Left Atrial Platelet Activity With Rheumatic Mitral Stenosis*

Correlation Study of Severity and Platelet P-Selectin Expression by Flow Cytometry

Mien-Cheng Chen, MD; Chiung-Jen Wu, MD; Hon-Kan Yip, MD; Hsueh-Wen Chang, PhD; Chih-Yuan Fang, MD; Teng-Hung Yu, MD; and Morgan Fu, MD

Background: Previous studies have demonstrated that platelet activation, evaluated by measuring the secretory substances of platelets (ie, platelet factor 4 and β-thromboglobulin), occurs in the peripheral blood of patients with rheumatic mitral stenosis (MS). However, the differences in platelet activation between peripheral and atrial blood, and the relationship between regional left atrial platelet P-selectin expression and the severity of MS have never been investigated.

Methods and results: A total of 16 patients with symptomatic MS undergoing percutaneous transluminal mitral valvuloplasty were studied (group 1). The fractions of platelets expressing P selectin in the prevalvuloplasty left atrial, right atrial, peripheral venous, and arterial blood were determined by flow cytometry. The mitral valve area was calculated by means of the Doppler pressure half-time method. Peripheral venous platelet activity also was evaluated in 23 control patients (including 15 healthy volunteers who were in sinus rhythm [group 2] and 8 patients who had chronic lone atrial fibrillation [group 3]). The fraction of peripheral venous platelets expressing P selectin among group 1 patients was significantly higher than that of group 2 or 3 patients (p < 0.008). In group 1 patients, the fraction of platelets expressing P selectin in the left atrium was significantly higher than that in the right atrium, the femoral vein, or the femoral artery (p < 0.01). Correlation analysis demonstrated that there was a significantly direct relationship between the severity of MS and the fraction of left atrial platelets expressing P selectin (p = 0.01; r = −0.620). The fraction of peripheral venous platelets expressing P selectin among group 2 patients did not differ from that of group 3 patients.

Conclusions: In patients with rheumatic MS, increased regional left atrial platelet P-selectin expression had a significantly direct relationship with the severity of MS. The increased regional left atrial platelet P-selectin expression was not reflected in peripheral venous blood samples.

(CHEST 2003; 124:1663–1669)

Key words: mitral stenosis; platelet; P selectin

Abbreviations: AF = atrial fibrillation; MS = mitral stenosis; PE = phycoerythrin; PTMV = percutaneous transluminal mitral valvuloplasty

A large body of epidemiologic data has indicated that patients with rheumatic mitral stenosis (MS), particularly those with atrial fibrillation (AF) or left atrial spontaneous echo contrast, are at risk for developing left atrial thrombus.1–6 AF itself has been shown to confer a hypercoagulable state.7,8 Jafri and associates9 also demonstrated that the coagulation system is activated in patients with MS and sinus rhythm, even in the absence of echocardiographically visualized thrombi. However, there are controversial issues relating to the reflection of atrial coagulation by peripheral blood, and the indexes of thrombogenesis between the left and right atria.10,11

*From the Division of Cardiology (Drs. Chen, Wu, Yip, Fang, Yu, and Fu), Department of Internal Medicine, Chang Gung Memorial Hospital, Kaohsiung, Taiwan, Republic of China; and the Department of Biological Sciences (Dr. Chang), National Sun Yat-Sen University, Kaohsiung, Taiwan, Republic of China. This study was supported by grant No. CMRP1139 from Chang Gung Memorial Hospital, Chang Gung University. Manuscript received December 16, 2002; revision accepted March 25, 2003.

Reproduction of this article is prohibited without written permission from the American College of Chest Physicians (e-mail: permissions@chestnet.org).

Correspondence to: Mien-Cheng Chen, MD, Division of Cardiology, Department of Internal Medicine, Chang Gung Memorial Hospital, 123, Tzu Pei Rd, Niao Sung Hsiang, Kaohsiung Hsien 83301, Taichung, Republic of China; e-mail: chenmien@ms76.hinet.net
Evidence has been shown that shear stresses in turbulent flow occur as a result of the induction of platelet activation by stenotic valves. Previous studies have demonstrated that platelet activation occurs in the peripheral blood of patients with MS. Therefore, hemodynamic effects may play a role in the activation of platelets in patients with MS. However, in these studies, platelet activation was evaluated by measuring the secretory substances of platelets that are plasma markers for evaluating platelet activation and that do not reflect changes in individual platelets. On the other hand, the measurement of key biochemical markers, such as P selectin (or CD62p antigen), a biologically relevant molecule that is released to the surface of platelet from alpha granules on activation, by flow cytometry allows us to see changes in individual platelets long before they can be detected in physiologic assays. In addition, the differences in platelet activation between the blood from the left and right atria and between peripheral and atrial blood have never been investigated. Accordingly, we undertook the present study to investigate the differences in platelet activation between blood from the left and right atria and between peripheral and atrial blood with the use of flow cytometry in patients with moderate-to-severe MS, and to determine whether there was a correlation between the severity of MS and regional left atrial platelet activation.

