

Measurement of Platelet-derived Microparticle Levels in the Chronic Phase of Cerebral Infarction Using an Enzyme-linked Immunosorbent Assay

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Assessment of platelet function is a critical component of the treatment and secondary prevention of cerebral infarction, and measurement of platelet-derived microparticle (PDMP) levels using flow cytometry may be a good indicator of platelet function. However, the flow cytometric analysis is not feasible in a variety of clinical situations. The goal of the present study was to measure PDMP levels using an enzyme-linked immunosorbent assay (ELISA) in chronic cerebral infarction patients and to determine the utility of PDMP level measurement for the monitoring of the effect of cilostazol and aspirin.

A crossover study was performed using 4-weeks of aspirin (100 mg/day) and 4-weeks of cilostazol (200 mg/day) in 18 patients. PDMP levels were also measured in 20 volunteers as controls. Experiments demonstrated that PDMP levels were significantly higher in chronic cerebral infarction patients (median 8.8 U/ml, interquartile range 5.1-14.9 U/ml, n=18) than in controls (median 5.5 U/ml, interquartile range 5.0-8.2 U/ml, n=20) ($P=0.047$). PDMP levels did not decrease after therapy with either aspirin (median 10.9 U/ml, interquartile range 6.2-17.9 U/ml, n=12) or cilostazol (median 9.2 U/ml, interquartile range 6.1-14.3 U/ml, n=12) compared with baseline PDMP levels in the 12 patients who completed this trial (median 11.4 U/ml, interquartile range 5.2-23.7 U/ml, n=12). There were no significant differences in PDMP levels between aspirin and cilostazol ($P=0.61$). In conclusion, PDMP levels as measured by ELISA were increased in patients with chronic cerebral infarction regardless of the anti-platelet therapy. This methodology may be a useful strategy of assessing platelet function in chronic cerebral infarction patients.

Platelet activation is an important component of the pathophysiology of ischemic stroke (5, 6, 8, 20), and anti-platelet therapy plays an important role in the secondary prevention of cerebral infarction. However, there is no commonly accepted method of evaluating modulation of platelet function by anti-platelet agents. For example, platelet activation has been measured in the context of the chronic phase of cerebral infarction using various methods, including aggregometry, plasma levels of platelet release products, and urinary excretion of thromboxane metabolites (5, 6, 8, 9, 20, 21, 22). However, these studies either require too many resources for routine clinical use or have not shown consistent results in clinical studies.

Activated platelets shed platelet-derived microparticles (PDMP) in response to certain stimuli. Recently, PDMP levels have been measured in the context of various diseases, including acute coronary syndrome (12), pulmonary embolism (7), arteriosclerosis (13), diabetes mellitus (14), and inflammatory bowel disease (1). In fact, several studies using flow cytometry have reported that PDMP levels were significantly elevated in the acute and chronic phases of ischemic stroke (4, 10). Therefore, the goal of the present study was to measure PDMP levels using an enzyme-linked immunosorbent assay (ELISA) method in patients in the chronic phase of cerebral infarction and to determine the utility of PDMP level measurement for monitoring of the anti-platelet effect of cilostazol and aspirin.

MATERIALS AND METHODS

Patients and protocols

The characteristics of the patients and volunteers as controls are summarized in Table 1. Eighteen patients with the chronic phase of cerebral infarction (14 men and 4 women, 67.3±9.6 yr) were studied. 20 age-matched healthy volunteers (10 men and 10 women, 61.3±8.7 yr) served as the controls. Study protocols conformed to the ethical guidelines of the 1975 Declaration of Helsinki and were approved by the ethics committee of Kobe University, and informed consent was obtained from all subjects included in the study.

Clinical diagnoses of the stroke patients included: lacunar infarction (n=5), cerebral atherothrombosis (n=7), multiple old lacunar infarctions (n=5), and vertebral artery dissection (n=1). The onset of stroke was greater than 3 months before entry into the study for all patients. Cerebrovascular risk factors of the stroke patients were as follows: hypertension (n=14), hyperlipidemia (n=13), diabetes mellitus (n=8), and current smoker (n=8). None of the patients had experienced inflammatory or cardiovascular disease within the previous 3 months, and none of the patients had clinically detectable renal, hepatic, infectious, or malignant disease. Because all patients had received anti-platelet therapy before enrolling in the study, baseline sample collections were performed after a 2-week wash-out period.

