Overproduction of Platelet Microparticles in Cyanotic Congenital Heart Disease With Polycythemia

Hitoshi Horigome, MD,* Yuji Hiramatsu, MD,† Osamu Shigeta, MD,† Toshiro Nagasawa, MD,‡ Akira Matsui, MD*

Tsukuba, Japan

OBJECTIVES
We sought to clarify the role of platelets in the pathogenesis of abnormal coagulation in patients with cyanotic congenital heart disease (CCHD) with polycythemia; we evaluated the production of platelet microparticles (MPs), platelet degranulation and aggregation response, as well as the correlations of these variables with polycythemia.

BACKGROUND
A shortened life span and suppressed aggregability of platelets are well known in patients with CCHD. Although platelet MPs are overproduced and play an important role in the coagulation process in various hematologic and cardiovascular disorders, the production of MPs remains to be elucidated in CCHD.

METHODS
We studied 19 patients (age 4.9 to 32.8 years, median 16.1) with CCHD complicated by cyanotic congenital heart disease (CCHD). Flow cytometry, using monoclonal antibodies, showed the presence of MPs as particles positive for the surface antigen (glycoprotein IIb/IIIa) specific to platelets, and platelet alpha-degranulation was recognized as platelets positive for the surface antigen of P-selectin. Platelet aggregation was assessed as the response to adenosine diphosphate (ADP). Relationships between these indexes and hematocrit (Hct) values were also evaluated.

RESULTS
Production of MPs correlated positively with Hct and markedly increased at Hct values above 60% in patients with CCHD. Surface P-selectin and the mean platelet volume in patients with CCHD were comparable with those in patients with ACHD. The platelet aggregation response to ADP significantly and negatively correlated with Hct. In two subjects who showed hemoptysis and underwent phlebotomy, MPs were reduced 6 h after the procedure. Platelet MPs are overproduced in patients who have CCHD with polycythemia, probably due to a high shear stress derived from blood hyperviscosity. Circulating incompetent platelets, which have already been activated, as well as MPs, might play an important role in the coagulation abnormalities identified in such patients. (J Am Coll Cardiol 2002;39:1072–7)

CONCLUSIONS
Platelet MPs are overproduced in patients who have CCHD with polycythemia; we evaluated the production, platelet degranulation and platelet aggregation, using a flow cytometric technique, we investigated MP croparticles might play an important role in abnormalities of the clotting system in CCHD. In the present study, by using a flow cytometric technique, we investigated MP production, platelet degranulation and platelet aggregation, and their relation to polycythemia in patients with CCHD and Eisenmenger syndrome.

It has been recognized for decades that patients with cyanotic congenital heart disease (CCHD) show a significant bleeding tendency (1). Adult and adolescent patients unsuitable for radical surgery for heart defects may develop serious polycythemia and require treatment with hematologic products (2). This hemorrhagic diathesis is attributed to various hemostatic defects, including thrombocytopenia (3,4), shortened platelet life-time (5,6), suppressed platelet aggregation (3), deficiency of clotting factors, such as von Willebrand factor (4,7), and increased vascularity associated with nitric oxide or prostacyclin released from the vascular endothelium, probably triggered by high shear stress due to hyperviscosity (8,9). Although patients with CCHD may also be considered susceptible to thrombosis and organ infarction (10) due to hyperviscosity, these complications are limited to those with relative iron deficiency (11). As to platelet function, we are aware of only a few reports demonstrating augmented platelet activation in patients with CCHD (12,13). Moreover, the mechanisms by which thrombocytopenia and platelet dysfunction occur have not been fully elucidated.

Recently, platelet-derived microparticles (MPs) were identified as a sensitive variable of platelet activation. These MPs are formed from the platelet surface membrane by an exocytic shedding process (14), by agonists such as thrombin and collagen (15), and have an average diameter of 0.1 μm (16). Increased production of MPs has been reported in various conditions or modalities, such as disseminated intravascular coagulation (17), myocardial infarction (18), coronary angiography (18), unstable angina (19), transient ischemic attack (20) and diabetes mellitus (21). High or prolonged shear stress of the platelet surface, such as in cardiopulmonary bypass (22) and arterial stenosis (23,24), is another important stimulus for MP production. Shear stress increases in proportion to increases in blood viscosity. However, production of MPs in this peculiar condition of CCHD with polycythemia has not been investigated. Microparticles might play an important role in abnormalities of the clotting system in CCHD. In the present study, by using a flow cytometric technique, we investigated MP production, platelet degranulation and platelet aggregation, and their relation to polycythemia in patients with CCHD and Eisenmenger syndrome.