Materials and Methods

Study Population

Sixteen patients who had symptomatic rheumatic MS without significant mitral, tricuspid, or aortic regurgitation, and a left atrial thrombus, and had undergone percutaneous transluminal mitral valvuloplasty (PTMV) were studied (group 1). There were 2 men and 14 women, ranging in age from 41 to 72 years (mean ± SD age, 56.5 ± 12.0 years). Thirteen patients had chronic AF, and 3 patients were in sinus rhythm. Five patients had a history of cerebral thromboembolism. Twelve patients were in New York Heart Association functional class III, and 4 patients were in New York Heart Association functional class II. No patients had a history of malignancy, inflammatory disease, collagen vascular disease, renal or liver disease, diabetes mellitus, hypertension, hyperlipidemia, infectious disease, deep venous thrombosis, or pulmonary embolism or had recently undergone surgery.

Peripheral venous platelet activity also was evaluated in 23 control patients. The group of control patients included 15 healthy volunteers who were in sinus rhythm (group 2) and 8 patients who had chronic lone AF without systemic disease or structural heart disease (group 3). In group 3, 2 patients had a history of systemic arterial thromboembolism. None of the control patients had a history of active malignancy, inflammatory disease, renal or liver disease, diabetes mellitus, hypertension, hyperlipidemia, deep venous thrombosis, or pulmonary embolism or had recently undergone surgery.

Informed consent was obtained from all study subjects. The study protocol was approved by the Institutional Review Committee on Human Research in our institution.

Doppler Echocardiography and Medications

In patients with rheumatic MS, transthoracic echocardiographic examinations were performed on the day of PTMV and before the valvuloplasty procedure with a 2.5-MHz transducer attached to a commercially available Doppler echocardiography machine (Sonos 5500; Hewlett-Packard; Palo Alto, CA) to assess left atrial dimension and mitral valve area. M-mode measurements were performed according to the recommendation of the American Association of Echocardiography. The mitral valve area was calculated by means of the Doppler pressure half-time method, and the reliability of this method in the measurement of mitral valve area has been confirmed in previous studies.

The severity of mitral, tricuspid, and aortic insufficiency was determined by Doppler color-flow mapping. The absence of a left atrial cavity or an appendageal thrombus was confirmed by transesophageal echocardiography.

In group 1 patients, warfarin therapy was discontinued for at least 3 days before the patient underwent PTMV and was administered on the second day after PTMV. Heparin, 5,000 U, was administered into the left atrium after transseptal puncture in each patient. Therapy with diuretic agents was discontinued on the day of PTMV. In group 3 patients, therapy with aspirin was discontinued for at least 7 days and warfarin was discontinued for at least 3 days before the study.

Valvuloplasty Procedure

PTMV was performed by the transseptal approach with the use of an Inoue balloon catheter. Details of the procedure have been described previously. In brief, an Inoue balloon catheter was inserted into the left ventricle via the transseptal approach. The distal half of the balloon was inflated in this position, and the balloon was pulled back to the mitral valve orifice. The balloon was then fully inflated and pulled back to the left atrium before being deflated. When additional balloon dilatation was required, the same procedure was repeated. Invasive pressure measurements were performed immediately before and after valvuloplasty.

Blood Sample Collection and Assessment of Platelet Activity

Blood samples were obtained in the fasting, nonsedative state between 9:00 and 10:00 AM in the control and study groups to exclude the possible influence of circadian variations. In group 1 patients, blood was obtained from the femoral vein and artery through introducer sheaths immediately after the puncture. Right atrial blood was obtained through a balloon catheter, and left atrial blood was obtained immediately after transseptal puncture before heparin administration. The first 3 mL blood was discarded, and the following 3 mL was drawn into an evacuated tube containing 3.8% buffered sodium citrate (Vacutainer; Becton Dickinson; Franklin Lakes, NJ). In the subjects in groups 2 and 3, blood was obtained under minimal tourniquet pressure from the antecubital vein using a sterile 22-gauge needle and syringe in a single attempt. The first 3 mL blood was discarded, and the following 3 mL was drawn into a Vacutainer containing 3.8% buffered sodium citrate. Blood samples with gross hemolysis were discarded. Mixtures of blood and sodium citrate were centrifuged (model 5400; Kubota Corp; Tokyo, Japan) for 15 min at 1,500 revolutions per minute at room temperature. The supernatant platelet-rich plasma was used to...
assess the platelet activity. The time between blood collection and antibody labeling was standardized to within 1 h.

