Circulating Platelet-Neutrophil Aggregates Play a Significant Role in Kawasaki Disease

Kentaro Ueno, MD; Yuichi Nomura, MD; Yasuko Morita, MD; Taisuke Eguchi, MD; Kiminori Masuda, MD; Yoshifumi Kawano, MD

**Background:** Circulating platelet-neutrophil aggregates play a crucial role in amplifying acute inflammation and could promote adverse effects involving vascular injury. The aim of this study was to evaluate the role of platelet-neutrophil aggregates in Kawasaki disease (KD).

**Methods and Results:** Forty patients with KD (30 intravenous immunoglobulin [IVIG] responders and 10 IVIG non-responders), 7 febrile patients with bacterial infections, and 9 normal volunteers were analyzed. Thirty-three patients with KD were treated with IVIG, and 7 were treated with IVIG plus prednisolone. We evaluated the rate of platelet-neutrophil aggregates and measured the platelet factor 4 (PF4) and β-thromboglobulin (β-TG) levels. The rate of platelet-neutrophil aggregates was significantly higher in patients with KD than those with bacterial infection and normal volunteers. The rate of platelet-neutrophil aggregates was significantly higher in patients with coronary artery abnormalities (CAA) than in those without CAA, and was correlated with PF4 and β-TG levels in patients with KD. Comparing time-course analysis, the rate of platelet-neutrophil aggregates was significantly decreased in patients treated with IVIG plus prednisolone than in those treated with IVIG alone.

**Conclusions:** The findings demonstrate that platelet-neutrophil aggregates are significantly present in higher rates and are closely related to pathological developments of CAA in KD. Additional prednisolone treatment for patients in the acute phase of KD could suppress platelet-neutrophil aggregates, indicating that platelet-neutrophil aggregates would inhibit amplified reciprocal vascular inflammatory activation. (Circ J 2015; 79: 1349–1356)

**Key Words:** Coronary artery abnormalities; Kawasaki disease; Platelet-neutrophil aggregates; Prednisolone; Vasculitis

Kawasaki disease (KD) is an acute febrile illness characterized by systemic vasculitis, and may lead to coronary artery abnormalities (CAA). High-dose intravenous immunoglobulin (IVIG) treatment effectively resolves the inflammation and reduces the occurrence of CAA in patients with KD. However, approximately 10–20% of patients with KD have sustained or recurrent fever after IVIG, and are at risk of developing CAA. Primary treatment with a combination of IVIG plus prednisolone (PSL) recently showed a significant advantage over IVIG alone for the prevention of CAA, reducing the need for additional rescue treatments. Platelets are involved in hemostasis, wound healing, and inflammation. Platelet activation at the site of inflamed endothelium contributes to vascular inflammation and vascular wall remodeling. Released chemokine from activated platelets, such as platelet factor 4 (PF4), CXCL7 and β-thromboglobulin (β-TG), have important effects on vascular inflammation. Vascular injury may lead to increased platelet activation with neutrophil and monocyte infiltration, as well as increased platelet adhesion and aggregation via the release of inflammatory cytokines with mutually facilitated. Circulating platelet-neutrophil aggregates amplify acute inflammation and exhibit a hyper-reactive response that could promote the development of thrombotic and inflammatory disease, obstruct the flow of coronary microvessels, and contribute to vascular inflammation and tissue injury. Likewise, activated platelets and neutrophils have been demonstrated during the acute phase of KD-associated inflammation and may be contribute to the occurrence of CAA. Therapeutic inhibition of platelet-neutrophil aggregates reduces neutrophil recruitment and permeability and may help to attenuate organ damage and mitigate the inflammatory process. Prednisolone can cause the inhibition of platelet adhesion, spreading, aggregation, thrombus formation and the interaction of platelets with monocytes through regula...
tion of P2Y12 receptor signaling, which is the main platelet receptor responsible for ADP induced platelet activation. Thus, PSL has a role in helping to control vascular and thrombotic diseases.3-5 The use of PSL as part of a combination treatment may have a beneficial effect on terminating the inflammatory process by its anti-inflammatory property, which suppresses immune cell activation, proliferation, and cytokine production, as well as by its ability to decrease endothelial expression of cellular adhesion molecules in the acute phase of KD. However, previous studies have suggested that the use of corticosteroids for patients with KD should be limited because such treatment may be linked to a higher incidence of CAA and impaired vascular remodeling.22,23 The main benefit of corticosteroid combination treatment is considered to be early suppression of the vasculitis that precedes vascular remodeling.3 In this study, we evaluated the physiological activity of platelet-neutrophil aggregates and platelet function in patients with KD.

