**Background:** Little is known about the platelet dynamics and the effect of antiplatelet therapy in Kawasaki disease (KD). The aim of this study was to clarify platelet activation dynamics in acute-phase KD patients by assaying platelet-derived microparticles (PDMPs).

**Methods and Results:** The PDMP level in 18 patients with acute KD was measured on ELISA. Of the 18 patients, 14 were receiving oral aspirin and i.v. immunoglobulin (IVIG) and 4, oral aspirin alone. Blood samples were drawn before, immediately after, and 10–14 days after IVIG infusion; thereafter, at 1, 2, and 3 months after the onset of disease. PDMP level before aspirin treatment was significantly higher in acute-phase KD patients than in the control subjects with common febrile diseases (P<0.01). In the acute-phase KD patients, IVIG significantly decreased PDMP level; the PDMP level was not lower on the similar day of KD in the patients who did not receive IVIG. Eight patients’ PDMP level rebounded after aspirin was discontinued.

**Conclusions:** Platelets are activated during acute-phase KD, which confirms the importance of antiplatelet therapy. In addition, platelet activation continues as long as 2 or 3 months after the acute phase, the time at which aspirin is commonly discontinued, and the timing of aspirin discontinuation should therefore be evaluated in each individual patient. (Circ J 2014; 78: 188–193)

**Key Words:** Antiplatelet therapy; Kawasaki disease; Platelet-derived microparticle

Kawasaki disease (KD) is a form of systemic panvасulitis. Typically, vasculitis damages vascular endothelial cells and causes loss of function, including anti-thrombotic action. The acute phase of KD is therefore thought to involve platelet activation, and antiplatelet therapy is included in the treatment protocol for this phase.

Based on this hypothesis, antiplatelet therapy is a common component of KD treatment strategies. The actual evidence for platelet activation, however, is limited to a few studies in which platelet aggregation tests showed increased aggregation capacity. Therefore, antiplatelet agent treatment is only a so-called empiric therapy, being based mainly on the coronary artery thrombotic occlusions that are often confirmed on autopsy after acute phase deaths. In other words, we are routinely using antiplatelet therapy although we do not understand the dynamics of platelet activation.

In this study, we therefore measured the levels of platelet-derived microparticles (PDMPs), which have recently received attention as a marker of platelet activation, in order to elucidate the platelet activation dynamics in KD.

PDMPs have recently been reported as a platelet activation marker, and the PDMP level can therefore be used as an index of platelet activation. PDMPs are endoplasmic reticulum-derived vesicles ranging from 0.02 to 0.1 μm in size that are discharged from platelets upon activation by some sort of stimulus. PDMPs have phospholipids with procoagulant activity on their surfaces and contain platelet granule components. PDMPs are not merely debris discharged from activated platelets but also possess intrinsic procoagulant activity and can activate platelets, white blood cells, and vascular endothelial cells to promote cell adhesion and contribute to blood coagulation. In addition, a vicious circle can develop as follows; cells activated by PDMPs produce inflammatory cytokines, which induce monocytes, macrophages, neutrophils, and vascular endothelial cells to express tissue factors, which promote the generation of thrombin, which mediates further activation of platelets, and which generates more PDMPs. Consequently, PDMPs are considered as important functional particles involved in the pathogenesis of vascular inflammation; in other words, PDMPs are a marker of both platelet activation and vascular inflammation.

Accordingly, the measurement of PDMP level in patients with KD with panvasculitis should allow us to examine the dynamic...
ics of both platelet activation and the vasculitis itself. The aim of this study was to prove that a state of platelet activation exists in patients with KD; to determine the clinical significance of antiplatelet therapy; and to demonstrate the usefulness of PDMP level in evaluating the degree of vasculitis and the effect of antiplatelet therapy in KD.

**Methods**

**Subjects**

The subjects were 18 patients with KD in the acute phase. According to the treatment protocol for the acute phase, 14 patients (patients 1–14; mean age, 2 years 7 months ± 2 years) received i.v. immunoglobulin (IVIG; 2 g/kg) and oral aspirin as antiplatelet agents, while 4 patients (patients 15–18; mean age, 1 year 7 months ± 1 year) received oral aspirin alone. The dose of aspirin was 30–50 mg·kg⁻¹·day⁻¹ during having fever, and we changed the dose (5 mg·kg⁻¹·day⁻¹) when the patients became afebrile. Aspirin was discontinued after 2 or 3 months. These subjects were compared with 33 age-matched patients with common febrile diseases as controls. All of the subjects were confirmed to have KD, with no questionable or incomplete diagnoses. Of the 14 subjects who received IVIG, none exhibited symptoms refractory to this treatment; the fever abated within 48 h after the start of the IVIG infusion in all subjects, and no subject developed any coronary artery disorder.