METHODS
Patients and blood sampling. We studied 19 patients (age 4.9 to 32.8 years, median 16.1) with CCHD complicated by
polycythemia (hematocrit [Hct] >45%, range 46.2% to 72.9%, mean [±SD] 57.9 ± 8.3%). None of the patients had undergone a radical operation for a heart defect due to hypoplastic pulmonary arteries or high pulmonary vascular resistance for the Fontan circulation (n = 13) or due to Eisenmenger syndrome (n = 6; 4 of them associated with Down syndrome, comprising the CCHD group). None of these patients had received antiplatelet drugs within two weeks before blood sampling. Twenty-one age-matched subjects with acyanotic congenital heart disease (ACHD) without polycythemia were also included in the present study (ACHD group). The Hct value of the ACHD group ranged from 29.0% to 41.4% (mean 36.2 ± 4.0%). We chose patients with ACHD, instead of healthy subjects, to compare with patients with CCHD, because these patients frequently have stenotic valvaral or vascular lesions, which could potentially activate platelets or produce MPs in association with turbulent flow. The characteristics of both groups are presented in Table 1.

The study protocol was approved by the Human Ethics Review Committee of Tsukuba University Hospital, and a signed consent form was obtained from each patient or from the parent(s) when the patient was under 20 years of age. Blood samples were collected from the antecubital vein using a tightly fitting tourniquet. The first sample was drawn into a syringe containing ethylenediamine tetra-acetic acid for measurement of the whole blood cell count and into a syringe containing sodium citrate for measurement of d-dimer, which reflects fibrinolysis of fibrin, but not fibrinogen. The second sample was gently introduced into a syringe containing 1/10 volume of acid-citrate-dextrose solution (6.8 mmol/l citric acid, 11.2 mmol/l trisodium citrate and 24 mmol/l glucose), to avoid in vitro platelet activation, and used for flow cytometry. Then, blood samples for platelet aggregation were obtained on 3.8% solution of sodium citrate. Hematocrit, mean corpuscular volume (MCV), platelet count and mean platelet volume (MPV) were measured by Cell-Dyn (Model 4000, Dainabot Inc., Tokyo, Japan).

### Measurement of platelet aggregation to adenosine 5'-diphosphate (ADP)
Platelet aggregation to ADP was measured using the NBS aggregometer (Model PAC-4S, NBS Hema Tracer, Tokyo, Japan). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared from the citrated samples by differential centrifugation at 150 g for 10 min and 15,000 g for 5 min, respectively. Before the studies of aggregation, the platelet count of PRP was adjusted to 150,000/μl by dilution with PPP. Platelet aggregation was assessed with the use of 10 μg ADP and expressed as percent light transmittance.

### Flow cytometric analysis of CD62 and CD41b expression
Platelet surface antigen was stained with fluorescein isothiocyanate-labeled or phycoerythrin-labeled monoclonal antibody (mAb) and analyzed by flow cytometry (FACsort, Becton Dickinson, San Jose, California). The mAbs used in the present study were CD62 (Becton Dickinson) and CD41b (TP80, Nichirei, Tokyo, Japan). Twenty microliters of each fixed PRP was incubated with 5 μl of 5-times diluted mAb in the dark at room temperature, to allow for antibody binding. CD62 recognizes expression of the platelet surface antigen, P-selectin, which reflects platelet alpha-granule secretion. Microparticles were identified by gating on CD41b-positive events, which reflect glycoprotein IIb/IIIa, and differentiated from normal-sized platelets by forward scatter size analysis. Ten thousand positive platelet events were analyzed, and MPs were reported as a percentage of the total platelet events. Details of the method have been described in our previous report (25).

### Phlebotomy
Phlebotomy was performed in two patients (17 and 22 years old) in the CCHD group who showed repeated hemoptysis. Their Hct values were 69.9% and 72.8%, respectively. The phlebotomized blood volume was 300 and 330 ml, respectively, and the same volume of saline solution was replaced in each patient. The platelet count and MP values were measured before and 6 h, 24 h, 5 days and 3 weeks after phlebotomy.