Platelet activity was determined with respect to alpha granule degranulation (surface expression of P selectin or CD 62p antigen). The 10-μL aliquots of platelet-rich plasma were placed in 5-mL polystyrene tubes (Falcon; Becton Dickinson), which contained 90 μL diluted, sterile, phosphate-buffered solution (pH 7.4; sodium chloride, 137 mmol/L; potassium chloride, 2.7 mmol/L; phosphate buffer, 10 mmol/L) to prevent the aggregation of platelets, along with 5 μL fluorochrome-labeled antibodies. A fluorescein isothiocyanate-conjugated antibody to glycoprotein IIb/IIIa (CD61; Becton Dickinson) was used as an activation-independent marker of platelets.27,28 A phycoerythrin (PE)-conjugated anti-CD62 antibody (Becton Dickinson) was used to assess alpha granule degranulation. To assess the extent of the nonspecific association of protein with platelets, a control tube containing fluorescein isothiocyanate-conjugated CD61 and nonfractionated PE-conjugated IgG (Becton Dickinson) was used for each blood sample. The reaction mixture was incubated at room temperature for 30 min in a dark room. Then, the antibody-bound platelets were fixed with 1% solution of paraformaldehyde, and platelet activity was assessed immediately after fixation to avoid further activation.29 The association of ligands with platelets was determined with a fluorescence-activated cell sorter (FACSCalibur system; Becton Dickinson). Platelets were identified with flow cytometry on the basis of size and antibody labeling was standardized to within 1 h.

Platelet activity was determined with respect to alpha granule degranulation (surface expression of P selectin or CD 62p antigen). The 10-μL aliquots of platelet-rich plasma were placed in 5-mL polystyrene tubes (Falcon; Becton Dickinson), which contained 90 μL diluted, sterile, phosphate-buffered solution (pH 7.4; sodium chloride, 137 mmol/L; potassium chloride, 2.7 mmol/L; phosphate buffer, 10 mmol/L) to prevent the aggregation of platelets, along with 5 μL fluorochrome-labeled antibodies. A fluorescein isothiocyanate-conjugated antibody to glycoprotein IIb/IIIa (CD61; Becton Dickinson) was used as an activation-independent marker of platelets.27,28 A phycoerythrin (PE)-conjugated anti-CD62 antibody (Becton Dickinson) was used to assess alpha granule degranulation. To assess the extent of the nonspecific association of protein with platelets, a control tube containing fluorescein isothiocyanate-conjugated CD61 and nonfractionated PE-conjugated IgG (Becton Dickinson) was used for each blood sample. The reaction mixture was incubated at room temperature for 30 min in a dark room. Then, the antibody-bound platelets were fixed with 1% solution of paraformaldehyde, and platelet activity was assessed immediately after fixation to avoid further activation.29 The association of ligands with platelets was determined with a fluorescence-activated cell sorter (FACSCalibur system; Becton Dickinson). Platelets were identified with flow cytometry on the basis of size and antibody labeling was standardized to within 1 h.

Continuous variables were described as the mean ± SD. Categoric variables were compared using the Fisher exact test (two-tailed). The difference in P-selectin expression in peripheral venous platelets between patients who were in sinus rhythm and those with AF was compared using the Student t test. Continuous variables were compared using the one-way analysis of variance or the Kruskal-Wallis test when it was appropriate. The Tukey procedure was used for post hoc comparisons. The relationships between the P-selectin expression of platelets and the mitral valve area, transmitral pressure gradient, atrial pressure, or atrial dimension were performed with the Pearson correlation. Statistical analysis was performed using a statistical software program (SAS for Windows, version 8.02; SAS Institute, Cary, NC). A p value of < 0.05 was considered to be statistically significant.

RESULTS

Comparison of Baseline Characteristics and Peripheral Venous Platelet P-Selectin Expression Among the Three Groups

The baseline characteristics for each group are summarized in Table 1. There were no significant differences among the three groups in terms of the use of β-blockade, Ca-blockade, amiodarone, propafenone, and aspirin, and blood cell counts and biochemistry data. The duration of AF among group 1 patients did not differ from that of group 3 patients (not significant). Group 3 patients were significantly older than group 2 patients, and the use of warfarin therapy in patients from groups 1 and 3 was significantly more frequent than that in group 2 patients. In group 1 patients, the mean left atrial dimension was 46.8 ± 7.3 mm, the mitral valve area was 1.04 ± 0.17 cm², the prevalvuloplasty left atrial pressure was 23.3 ± 4.5 mm Hg, and the transmitral pressure gradient was 12.0 ± 4.5 mm Hg. All of the patients in group 1 had swirling spontaneous echo contrast only in the left atrium confirmed by transesophageal echocardiography.