**Methods**

**Patients**

This study was reviewed and approved by the Kagoshima University Ethics Committee in 2010. Patients with KD, disease controls, and normal volunteers were analyzed after written informed consent had been obtained from their parents. The clinical records of consecutive patients with definitive KD and disease controls who were referred and then admitted to Kagoshima Medical Association Hospital and Kagoshima City Hospital from April 2010 to June 2014 were included in this study. KD was defined using the Japanese criteria.24 The first day of illness was defined as the first day of fever. When the medical team considered the probability of KD to be high, treatment was initiated, even if the patient had not fully met the criteria for KD. All patients with KD were treated with a single infusion of IVIG at 2 g/kg. The patients also received aspirin (30 mg/kg), the dose of which was decreased to 3–5 mg/kg per day after they were afebrile for at least 28 days to 2–3 months until the patients showed no evidence of coronary changes after fever onset. Patients who had resolution of fever (<37.5°C) within 24 h of finishing the initial IVIG treatment were considered to be IVIG responders. Patients who had persistent fever beyond 24 h or recrudescent fever associated with KD symptoms after an afebrile period were defined IVIG non-responders and were given additional IVIG treatments. Patients who fulfilled 5 points or higher predictive value of the risk score in the prediction of no response to initial IVIG were treated with IVIG plus PSL at 2 mg/kg per day in 3 divided doses; this dose was halved every 5 days.5 Scoring and cut-off values of the risk score were as follows: 2 points each for a serum sodium concentration of 133 mmol/L or less, 4 days or fewer of illness at diagnosis, aspartate aminotransferase concentration of 100 U/L or more, white blood cells representing neutrophils of 80% or greater; and 1 point each for a platelet count of 300 ×10^9/L or less, C-reactive protein concentration of 10 mg/dL or more, and age 12 months or younger.26 Patients who did not receive IVIG were excluded from this study.

Disease controls comprised febrile patients with the following bacterial infections: bacterial bronchitis, pneumonia, urinary tract infection, or bacteremia. Normal volunteers comprised children of the same age who visited our hospital for heart examination and did not exhibit any abnormalities in medical examination and preceding laboratory tests.

**Echocardiography**

Two-dimensional echocardiography was performed to evaluate cardiac function and the presence of CAA. Examination was performed before and after IVIG treatment at 2- to 3-day intervals during hospitalization and once a week at outpatient clinics until the end of the first month of illness. All coronary artery diameters (in mm) in patients with KD were transformed to CAA z-scores using the Japanese normal values of the coronary artery dimensions, as previously described.27 The maximum CAA z-score among the right, left main, and left anterior descending arteries during the first month of illness was used to evaluate patients for the presence of CAA. A maximum z-score of >2.5 was defined as the criterion for CAA.

