Increased Thrombogenesity in Patients With Cyanotic Congenital Heart Disease

Hidemi Kajimoto, MD; Makoto Nakazawa, MD; Kagari Murasaki, MD*; Yoshiki Mori, MD; Kenjiro Tanoue, MD*; Hiroshi Kasanuki, MD*; Toshio Nakanishi, MD

Background  The basic mechanisms of thromboembolism in cyanotic congenital heart disease (CCHD) have not been well clarified. P-selectin on the platelets reflects platelet activation. Thrombomodulin is a critical cofactor for thrombin-mediated activation of protein C and reflects the anticoagulant activity of the endothelium. The present study was performed to evaluate whether platelet activation exists in patients with CCHD.

Methods and Results  Platelet P-selectin as a marker of platelet activation, plasma thrombomodulin level and protein C activity as markers of anticoagulant activity of the endothelium and thrombin–antithrombin complex III (TAT) were examined in 35 patients with CCHD. Plasma thrombomodulin level (1.1±0.9 vs 2.2±0.3 FU/ml) and protein C activity (71.1±29.8 vs 117.8±24.8%) were significantly lower in patients with CCHD as compared with the control subjects. The levels of plasma TAT (255±811 vs 1.9±0.9 ng/ml) and P-selectin on platelets (6.3±4.5 vs 3.3±0.3 mean fluorescence intensity) were significantly higher in the patients with CCHD than in the controls. Four of the CCHD patients who experienced thromboembolic events had elevated levels of platelet P-selectin (p=0.02) compared with CCHD patients without thromboembolic events.

Conclusion  Platelet activation exists in patients with CCHD and it may play an important role in the thromboembolic events in CCHD. (Circ J 2007; 71: 948 – 953)

Key Words:  Endothelium; Hypoxia; Thromboembolism

Cyanotic congenital heart disease (CCHD) is associated with an increased risk of stroke and thromboembolism! A recent study using contrast-enhanced computed tomography revealed a high prevalence of pulmonary thrombosis in patients with Eisenmenger syndrome! The precise mechanisms of the increased incidence of thromboembolism in patients with CCHD have not yet been determined, but endothelial dysfunction, hematic abnormalities and platelet activation may be underlying factors causing hypercoagulability and thromboembolism.

Platelets interact with endothelial cells, leukocytes and other platelets. Intravascular thrombus formation is enhanced by thrombin, which activates the coagulation cascade. platelets and the formation of neutrophil/platelet conjugates. P-selectin is an adhesion molecule found in the secretory granules of platelets and Weibel–Palade bodies of endothelial cells, and is mobilized to the plasma membrane on activation. P-selectin expressed on platelets may be a direct inducer of pro-coagulant activity associated with vascular and thrombotic diseases. Although P-selectin is likely to play an important role in the thrombus formation, there have been only a few studies evaluating P-selectin in CCHD.

Thrombomodulin is expressed mainly on the internal surface of vessels. Endothelial thrombomodulin is a key component of the protein C anticoagulant pathway that facilitates the activation of protein C by thrombin. Therefore, thrombomodulin acts as an intrinsic anticoagulant barrier between the blood and the endothelium. The plasma thrombomodulin level was elevated in disseminated intravascular coagulation and atheromatous arterial disease. It may initially increase with acute vascular injury but decrease with subsequent downregulation of its production during chronic vessel injury. Several reports have shown that reduced thrombomodulin enhances thrombus formation. We hypothesized that the decreased expression of protein C and the increased expression of P-selectin on platelets due to a reduced thrombomodulin level may contribute to the formation of thrombi in patients with CCHD. To test this hypothesis, we measured plasma levels of thrombomodulin and protein C, and the expression of P-selectin on platelets in patients with CCHD. The plasma thrombin–antithrombin complex III (TAT) level was also measured to evaluate coagulability.

Methods

Patients  We enrolled 35 patients with CCHD. The inclusion criterion was the presence of cyanosis due to right-to-left shunt in patients with congenital heart disease. The demography of the patients is summarized in Table 1. There were 19 men and 16 women, with a mean age of 13±11 years, ranging from 1 to 37 years. Twenty-five patients had undergone palliative operations (Balock-Taussig shunt or bidirectional Glenn). Eight patients underwent definitive operations (Fontan operation in 7 and Rastelli operation in 1), but the
right-to-left shunt developed after the operation. One patient with Tetralogy of Fallot was waiting for intracardiac repair and one patient did not have any operation due to severe pulmonary hypertension. Most of the patients who had a Blalock-Taussig shunt or bidirectional Glenn operation were on aspirin or a combination of aspirin and warfarin, heparin or ticlopidine. Only 2 of 7 patients were on anti-platelet drugs after the Fontan operation.