Then, a crossover study using aspirin and cilostazol was performed to compare PDMP levels before and after anti-platelet therapy. Enrolled patients were randomly divided into two groups, and one of the groups received a 4-week course of aspirin (100 mg/day) followed by a 4-week course of cilostazol (200 mg/day), while the other groups received the drugs in the opposite order.

Sample collections were performed after the 2-week washout period and on the last day of each 4-week treatment period (i.e., at the crossover point and after completion of the second anti-platelet regimen at 8 weeks). There was no wash-out period between cross-over of the test drugs.

Blood sampling and sample preparation

Two milliliters of blood were obtained via a 21-G needle inserted into a forearm vein. Samples were mixed with a 1:10 volume of acid citrate dextrose/ethylene diaminetetraacetate (ACD/EDTA) (1.0 g EDTA-2Na, 2.2 g trisodium citrate, 0.807 g citric acid, 2.2 g dextrose in 100 ml of distilled water, nipro neotube, NIPRO, Japan). Platelet-rich plasma (PRP) was prepared by centrifugation at 150 ×g for 20 min at room temperature and stored at -80°C until next procedure was performed. PRP was prepared by centrifugation at 8000 ×g for 5 min at room temperature to obtain platelet-poor plasma (PPP). PPP was subsequently used for ELISA analysis of PDMP levels.

Measurements of PDMP

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PDMP levels were determined by an ELISA system based on the modified method reported by Osumi et al (18). One hundred microliters of purified GPIX antibodies (KMP-9) was added to each well of 96-well microtiter plates (MaxiSorp, Nunc) and incubated for 18 h at 4°C. After 3 washes with 50 mM Tris-saline (pH 8.0), 350 µl of blocking buffer was added to each well and incubated at least 18 h at 4°C. PRP samples from several healthy volunteers were combined and adjusted to 250,000 platelets/µl and used as a standard for ELISA. One unit/milliliter of PDMP was defined as 24,000 platelets/ml of solubilized platelet in this ELISA system. This ELISA kit is available from JIMRO Co. Ltd, (Takasaki Japan) commercially. Fifty microliters of pretreatment solution added to each well and 50 µl of PDMP samples or standards was added to each well and incubated for 3 h at 25°C on a plate shaker (200 rpm). Plates were washed 3 times with 350 µl/well of wash buffer (0.05% Tween 20 in PBS). One hundred microliters of peroxidase-conjugated GPIb antibody (NNKY5-5) was added to each well and incubated for 1 h at 25°C on a plate shaker. After each well was washed 3 times with 350 µl of wash buffer and then incubated with 100 µl of peroxidase substrate solution (TMB plus, DakoCytomation) for 20 min at room temperature. After this incubation, 100 µl of stop solution (1mol/L H₂SO₄) was added to each well, and the absorbance was measured with an EIA reader at 450 nm. PDMP levels <5.0 U/ml were defined as 5.0 U/ml in this study.

Statistical analyses

All data related to the PDMP are shown as median and interquartile range for PDMP levels. Statistical analysis was performed using the Mann-Whitney U-test for comparison between baseline PDMP levels in the cerebral infarction patients and those in the control subjects, and the Wilcoxon signed-ranks test was used to compare the PDMP levels between before and after anti-platelet therapy with aspirin or cilostazol. Wilcoxon signed-ranks test was also used to compare between PDMP levels with 4-weeks course of aspirin and those with 4-weeks course of cilostazol. All analyses were conducted using StatView version 5.0 software (SAS, Cary, NC, USA).

Differences resulting in two tailed *p*-values less than 0.05 were considered to be statistically significant.

Table1: Clinical characteristics of study patients and controls

	Chronic cerebral infarction (n=18)	Control (n=20)
Gender (M/F)	14/4	10/10
Age(years)	67.3±9.6	61.3±8.7
BMI(kg/m ²)	23.9±2.1	22.1±2.4
No. of hypertention	14	7
No. of diabetes mellitus	8	1
No. of hyperlipidemia	13	6
No. of current smokers	8	8

Mean ±SD values are shown.