### Statistical analysis
Data are expressed as the mean value ± SD. Differences in Hct, platelet count, ADP aggregation of platelets, MPV, d-dimer, MPs and P-selectin expression between the two groups were compared by using the Student unpaired t test. Correlations between the Hct value and values of MPs, P-selectin, platelet aggregation to

### Abbreviations and Acronyms
- ACHD = acyanotic congenital heart disease
- ADP = adenosine 5'-diphosphate
- CCHD = cyanotic congenital heart disease
- Hct = hematocrit
- mAb = monoclonal antibody
- MCV = mean corpuscular volume
- MP = microparticle
- MPV = mean platelet volume
- PPP = platelet-poor plasma
- PRP = platelet-rich plasma

### Table 1. Laboratory Data in Patients With Cyanotic and Acyanotic Congenital Heart Disease

<table>
<thead>
<tr>
<th></th>
<th>CCHD Group (n = 19)</th>
<th>ACHD Group (n = 21)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>15.8 ± 7.98</td>
<td>14.8 ± 8.22</td>
<td>*</td>
</tr>
<tr>
<td>Range</td>
<td>4.9–32.8</td>
<td>5.2–28.8</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>19.1 ± 2.37</td>
<td>12.9 ± 1.35</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>57.9 ± 8.26</td>
<td>36.2 ± 3.99</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MCV (μm³)</td>
<td>94.6 ± 9.04</td>
<td>86.5 ± 4.85</td>
<td>0.005</td>
</tr>
<tr>
<td>D-Dimer (ng/ml)</td>
<td>74.0 ± 44.8</td>
<td>60.6 ± 23.5</td>
<td>0.325</td>
</tr>
<tr>
<td>Platelet count (×10⁹/μl)</td>
<td>204 ± 59.0</td>
<td>271 ± 63.8</td>
<td>0.002</td>
</tr>
<tr>
<td>MPV (μm³)</td>
<td>8.5 ± 0.89</td>
<td>8.2 ± 0.67</td>
<td>0.287</td>
</tr>
<tr>
<td>MPs (%)</td>
<td>9.5 ± 9.0</td>
<td>2.6 ± 1.2</td>
<td>0.001</td>
</tr>
<tr>
<td>P-selectin (%)</td>
<td>6.9 ± 2.3</td>
<td>9.6 ± 5.7</td>
<td>0.217</td>
</tr>
<tr>
<td>ADP aggregation (%)</td>
<td>31 ± 18</td>
<td>67 ± 12</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data are expressed as the mean value ± SD.
*Matching variables.  
ACHD = acyanotic congenital heart disease; ADP = adenosine 5'-diphosphate;  
CCHD = cyanotic congenital heart disease; MCV = mean corpuscular volume;  
MPs = microparticles; MPV = mean platelet volume.
ADP, platelet count and MPV were analyzed by simple regression analysis. A p value <0.05 was considered significant.

RESULTS

The mean values of Hct and MCV in the CCHD group were higher than those in the ACHD group. The platelet count and ADP-induced platelet aggregation in the CCHD group were significantly lower than those in the ACHD group, but the values of MPV and D-dimer were not different between the two groups (Table 1). Both platelet count and platelet aggregation correlated negatively with the Hct value when the entire data of both groups were included in the analysis (Figs. 1 and 2). This significant correlation was observed in the CCHD group, as well. Flow cytometric evaluation revealed a significant increase in the production of platelet MPs in the CCHD group, which correlated with the Hct value (Figs. 3 and 4). In particular, MP formation increased markedly when Hct was >60%. Microparticles also tended to increase inversely with the platelet count, but the correlation between the two variables was not significant. In contrast, the proportion of P-selectin–positive platelets in the CCHD group was comparable to that in the ACHD group and did not show any correlation to the Hct value.

Figure 5 shows the effects of phlebotomy on the platelet count and MPs in two patients. The Hct values were reduced from 69.9% (before phlebotomy) to 65.9% (6 h after phlebotomy) in one patient, and from 72.8% to 69.1% in the other. Microparticles were reduced and the platelet count was increased at 6 h after the procedure, and these effects were maintained over several days.

DISCUSSION

Platelet MPs in CCHD. Thrombocytopenia and suppressed platelet aggregation are known factors underlying the bleeding tendency in patients with CCHD and Eisenmenger syndrome (3,7,26). The platelet count correlates negatively with the Hct value (7). In the present study, our results showed a significant correlation between the platelet MP count and Hct value. In particular, MP production increased markedly at Hct levels above 60% to 65%. This finding is consistent with the conventional criterion that sets the Hct level over 65%, at which blood hyperviscosity reaches a critical level and phlebotomy is indicated (3,7), although the Hct level alone is not, by itself, an indication for the procedure. Increased production of MPs is probably associated with activation of platelets induced by high shear stress (23,24,27) generated by blood hyperviscosity. It has been demonstrated that, in adult patients with coronary artery disease, vascular stenosis generates turbulent flow and high shear stress of the platelet surface, resulting in overproduction of MPs (23). Formation of MPs is increased with prolongation of the shear stress (24). Further, we demonstrated that MPs decreased after phlebotomy in association with a reduction in Hct, although this was studied in only two patients who underwent phlebotomy,
because this procedure is only recommended for patients with unstable or progressive polycythemia.