Five patients in the present study developed thromboembolic events and all except one had been on aspirin (Table 1). In patients 31, 33 and 34, thromboembolic events occurred 1–3 months after the blood test was performed. In patient 32, the blood test was performed 18 days after the thromboembolic event. In patient 35, thrombus was observed in the right atrium at the time of the blood test.

Twelve healthy subjects without obvious cardiopulmonary disease (5 men and 7 women, mean age 24±5 years, range 18 to 33 years) served as controls. All subjects gave

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**Table 1 Demographic Data of Patients**

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<thead>
<tr>
<th>Patients no.</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Previous operation</th>
<th>Medication</th>
<th>PC activity (%)</th>
<th>TAT (ng/ml)</th>
<th>TM (FU/ml)</th>
<th>P-selectin (MFI)</th>
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**Group 2. Patients with thromboembolic events**

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<th>Medication</th>
<th>PC activity (%)</th>
<th>TAT (ng/ml)</th>
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**Group 1. Patients without thromboembolic events**

PC, protein C; TAT, thrombin-antithrombin III complex; TM, thrombomodulin; Hb, hemoglobin; Hct, hematocrit; SRV, single right ventricle; BTS, Blalock-Taussig shunt; PAB, pulmonary artery band; RV, right ventricle; VSD, ventricular septal defect; BDG, bidirectional Glenn procedure; ND, not determined; DORV, double outlet right ventricle; SRV, single right ventricle; SLV, single left ventricle; cTGA, corrected transposition of the great arteries; AVSD, atrioventricular septal defect; PA, pulmonary atresia; PA/IVS, pulmonary atresia with intact ventricular septum; TA, tricuspid atresia; AP, aortopulmonary; IAA, interrupted aortic arch; TGA, transposition of the great arteries; TOF, tetralogy of Fallot.

*Not significantly different from that value of group 1. **significantly (p<0.02) different from that value of group 1.*
informed consent for the present study.

Methods

A monoclonal antibody against P-selectin (AK4, mouse IgG1) was obtained from Becton Dickinson Inc. We tested the blood samples from patients under stable conditions. No patients had undergone cardiac operation or cardiac catheterization within one month of sampling. Whole blood was obtained from an antecubital vein without venous stasis using 22-gauge needle. The first 3 drops of blood was discarded before the collection of blood used for analysis and the blood was added to 0.38% trisodium citrate. Immediately after the collection of the blood, we evaluated the expression of P-selectin on platelets as follows. Samples were analyzed on a FACS caliber flow cytometer with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). A gate was set around the platelets, and 10,000 platelets were counted in each sample. The platelets were incubated with fluorescein isothiocyanate-conjugated anti-P-selectin monoclonal antibody (AK4) at room temperature for 30 min, then fixed in 1% (vol/vol) paraformaldehyde and analyzed using flow cytometry. Platelet P-selectin was expressed as mean fluorescence intensity (MFI).

We measured plasma thrombomodulin, protein C activity, TAT, whole-blood hemoglobin and hematocrit levels using standard clinical laboratory methods. When the values of platelet P-selectin, plasma thrombomodulin, protein C activity and/or TAT were markedly abnormal, we re-evaluated those data one week later and confirmed that the data were indeed abnormal (data not shown).

Statistical Analysis

Data were expressed as mean±SD. The association between inhibition of thrombomodulin synthesis, the expression of P-selectin on platelets and thromboembolism were analyzed using one-factorial ANOVA and Bonferroni’s test. Baseline characteristics of the patients with and without CCHD were evaluated using Mann-Whitney’s U-test for non-continuous variables. Multiple linear regression analysis was used to evaluate the possible clinical significance of plasma thrombomodulin, the expression of P-selectin on platelets, TAT and protein C. A level of p<0.05 was accepted as statistically significant.