**Blood Sampling**

Whole blood samples were collected into 3.2% sodium citrate tubes before the first IVIG administration and the following day after completion of the initial IVIG administration (median, 2 days after IVIG) in patients with KD, and before antibiotic treatment for patients with bacterial infections. The platelet-neutrophil aggregates were evaluated within 4 h after the blood had been drawn. The expression of integrin CD11b, a cell-surface adhesion molecule on peripheral neutrophils, and CD41, a cell-surface antigen of human platelet membranous glycoprotein IIb/IIIa, were measured by flow cytometry (Beckman Coulter, Fullerton, CA, USA). Briefly, after a 30-min incubation of 50 μL of sodium citrate-anticoagulated whole blood with 10 μL of mouse antihuman CD11b-R-phycocerythin (CD11b-PE) antibody and 10 μL of mouse antihuman CD41-fluorescein isothiocyanate (CD41-FITC) antibody, 250 μL of lysis buffer (OptiLyse C; Beckman Coulter, Fullerton, CA, USA) was added. The mixture was incubated for 15 min. A total of 250 μL of phosphate-buffered saline was added, and this was followed by another 15-min incubation. Neutrophils were gated based on forward and side scatter profiles, which represent size and granularity, respectively. Cells were analyzed for CD11b and CD41 expression using an EPICS XL flow cytometer (Beckman Coulter, Miami, FL, USA). All incubations were performed at room temperature in the dark.

Blood samples were also collected into tubes containing citrate, theophylline, adenosine, and dipyridamole. Analyses of PF4 and β-TG were conducted using platelet-poor plasma centrifuged at 2,000 g for 30 min at 4°C. Platelet-poor plasma was immediately frozen at −80°C and measured in duplicate using enzyme-linked immunosorbent assay. The clinical protocols for the measurement of PF4 and β-TG levels were approved by the institutional review board of Kagoshima University Hospital.

**Statistical Analysis**

Continuous variables are summarized by median values and interquartile ranges (IQR; 25th, 75th percentile). Categorical variables are presented as frequencies and percentages. Baseline comparisons between patients were performed by using a Mann-Whitney U test or a Fisher’s exact test when appropriate. Simple linear regression analysis was used to evaluate correlations between parameters. Correlations between platelet-neutrophil aggregates and PF4 or β-TG levels were analyzed by using correlation coefficients. All statistical analyses were performed by using the SPSS statistical software package, version 17.0 J (SPSS Japan Inc, Tokyo, Japan). Two-tailed values of P values <0.05 were considered statistically significant.
Platelet-Neutrophil Aggregates in Kawasaki Disease

2 patients were treated with IVIG treatment twice and had transient CAA that resolved during the first month after the onset of KD symptoms. The remaining patient was treated with IVIG plus PSL at first, but then subsequently needed additional IVIG treatment and had CAA that resolved spontaneously within 6 months after the onset of KD symptoms. Seven patients with KD who fulfilled the risk score criterion were treated with IVIG plus PSL.

No significant differences were observed in sex or laboratory data, with the exception of the sodium levels. The baseline white blood cell count and C-reactive protein level were not significantly different.

Results

Comparison of Clinical Characteristics and Laboratory Findings Between Patients With KD and Patients With Bacterial Infection

During the study period, we evaluated 40 patients with KD (median, 1.75 years, IQR 0.94–2.30), 7 febrile patients with bacterial infections (median 1.82, IQR 0.86–2.22), and 9 normal volunteers (median, 2.1 years, IQR 1.0–3.2). The clinical characteristics and laboratory findings of the KD group and bacterial infection group are shown in Table 1. Of the 40 patients with KD, 30 were defined as IVIG responders and the remaining 10 were defined as IVIG non-responders. CAA were found in 3 IVIG non-responder patients with KD (7.5%). Of those, 2 patients were treated with IVIG treatment twice and had transient CAA that resolved during the first month after the onset of KD symptoms. The remaining patient was treated with IVIG plus PSL at first, but then subsequently needed additional IVIG treatment and had CAA that resolved spontaneously within 6 months after the onset of KD symptoms. Seven patients with KD who fulfilled the risk score criterion were treated with IVIG plus PSL. No significant differences were observed in sex or laboratory data, with the exception of the sodium levels. The baseline white blood cell count and C-reactive protein level were not significantly different.