**Platelet activation and P-selectin.** There are a few reports supporting platelet activation in cyanotic heart disease. Adatia et al. (12) showed changes in the ratio of biosynthesized prostacyclin to that of thromboxane A2 in children with CCHD, which was speculated to contribute to thrombus formation. Olgun et al. (13) studied platelet activation by flow cytometry using mAb against P-selectin, similar to our study. They reported that platelets positive for surface P-selectin, indicating platelet degranulation, were increased in patients with congenital heart disease, especially CCHD, as compared with healthy children. In contrast to their results, P-selectin–positive events were not different between the CCHD and ACHD groups in the present study, consistent with the results of Levin et al. (28). Furthermore, there was no significant correlation between P-selectin–positive events and the Hct value. It is possible that this difference is partly due to the method used for blood sampling when two different studies are compared with each other. Even if the investigators collected blood samples as gently as possible, the blood extraction maneuver itself might have activated platelets in patients with polycythemia. However, we speculate that it is not easy to recognize platelet activation by the flow cytometric technique for alpha-granule secretion (anti–P-selectin), because the process is ongoing in this specialized condition of slowly progressing or stable polycythemia. The index alpha-degranulation has been demonstrated to reach its peak value in 10 min after activation (29). Furthermore, Michelson et al. (30) reported that circulating degranulated platelets rapidly lose surface P-selectin to the plasma pool, but continue to circulate. These findings endorse our results that circulating P-selectin–positive platelets are not significantly increased in patients with CCHD. Platelets that continue to circulate after degranulation, a kind of “incompetent platelets” after activation, might account for the suppressed platelet aggregation response in CCHD. Furthermore, MPV values in the CCHD group were comparable with those in the ACHD group. This means that the cause of suppressed platelet aggregation does not originate from the process of platelet production (fragmentation of megakaryocytes). Even in patients with CCHD, it is probable that normal-sized, normally functioning platelets are first generated and introduced into the circulation. In other words, circulation of “incompetent platelets” after activation by high shear stress might be interpreted as a physiologic adjustment, so that formation of massive thrombi is precluded.
Platelet MPs as procoagulant. Although the exact function of MPs and their life span have not been clarified, there seems to be a consensus that MPs are an index of platelet activation (23,24,27). Compared with P-selectin on the platelet surface, MPs may remain in the blood for a longer period after platelet activation; thus, MPs could be a useful index of platelet activation in chronic processes. In contrast, recent studies support the concept that MPs also play an important role in the normal coagulation process. Miyazaki et al. (27) reported a close relationship between platelet procoagulant activity and the shedding of procoagulant-containing MPs by platelets. Indeed, MPs were demonstrated to express factor Va and Xa activities and exhibit procoagulant surface properties (15,31). Furthermore, platelet MPs have been suggested to bind to subendothelial materials and act as a substrate for further platelet binding, thus promoting platelet adhesion (32,33). Clinically, Holme et al. (23) reported that local generation of MPs in atherosclerotic small arteries or arterioles may promote acute arterial occlusion through procoagulant activity. Wenche et al. (34) showed significantly increased MPs in patients with idiopathic thrombocytopenic purpura complicated by stroke. The d-dimer values, which reflect fibrinolysis of fibrin, were not different between the two groups in the present study. It is possible that this result is influenced by the chronic process of thrombus formation in polycythemia. Although there were no clinically apparent thrombotic episodes in our patients who had CCHD with a high level of MPs, we should be aware of possible procoagulant activities of MPs and a predisposition to thromboembolism in the presence of a bleeding tendency in CCHD.

Conclusions. We have demonstrated in the present study that platelet MPs are produced abundantly at Hct levels over 60% to 65%. Overproduction of MPs and suppression of platelet aggregation, both related to hyperviscosity, may play an important role in the pathogenesis of hemostatic defects and coagulation abnormalities in patients with CCHD.

Acknowledgment
The authors are grateful to Dr. Hiroaki Nishikawa, University of Tsukuba, for expert statistical assistance.

Reprints requests and correspondence: Dr. Hitoshi Horigome, Department of Pediatrics, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan. E-mail: hhorigomi@md.tsukuba.ac.jp.

REFERENCES
both platelet aggregation and shedding of procoagulant containing microparticles. Blood 1996;88:3456–64.