Results

Representative fluorescence histograms of P-selectin on platelets are shown in Fig 1A. The levels of P-selectin on platelets were greater in the patients than in the normal subjects (6.3±4.5 vs 3.3±0.3 MFI; p<0.01) (Fig 2D). The plasma thrombomodulin level and protein C activity were lower in the patients than in the normal subjects (1.1±0.9 vs 2.2±0.3 FU/ml; p<0.01, and 71.1±29.8 vs 117.8±24.8; other values are shown in Fig 2).
p<0.01, respectively) (Figs 2A, B). The levels of TAT were greater in the patients than in the normal subjects (255±811 vs 1.9±0.9 ng/ml; p<0.01) (Fig 2C). There was a significant positive correlation between P-selectin and TAT levels at baseline in the patients (r=0.5; p<0.01). Platelet P-selectin and TAT levels did not correlate with arterial oxygen saturation, hemoglobin or hematocrit values in the patients with CCHD.

P-selectin on platelets were elevated in the patients with thromboembolic events as compared with the patients without such events (10.9±8.5 vs 4.6±2.5 MFI; p=0.02) (Table 1). Similarly, the patients with thromboembolic events had increased plasma levels of TAT compared to those without (1,100±1,734 vs 83±350 ng/ml; p=0.003). The plasma thrombomodulin level and protein C activity in the patients with thromboembolic events were not significantly different from the values in patients without such events. In addition, whole blood hemoglobin and hematocrit levels were not significantly different between patients with and without thromboembolic events.

Discussion

Platelet P-Selectin

P-selectin is an adhesion molecule found in the secretory granules of platelets and Weibel-Palade bodies of endothelial cells, and is mobilized to the plasma membrane on activation.11,20-28 Activated platelets expressing P-selectin on the surface release their granule contents, facilitating the adhesion of platelets and neutrophils to the endothelium and causing platelet aggregation and enlargement of thrombi through recruitment of leucocytes and platelets.8 Thus, P-selectin expressed on platelets is likely to play an important role in thrombus formation.

Horigome et al measured plasma levels of P-selectin and showed that plasma level of P-selectin was elevated in CCHD.12 Plasma P-selectin may be attributed to increased release from platelets or from the endothelium and, therefore, the exact mechanisms for the increased levels of plasma P-selectin remained unclear. The present study demonstrated directly that P-selectin expression on the platelets is elevated, indicating that platelet activation does exist in CCHD. Although many patients were receiving an antiplatelet drug (aspirin or ticlopidine) or a combination of an antiplatelet and an anticoagulant drug (heparin or warfarin), platelet P-selectin was elevated in many patients in the present study. Previous studies failed to demonstrate elevated platelets P-selectin in patients with CCHD.4-25 The reason for that is not clear, but different methods of platelet P-selectin measurement may be partly responsible, since P-selectin is rapidly mobilized to the platelet surface and lost into the plasma.26

Recently Kario et al demonstrated that endothelial cell damage is a potential risk factor for cerebral infarction.27 It was also reported that the increased expression of platelet P-selectin associated with a reduced NO level, a marker of endothelial dysfunction, was a risk factor for cerebral infarction in patients with atrial fibrillation.24 Elevated plasma P-selectin has been reported in patients with congestive heart failure.28 Primary pulmonary hypertension23 and CCHD25-29. The platelet activation under these conditions may be due to increased shear stress and/or endothelial dysfunction, but the precise mechanisms for the platelet activation in patients with CCHD remain unclear.30 Horigome et al reported that the hematocrit value showed a positive correlation with soluble P-selectin, and speculated that increased shear stress due to hyperviscosity may be a major factor causing platelet activation.11 In the present study, however, a significant correlation between the hematocrit value and platelet P-selectin was not observed. Furthermore, hemoglobin and hematocrit levels were not significantly different between patients with and without thromboembolic events. The role of hyperviscosity in platelet activation in CCHD should be studied further.

Endothelial Dysfunction in CCHD

Thrombomodulin is expressed mainly on the surface of vascular endothelial cells. Endothelial thrombomodulin is a key component of the protein C anticoagulant pathway, which facilitates the activation of protein C by thrombin.24,19,31-34 Activated protein C is known to inhibit clotting factors V and VIII.31-34 Therefore, thrombomodulin acts as an intrinsic anticoagulant barrier between the blood and the endothelium, preventing blood from clotting on the internal surface of vessels.35 The plasma thrombomodulin level initially increases with acute vascular injury, but it may decrease with subsequent downregulation of its production during chronic vessel injury.36,37 It was reported that downregulated gene expression of thrombomodulin by rapid atrial pacing induced a local coagulation imbalance on the internal surface of the atrial cavity, leading to atrial intramural thrombus formation.38 Downregulation of this molecule may, therefore, disturb the optimal coagulation balance and promote thrombogenesis.