Data are expressed as median values and interquartile range (25th, 75th percentile). CAA, coronary artery abnormalities; IVIG, intravenous immunoglobulin; Responder, IVIG responder; Non-responder, IVIG non-responder.
Comparison of Clinical Features and Laboratory Findings Between IVIG Responders and Non-Responders

The clinical characteristics and laboratory findings of the IVIG responders and non-responders are shown in Table 2. No significant differences were observed for age, sex, or day of illness at IVIG initiation between the 2 groups. However, the duration of fever and maximum CAA z-score were significantly different between the groups.

Increased Rates of Platelet-Neutrophil Aggregates in Patients With KD

In neutrophil gating, 99% of the cells were positive for the neutrophil marker, CD 11b-PE (Figure 1A). Platelet-neutrophil complexes within neutrophils (upper right quadrant) were identified by the binding of CD11b and the platelet-specific antibody, CD41-FITC, in normal volunteers (Figure 1B). Time-course evaluation of the rates of platelet-neutrophil aggregates in patients with KD showed a trend toward higher rates within 2 or 3 days after IVIG administration than before IVIG administration, with a subsequent decrease in the convalescent stage (Figure 1C). The rates of platelet-neutrophil aggregates were significantly higher in patients with KD than in both patients with bacterial infection and normal volunteers (Figure 1B). Compared with the rate of platelet-neutrophil aggregates before IVIG treatment between IVIG responders and IVIG non-responders, the rate of platelet-neutrophil aggregates were higher in the non-responders than in the responders, but these differences were not statistically significant (P=0.150).

Relationship Between Rates of Platelet-Neutrophil Aggregates and CAA

We also examined the rates of platelet-neutrophil aggregates before and after IVIG treatment and the rate of CAA. The rate of platelet-neutrophil aggregates before IVIG treatment was significantly higher in patients with CAA than in those without CAA (before IVIG, P=0.011) (Figure 3A). No relationship was observed between the rate of platelet-neutrophil aggregates and CAA after IVIG treatment (P=0.11).
Platelet-Neutrophil Aggregates in Kawasaki Disease

**Relationship Between Rates of Platelet-Neutrophil Aggregates and PF4 and β-TG Levels**

The PF4 and β-TG levels were obtained from 25 patients with KD before IVIG treatment. The rates of platelet-neutrophil aggregates in patients with KD were moderately correlated with the PF4 and β-TG levels (r=0.636, P<0.01 and r=0.448, P=0.025, respectively) (Figure 3B).

**Prednisolone Decreased Rates of Platelet-Neutrophil Aggregates in Whole Blood of Patients With KD**

We compared the time-course of the rates of platelet-neutrophil aggregates between IVIG treatment alone (12 IVIG responders, 3 IVIG non-responders) and IVIG plus PSL treatment to evaluate the effects of PSL as a part of primary combination treatment on the rate of platelet-neutrophil aggregates during the acute phase of KD. A comparison of clinical characteristics and laboratory findings between the patients who have elevated platelet-neutrophil aggregate after IVIG treatment and the patients who have decreased platelet-neutrophil aggregates after IVIG treatment in IVIG treatment alone group, found that the duration of fever was longer, and the C-reactive protein levels and the maximum CAA z-score was higher in the former than in the latter, but these differences were not statistically significant (P=0.204, P=0.371, P=0.269). The rates of

![Figure 2. Comparison of rates of platelet-neutrophil aggregates in patients with Kawasaki disease (KD), patients with bacterial infection, and normal volunteers. Platelet-neutrophil aggregates were significantly higher in patients with KD than in patients with bacterial infection and normal volunteers. **Significant difference between groups (P<0.01).](image)