Written consent to participate in the study was obtained from all of the patients’ guardians as their representatives. This study obtained Ethics Committee approval in University Hospital, Kyoto Prefectural University of Medicine.

**Blood Sampling**

Two milliliters of blood were obtained via a 21-G needle and mixed with a one-tenth volume of acid citrate dextrose/ethylene diamine tetraacetate (ACD/EDTA, Nipro Neotube; Nipro, Japan). The samples were centrifuged at 8,000–10,000×g to obtain platelet-poor plasma (PPP). All procedures were performed at room temperature. The samples were stored in deep-freeze (from −40 to −80°C) until analysis.

Blood samples were collected from acute-stage patients at the following times: (1) before IVIG; (2) 2 days after IVIG; and (3) 10–14 days after IVIG.

**Figure 1.** Comparison of platelet-derived microparticle (PDMP) level between controls (common febrile diseases) and Kawasaki disease (KD) patients. PDMP level was significantly higher in the KD patients (P=0.0001).

**Figure 2.** Dynamics of platelet-derived microparticle (PDMP) level in Kawasaki disease patients after i.v. immunoglobulin (IVIG). (a) before IVIG; (b) 2 days after IVIG; (c) 10–14 days after IVIG.
The ELISA used is now available as a kit from Ootsuka Pharmaceutical (Tokyo, Japan). Fifty microliters of pretreatment solution and 50 µl of a PPP (PDMP sample) or standard were added to each well of a 96-well plate (3) 10–14 days after IVIG; (4) 1 month; (5) 2 months; and (6) 3 months after the onset of disease. In patients treated only with aspirin, samples were collected at (1’) day 5; (2’) day 7; and (3’) day 14–21 after onset. These time points (1’–3’) were almost the same as time points (1–3).
and incubated for 3 h at 25°C on a plate shaker (200 rpm). The 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 was added to each well and incubated for 1 h at 25°C on a plate shaker. Each well was washed 3 times with 350 µl of wash buffer and then incubated with 100 µl of peroxidase substrate solution for 20 min at room temperature. Finally, 100 µl of stop solution was added to each well, and the absorbance was measured with an microplate reader at 450 nm.

Statistical Analysis

Inter-group differences in continuous variables were evaluated using the Wilcoxon signed rank test, because the data were not normally distributed. The intra-group differences among the time points were analyzed using Mann-Whitney test. P<0.05 was considered statistically significant.

Results

We first compared PDMP level between the patients with acute-phase KD (43.9±13.5 U/ml) and those with common febrile diseases (15.4±6.8 U/ml). The PDMP level was significantly higher in the patients with acute-phase KD (P=0.0001; Figure 1). Second, we examined the PDMP dynamics during the acute phase of KD and found no statistically significant reduction in the PDMP level immediately after IVIG treatment (27.8±18.3 U/ml) compared with the pre-IVIG level (33.5±16.6 U/ml; Figure 2). Even before IVIG treatment, however, the PDMP level was significantly lower in the patients who had already received aspirin (20.3±8.3 U/ml) than in those who had received no anti-platelet therapy (43.4±14.3 U/ml; P=0.0027; Figure 3). The PDMP level 10–14 days after IVIG treatment (21.9±8.5 U/ml) was significantly lower than the pre-IVIG level (P=0.0009; Figure 2).

Third, in consideration of the fact that the platelet count changes greatly during the acute phase of KD, we evaluated the dynamics of the PDMP level per 10,000 platelets. The PDMP level per 10,000 platelets before IVIG infusion was 1.00±0.39 U/ml. The PDMP level of the patients who started receiving aspirin before IVIG (0.65±0.19 U/ml) was lower than that of the patients who received no aspirin before IVIG (1.26±0.26 U/ml; P=0.0007; Figure 4). The PDMP levels both immediately (0.65±0.24 U/ml) and 10–14 days after (0.49±0.25 U/ml) IVIG treatment were significantly lower than the pre-IVIG level (P=0.0012 and P=0.0011, respectively; Figure 5).