The lower plasma thrombomodulin level and protein C activity (Fig 2A) suggest the presence of impaired endothelial function in patients with CCHD. Previous studies also showed decreased plasma thrombomodulin levels in patients with CCHD. These data are in agreement with the study by Ferreiro et al showing that nitric oxide synthesis activity was blunted in patients with CCHD, suggesting that the endothelial function is impaired.39

The endothelium may be damaged by the increased shear stress on the vessel wall caused by increased blood viscosity and/or by chronic hypoxemia in CCHD.3 It has been speculated that in patients with primary pulmonary hypertension, damage to the endothelium leads to a decreased thrombomodulin level and to platelet activation.23,36,37 It is likely that in patients with CCHD, plasma thrombomodulin level may be downregulated by its decreased production due to chronic endothelial injury and persistent hypoxemia.36,37

Since we did not measure protein C antigen, we cannot exclude the possibility that protein C antigen production in the endothelium or liver is decreased in CCHD patients. However, since plasma thrombomodulin levels decreased, it is more likely that the decreased plasma protein C activity is due, at least in part, to the reduced activation by endothelial thrombomodulin, which in turn results from endothelial dysfunction.

Thromboembolic Events

The increased coagulability and platelet activation caused by the endothelial dysfunction may result in local thrombosis in CCHD. Furthermore, platelet P-selectin may be a direct inducer of pro-coagulant activity.11 A high incidence of thromboembolism and organ infarction has been reported in patients with CCHD. Candice et al reported that a pulmonary artery thrombus was noted in 21% of patients with Eisenmenger syndrome.2 The present study is the first
showing that platelet P-selectin levels were higher in pa-
tients with than in those patients without thromboembolic
events in CCHD, suggesting that elevated P-selectin may be a risk factor for cerebral thromboembolism in CCHD.

The effect of anti-platelet drugs on platelet P-selectin
level remains unclear. O’Connor et al showed that platelet
P-selectin was elevated in patients with congestive heart
failure despite aspirin therapy.27 In contrast, Serebruany et
al showed that the platelet P-selectin level in patients with
acute myocardial infarction who were taking aspirin was
lower than in patients who were not taking the drug.28 In
the present study, platelet activation was present even though
antiplatelet drugs had been administered.

In addition to elevated P-selectin on platelets, we showed
plasma levels of TAT in patients with thromboembolic
events were higher than in those without. Thus, elevated
P-selectin and TAT may indicate a high risk of thrombo-
embolism. This may suggest that aspirin alone cannot
always inhibit platelet activation and increased TAT levels
may be related to platelet activation. In order to prevent
thromboembolic events in patients with CCHD, laboratory
tests examining platelet activation and hypercoagulability
should be performed, and appropriate anti-platelet and anti-
coagulation therapy is required in each patient.

Some patients with elevated P-selectin and TAT levels
did not have thromboembolic events in the present study
(Table I, Figs 2C,D). The occurrence of thromboembolic
events may be multifactorial. It must be noted, however,
that thromboembolic events did occur in patients with
elevated P-selectin and TAT levels. We believe that it is
prudent to evaluate the presence of thrombus or to give
anti-platelet or anti-coagulant drugs in such patients.

Study Limitations

There are limitations in the present study. The study pop-
ulation was heterogeneous with respect to age, severity of
cyanosis, anticoagulation therapy and cardiac diagnosis.
Furthermore, a relatively small number of patients had an
episode of thromboembolic event and patients with elevated
P-selectin expression on the platelet did not necessarily
have episodes of thromboembolic events. The precise
mechanisms of thromboembolic events in CCHD remain
unclear and may be multi-factorial. Nevertheless, the pres-
ent study did show directly that platelet activation exists in
CCHD.

Conclusion

Platelet activation and endothelial dysfunction causing
pro-coagulation may exist in patients with CCHD, espe-
cially in patients with thromboembolic events.

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