RESULTS

Baseline PDMP levels were significantly higher in the chronic phase of cerebral infarction patients without medication (median 8.8 U/ml, interquartile range 5.1-14.9 U/ml, n=18) than in the control subjects (median 5.5 U/ml, interquartile range 5.0-8.2 U/ml, n=20, p=0.047) (Fig. 1). Six of 18 patients dropped out of the cross-over anti-platelet therapy trial because of headache with cilostazol administration (n=2), withdrawal of informed consent (n=1), admission to an outside hospital (n=1), or unknown reasons (n=2). In 12 patients who completed the crossover study, there were no significant differences in PDMP levels following a 4-week course of aspirin (median 10.9 U/ml, interquartile range 6.2-17.9 U/ml, n=12) when compared with a 4-week course of cilostazol (median 9.2 U/ml, interquartile range 6.1-14.3 U/ml, n=12, P=0.61). The PDMP levels did not decrease significantly after 4 weeks anti-platelet therapy with either aspirin or cilostazol when compared with baseline levels (median 11.4 U/ml, interquartile range 5.2-23.7 U/ml, n=12 vs. aspirin 4-weeks: p=0.48 vs. cilostazol 4 weeks: P>0.99) (Fig. 2).

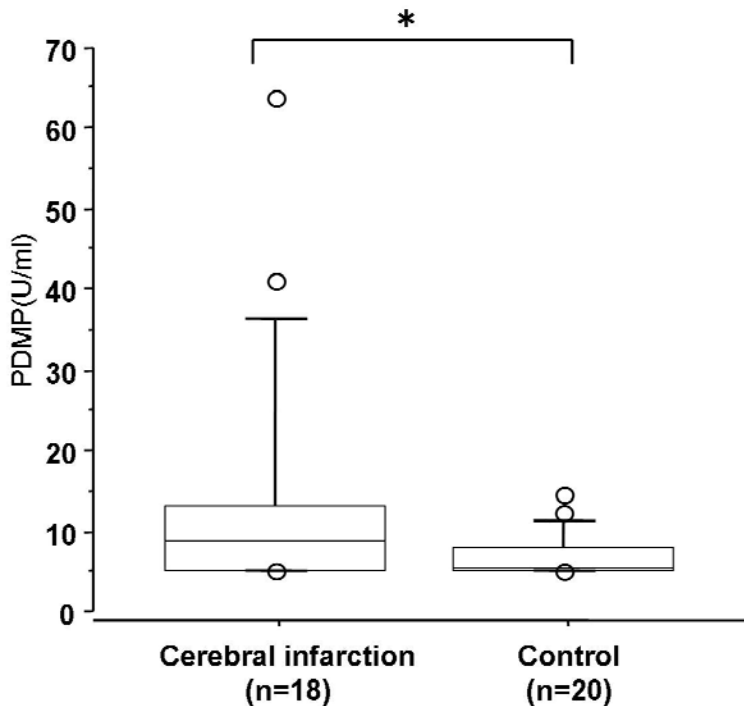


Figure 1: Baseline circulating platelet-derived microparticles (PDMP) levels in 18 chronic phase cerebral infarction patients and in 20 controls. Baseline PDMP levels were significantly higher in the chronic phase of the cerebral infarction patients when compared with controls (*: P=0.047). The PDMP levels were measured by the ELISA method. The box indicates the median±25 percentile. The lower bar indicates the 10th percentile, and the upper bar indicates the 90th percentile.