![Figure 3. (A) Relationships between platelet-neutrophil aggregates and coronary artery abnormalities (CAA). The rates of platelet-neutrophil aggregates were significantly higher in patients who showed CAA than in those who did not show CAA (median 64.0, IQR 58.6–64.2 vs. median 38.9, IQR 27.8–47.4, respectively; P=0.011). *Significant difference between groups (P<0.05). (B) Relationship between rates of platelet-neutrophil aggregates and platelet factor 4 (PF4) and β-thromboglobulin (β-TG) levels. The rates of platelet-neutrophil aggregates in patients with Kawasaki disease were moderately correlated with the PF4 and β-TG levels (r=0.636, P<0.01 and r=0.448, P=0.025, respectively). IVIG, intravenous immunoglobulin.)](image)
Platelet-neutrophil aggregates were significantly decreased in patients treated with IVIG plus PSL than in patients treated with IVIG alone (P=0.003 and 0.13, respectively) (Figure 4).

**Discussion**

In the present study, we demonstrated that the rates of platelet-neutrophil aggregates were significantly higher in patients with KD than in both patients with bacterial infection and normal volunteers. The rates of platelet-neutrophil aggregates before IVIG treatment were significantly higher in patients who showed CAA than in those who did not show CAA, and were correlated with the levels of PF4 and β-TG, which are released from activated platelets. Furthermore, PSL suppressed the rates of platelet-neutrophil aggregates in whole blood. Our findings indicate that platelet-neutrophil aggregates interact as a result of the vascular inflammatory process and might play a significant role in the severity of vasculitis in patients with KD.

Platelets have been shown to play a major role in a number of inflammatory diseases including rheumatoid arthritis, atherosclerosis, and sepsis. Enhanced platelet aggregation and activated platelet function have been noted during the acute phase of KD inflammation. In this study, we demonstrated that the rates of platelet-neutrophil aggregates were significantly higher in patients with KD than in patients with bacterial infection and normal volunteers. Approximately 10–20% of neutrophils circulate as platelet-neutrophil aggregates. Platelet-neutrophil aggregates can be mediated via P-selectin on activated platelets; P-selectin, in turn, recognized by PSGL-1 on leukocytes, which is the primary ligand for P-selectin and is constitutively expressed on the surface of circulating leukocytes. Platelet/neutrophil crosstalk are essential arms of the innate immune response, but may affect the development of thrombotic and inflammatory diseases. Platelets not only play a role in hemostasis, but also play an important role as amplifiers in acute inflammation via the release of substances with physiologic potential and by interaction with leukocytes and vascular endothelial cells involved in modulation of the inflammatory reaction. The interaction of platelets with neutrophils promotes the recruitment of neutrophils into inflammatory tissue; this is mainly mediated through P-selectin and β2 and β3 integrins and adherent platelets, which secrete neutrophil and endothelial cell activators to induce the production of inflammatory cytokines. Platelets are important amplifiers of acute inflammation. Platelets and neutrophils have the potential to promote an inflammatory process and together platelet-neutrophil aggregates can induce a faster and harder response. Our study indicates that vascular inflammation during the acute phase of KD is accompanied by platelet/neutrophil crosstalk reactivity and is distinct from bacterial infectious disease. In the early phase of KD, the possibility of collisions between platelets and neutrophils is promoted by the rheologic marginalization of neutrophils exiting the central core of the blood vessel. With further activation by inflammatory stimuli, platelet-neutrophil interactions are extensively formed, resulting in massive neutrophil migration and accumulation in vascular endothelial cells to cause tissue injury. Platelet-neutrophil aggregates are likely to be related to pathological developments associated with systemic vasculitis in conditions such as KD.

