelet aggregation and bleeding during

coronary bypass grafting and found that patients who had greater clopidogrel-induced platelet inhibition required a significantly higher number of platelet and red blood cell transfusions than patients with lower clopidogrel-induced platelet inhibition. This may support our major result, suggesting the possibility of using a platelet function assay as a tool for the risk stratification of bleeding events. Our finding that excessively suppressed platelet function rather than the number or amount of antiplatelet agents taken was associated with the incidence of bleeding may support the well-known wide interindividual variability in the response to antiplatelet agents, as characterized by

clopidogrel¹⁶⁻¹⁸⁾, suggesting the importance of assessing the *in vivo* pharmacological effects of these agents. Several investigators reported that increasing the dose or adding antiplatelet agents resulted in stronger platelet inhibition without increasing the number of bleeding complications^{8, 10, 19, 20)}. This seems slightly different from our major finding mentioned above. Possible explanations for the difference are that (1) the subjects included in our study were markedly older than those previous studies; (2) the subjects included in our study were all Japanese (Asian populations have a relatively high incidence of bleeding complications compared with other ethnic groups); (3) in our study, nonfatal bleeding was also considered a primary endpoint; and (4) the platelet function assay was not necessarily the same as those used in previous studies.

In agreement with previous research²¹⁻²³⁾, an advanced age and decreased eGFR were other independent predictors of bleeding events in our study. GFR is known to decrease with aging, so there would be a close relationship between GFR and age; however, adjustment for age hardly attenuated the association between eGFR and the risk of bleeding. There are reports that decreased GFR may influence platelet function^{24, 25)}, but this was not shown in our study.

We observed ischemic events more frequently in patients in whom the platelet aggregation rate induced by 8 μ M ADP was above the mean (47.3%) compared with subjects with a rate below the mean. In receiver operating characteristic curve analysis to distinguish patients taking dual antiplatelet agents (aspirin and ticlopidine) from those not taking antiplatelet drugs, the appropriate cut-off value for the aggregation rate induced by 8 μ M ADP was 47.3% (85% sensitivity and 81% specificity), which, interestingly, is the same as the mean value for the aggregation rate. Taking these results together, although the positive predictive value of platelet function for the incidence of ischemia is not high, dual antiplatelet therapy may reduce the risk of ischemic events^{26, 27)}, provided these dual antiplatelet agents act effectively.

Several studies have reported that platelet aggregation function assays were useful for detecting residual platelet reactivity or resistance to antiplatelet agents, which are strongly associated with ischemic events⁸⁻¹⁰⁾. In our study, we confirmed an additional finding, which is that platelet aggregation function assays are useful for detecting patients at high risk for bleeding events. In particular, whole blood aggregation analysis using a screen filtration pressure method has good reproducibility and easy handling^{12, 13)} and requires only a short time. Hence, we presume it would be suitable as a monitoring instrument in an

outpatient clinic.

In our study, subjects who took a combination of aspirin and warfarin had a significantly higher number of bleeding events than those who took no medication. Subjects who took other combinations of medications ($n = 57$), including 23 subjects who took various combinations of antiplatelet and anticoagulant agents, also had a relatively high number of bleeding events. These findings are consistent with the results of previous studies, which showed that the concomitant administration of antiplatelet and anticoagulant agents was associated with a relatively high risk of bleeding complications²⁸⁻³⁰⁾. From the results of our study, however, we were unable to conclude that the combined use of antiplatelet and anticoagulant agents was an independent predictor of bleeding events. This may be explained by the fact that the INR values tended to be lower in subjects who took both antiplatelet and anticoagulant agents than in those who took warfarin alone.

The major limitation of our study is that the statistical power was limited because of the relatively small number of bleeding or ischemic events observed. The reason for using 8 μ M ADP as an aggregation agonist was that among the different concentrations of ADP tried in a preliminary test, this concentration reflected the widest range of platelet functions. However, if other concentrations of ADP or other aggregation agonists, such as collagen or arachidonic acid, had also been used, further insights into this subject may have been obtained. Finally, the extent to which our *ex vivo* platelet aggregation function tests represent *in vivo* platelet activity remains unknown. To obtain additional evidence, randomized trials with a large number of subjects are needed to determine whether adjustments to the use of antiplatelet agents, based on findings using a whole blood aggregometer, reduce the risk of bleeding events.

In conclusion, a low platelet aggregation rate determined using a whole blood aggregometer was an independent predictor of bleeding events. Furthermore, a platelet function assay using whole blood aggregometry based on a screen filtration pressure method was useful for identifying patients at high risk for bleeding. Especially in elderly or subjects with decreased renal function, careful monitoring might be necessary in order to prevent overdosing with antiplatelet agents.

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Statement of conflicts of interest

The authors have no conflicts of interest.

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