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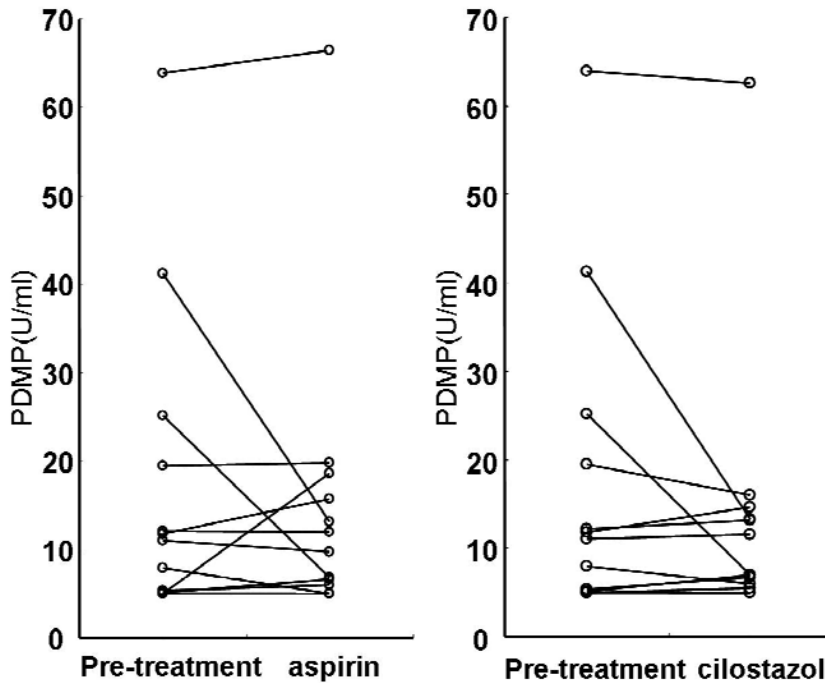


Figure 2: Changes in PDMP levels before and after anti-platelet therapies with aspirin (100 mg/day) or cilostazol (200 mg/day) in 12 patients in the chronic phase of cerebral infarction.

There were no significant differences between the PDMP levels when comparing baseline, post-aspirin, or post-cilostazol. Paired samples were obtained from 12 patients who completed this trial in the chronic phase of cerebral infarction. PDMP levels were determined by the ELISA method.

DISCUSSION

PDMP activates the coagulation cascade (19), promotes leukocyte and endothelial cell adhesion (15, 16), and may stimulate cytokine secretion and tissue factor expression in endothelial cells (2, 3, 14, 15), thereby increasing the risk of atherosclerosis and ischemic stroke. Therefore, PDMP levels may be a marker of the risk of stroke, and therapeutic reduction of PDMP levels may reduce the risk of subsequent stroke.

PDMP has been measured accurately using flow cytometry (17), but this method is not feasible in most clinical centers. The recent development of an ELISA for PDMP represents a viable alternative for the clinical measurement of PDMP levels (18). Indeed, ELISA can be performed after freezing the sample, allowing samples to be batched for central assays and thereby reducing costs.

Two reports (4, 10) have suggested that anti-platelet therapy has minimal effects on PDMP levels as measured by flow cytometry in the context of acute cerebral infarction. Specifically, PDMP levels were higher in patients with acute cerebral infarction than in controls, regardless of whether or not patients received anti-platelet therapy. Despite taking aspirin, PDMP levels were also higher in 7 of 19 patients with multi-infarct dementia (10). Cherian et al. reported that PDMP levels were similar when comparing patients in the acute and chronic phases of cerebral infarction (4). These observations are consistent with results

from the present study, which demonstrated that PDMP levels remained elevated in the chronic phase of cerebral infarction whether or not the patient received anti-platelet therapy. Furthermore, this is the first report to assess changes in PDMP levels before and after anti-platelet therapy during the chronic phase of cerebral infarction using the ELISA method.

The present study demonstrated that anti-platelet treatment did not significantly influence PDMP concentration. Thus, it is possible that serum anti-platelet medication levels are insufficient. Alternatively, persistent elevations in PDMP levels may result from high shear stress secondary to atherosclerotic cerebrovascular disease (11).

In two of three patients with very high concentrations of PDMP (>20 U/ml), PDMP levels decreased to less than 20 U/ml after institution of anti-platelet therapy. Therefore, anti-platelet therapy may reduce PDMP concentrations only in those patients with very high PDMP levels. Another patient (54 year-old man with atherothrombotic infarction) had extremely high PDMP concentrations (>60 U/ml) before and after anti-platelet therapy. This patient had multiple risk factors for cerebral infarction, including cigarette use, hyperlipidemia, and elevated BMI, which may have contributed to this persistently elevated PDMP level.

In conclusion, PDMP levels measured by ELISA were increased in patients with chronic cerebral infarction regardless of anti-platelet therapy. These data suggest that there is no practical rationale for measuring PDMP levels in patients in the chronic phase of cerebral infarction at the present time. Further study would be of benefit to determine whether patients with high PDMP levels would experience a high recurrence rate of cerebral infarction and whether an increased dose or combination of anti-platelet drugs can reduce PDMP levels.

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