Fourth, comparison between the patients who did and did not receive IVIG showed that respective PDMP level increased in all the patients who did not receive IVIG (n=4; Figure 6), and this trend was opposite to the dynamics in the patients who received IVIG. Furthermore, the PDMP level of the patients who received IVIG was significantly lower than that in the patients who did not receive IVIG.
besides procoagulant activity. Among these is increasing the expression of adhesion molecules by white blood cells or vascular endothelial cells, which may contribute to vascular inflammation. Conversely, increased expression of inflammatory cytokines has been reported to promote platelet aggregation and the generation of PDMPs. From this point of view, PDMP can be an index for evaluation of vasculitis.

In the present study, we used a sandwich ELISA based on antibodies against the platelet-specific markers CD42b (GPIb) and CD42a (GPIX), which was recently developed by Nomura et al., and obtained stable reproducibility. PDMPs are expected to become an even more useful marker now that standardization, which was an issue of flow cytometry, has become easy to do, and the reliability of the measured results has increased.

The mechanism of platelet activation in KD was theorized to proceed as follows: the intense inflammation during the acute phase damages the vascular endothelial cells, leading to loss of the anti-thrombotic function of the vascular endothelium and causing platelet activation. But there has been no actual evidence for this mechanism. Only the observation of thrombi, which are the final result of platelet activation, during autopsies of deceased patients with acute-phase KD supported this theory.

Previously, the representative report on platelet activation in the acute phase was the study by Taki et al that measured platelet aggregation capacity. They reported that platelet coagulant activity is elevated during the acute phase and might continue to increase for several months. That method, however, measures the reactivity of retrieved platelets and does not necessarily reflect their status in vitro. The present PDMP measurement method evaluates platelet activation in vitro and is therefore an extremely significant advance.

In the present study, the PDMP level of several patients without obvious coronary artery disorders rebounded when aspirin was discontinued after 2–3 months, which indicates that platelet activation may persist long past previous expectations. Aspirin who did not receive IVIG at the same time points (P=0.016; Figure 7). And even if PDMP level initially declined, 4 patients had recurrent rising PDMP level when aspirin was discontinued after 2 months, and so did another 4 when it was discontinued after 3 months (Figure 8).

**Discussion**

In the present study, PDMP level was elevated, indicating greater platelet activation in patients with acute-phase KD than in other febrile patients. Furthermore, the use of aspirin as an antiplatelet agent significantly changed PDMP level, which supports the value of antiplatelet therapy during the acute phase.

We used PDMP as an index of platelet activation to show that platelet activation is present during the acute phase of KD and that antiplatelet therapy is clinically significant. Although PDMP has recently received a great deal of attention as a marker of platelet activation in adults, there are no data on PDMP level in pediatric patients. PDMP can be expected to be a clinically useful index of platelet activation in pediatric patients in the future.

In adults, PDMP has been featured increasingly often in reports covering such diverse clinical areas as oncology, in addition to thrombotic diseases, and is a subject of great interest in the understanding of the pathogenesis of vascular disease. The mechanism by which PDMPs are generated has been previously described as follows: platelets are activated by some stimulus, causing phospholipids to be displayed on the platelet surface due to the flip-flop phenomenon; the catalytic activity of these phospholipids then increases the surface’s procoagulant activity. A portion of the platelet surface membrane separates and is released as a PDMP that has phospholipids on its surface and contains platelet granule components. Therefore, PDMPs have been reported to have stronger procoagulant activity (50- to 100-fold higher) than platelets.

Furthermore, discharged PDMPs have many other functions besides procoagulant activity. Among these is increasing the expression of adhesion molecules by white blood cells or vascular endothelial cells, which may contribute to vascular inflammation. Conversely, increased expression of inflammatory cytokines has been reported to promote platelet aggregation and the generation of PDMPs. From this point of view, PDMP can be an index for evaluation of vasculitis.

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Platelet-Derived Microparticles in KD

Conclusions

Platelets are activated during the acute phase of KD, which confirms the clinical importance of antiplatelet therapy in these patients. We also showed that IVIG, the standard treatment for acute-phase disease, may act secondarily to reduce platelet activation by suppressing inflammation. Furthermore, the timing of discontinuation of antiplatelet therapy should be determined on an individual basis.

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Disclosures

None.

References