The rates of platelet-neutrophil aggregates were significantly higher in patients who showed CAA than in those who did not show CAA, and were correlated with the levels of PF4 and β-TG; these parameters could be used as indices of platelet activation and membrane biocompatibility. During the platelet release reaction, PF4 and β-TG are released from the α-granule and can be used to monitor in vivo platelet activation. PF4 is rapidly taken up by endothelial cells, through a reversible binding of PF4 to heparin sulfates at the endothelial surface, and may be important in directing neutrophils to the endothelium prior to emigration. In the early stage of CAA development, an influx of neutrophils occurs in an affected coronary artery followed by a rapid transition to an influx of large mononuclear cells, lymphocytes, and plasma cells. Destruction of the internal elastic lamina occurs, followed by myofibroblast proliferation, which leads to the formation of a coronary aneurysm. In autopsied patients with KD complicated with CAA, multiple layers are present within the markedly thickened intima, which contains linearly arranged microvessel layers rich in smooth muscle cells and fibrous layers. Considering that platelets play a role as amplifiers of acute inflammation, reciprocal activation of platelets and neutrophils is a key factor in the severity of vasculitis. In adults with coronary artery disease, a large quantity of platelet-neutrophil aggregates is an important component of pro-inflammatory activity in the systemic inflammatory state. Platelet-neutrophil aggregates exhibit a hyper-reactive response that could increase the risk of thrombotic events, obstruct the flow of coronary microvessels, and contribute to vascular inflammation and tissue injury. Platelet-mediated neutrophil recruitment promotes the development of intimal hyperplasia at the site of vascular injury, and platelet-neutrophil aggregates contribute to clot formation via production of procoagulant tissue factor, which interacts with monocytes and neutrophils. Hence, platelet-neutrophil aggregates are among the essential components of various inflammatory diseases.
aggregates in patients with KD complicated with CAA have a greater potential for activation than those in patients without CAA and may contribute to thrombosis and prolonged inflammation, increasing the severity of coronary outcomes. In contrast, no significant differences in the rates of platelet-neutrophil aggregates were seen between the IVIG responders and non-responders before IVIG treatment. IVIG unresponsiveness may be influenced not only by the severity of vasculitis, but also by multiple factors such as the patient’s genetic background or immunological abnormalities. 40

In this study, we found that the rates of platelet-neutrophil aggregates were significantly decreased in patients treated with IVIG plus PSL than in those treated with IVIG alone. Platelet-neutrophil aggregates promote their respective activations, formation of thromboxane A2 (TXA2) and other inflammatory mediators; these lead to a broad range of cellular responses including integrin activation, neutrophil recruitment, platelet aggregation and increased vascular permeability, and can be involved in vascular injury. 39 Inhibition of platelet-neutrophil aggregates improved gas exchange, reduced neutrophil recruitment and permeability, and prolonged survival in acid-induced acute lung injury model mice. 41 PSL modulates platelet aggregation through mechanisms involving engagement of glucocorticoid receptor α on blood platelets and platelet-monocyte interactions via regulation of platelets. In addition, PSL inhibits ADP-induced platelet aggregation, platelet adhesion, and thrombus formation. 41,42 Furthermore, IVIG plus PSL as a primary treatment for KD rapidly reduces circulating inflammatory cytokines. 43 Considering the biological property of platelet and/or neutrophil interaction, primary treatment involving a combination of IVIG and PSL has the potential to suppress vasculitis in the early phase via reducing platelet-neutrophil aggregates, and helping to prevent vascular remodeling and controlling unwanted vascular thrombotic disease. Immediate treatment of the inflammation associated with vasculitis is important before pathological changes become irreversible in patients with KD, because a delay in the start of PSL treatment could contribute to critical vascular complications. Further studies are required to more fully evaluate the combination of IVIG plus PSL as a primary treatment for KD.

In conclusion, platelet-neutrophil aggregates are present in significantly higher rates and are closely related to pathological developments of CAA in patients with KD. Additional PSL treatment in the acute phase of KD could suppress platelet-neutrophil aggregates, indicating that platelet-neutrophil aggregates would inhibit amplified reciprocal vascular inflammatory activation.

Acknowledgments
We appreciate our patients and their parents for their commitment to this project. This study was supported in part by the Japan Kawasaki Disease Research Center.

Disclosures
Statements: Kentaro Ueno wrote the first draft of the manuscript. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

Conflicts of Interest: All authors have no conflicts of interest to disclose.

References

Platelet-Neutrophil Aggregates in Kawasaki Disease