The fraction of platelets expressing P selectin among group 1 patients was significantly higher than that among group 2 or 3 patients (p = 0.008) [Table 1], and the increased peripheral venous platelet activity of patients with rheumatic MS was evident both in patients who were in sinus rhythm and those with AF (4.5 ± 2.7% and 6.8 ± 7.4%, respectively). The fraction of platelets expressing P selectin in the peripheral venous blood of group 2 patients (1.7 ± 0.9%) was consistent with that found in a previous study (2.3%) using a 3.8% sodium citrate solution as an anticoagulant. The platelet activation with respect to the P-selectin expression of group 2 patients did not differ from that of group 3 patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group 1 (n = 16)</th>
<th>Group 2 (n = 15)</th>
<th>Group 3 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>56.5 ± 12.0</td>
<td>46.5 ± 16.9</td>
<td>65.8 ± 14.21</td>
</tr>
<tr>
<td>Men</td>
<td>12.5</td>
<td>53.3</td>
<td>62.51</td>
</tr>
<tr>
<td>Duration of AF, mo</td>
<td>49.9 ± 43.3</td>
<td>33.7 ± 35.9</td>
<td></td>
</tr>
<tr>
<td>History of embolism</td>
<td>31.3</td>
<td>0.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digitalis</td>
<td>62.5‡</td>
<td>0.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Warfarin</td>
<td>93.8†</td>
<td>0.0</td>
<td>87.5§</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.0</td>
<td>0.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>6.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Propafenone</td>
<td>0.0</td>
<td>0.0</td>
<td>12.5</td>
</tr>
<tr>
<td>β-blockade</td>
<td>18.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Ca-blockade</td>
<td>18.8</td>
<td>0.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Platelets, 10⁹ cells/mL</td>
<td>19.7 ± 5.2</td>
<td>24.3 ± 4.3</td>
<td>20.7 ± 8.0</td>
</tr>
<tr>
<td>Hemoglobin, g/mL</td>
<td>12.4 ± 1.7</td>
<td>13.9 ± 2.5</td>
<td>13.0 ± 2.6</td>
</tr>
<tr>
<td>Leukocytes, 10⁵ cells/mL</td>
<td>6.5 ± 2.0</td>
<td>6.3 ± 1.6</td>
<td>5.9 ± 2.5</td>
</tr>
<tr>
<td>Calcium, mg/dL</td>
<td>8.9 ± 0.4</td>
<td>9.1 ± 0.4</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>1.0 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>CD62p in the PVB, %</td>
<td>6.3 ± 6.7‡</td>
<td>1.7 ± 0.9</td>
<td>1.3 ± 0.8</td>
</tr>
</tbody>
</table>

*pValues given as mean ± SD or %. PVB = peripheral venous blood. 
†p < 0.01 (group 2 vs 3). 
‡p < 0.03 (group 1 vs group 3). 
§p < 0.04 (group 1 vs groups 2 and 3). 
¶p < 0.0001 (group 1 or 3 vs group 2). 
‖p = 0.008 (group 1 vs groups 2 and 3, by analysis of variance).
**Left Atrial Platelets P-Selectin Expression**

Analysis of the prevallvuloplasty data of the 16 patients with rheumatic MS revealed that the fraction of platelets expressing P selectin in the left atrium (13.2 ± 11.6%) was significantly higher than that in the right atrium (5.4 ± 3.8%), femoral vein (6.3 ± 6.7%) or femoral artery (5.4 ± 4.3%) (p < 0.01) [Fig 1].

**Correlation of Mitral Valve Area and Left Atrial Platelet P-Selectin Expression**

Correlation analysis demonstrated that there was a significantly direct relationship between the severity of MS and the fraction of left atrial platelets expressing P selectin (ie, the smaller the mitral valve area, the higher the fraction of left atrial platelets expressing P selectin; p = 0.01; r = −0.620) [Fig 2]. However, there was no significant correlation between left atrial platelet P-selectin expression and left atrial dimension (p value, not significant; r = 0.205), prevallvuloplasty left atrial pressure (p value, not significant; r = 0.388), or transmitral pressure gradient (p value, not significant; r = 0.342). On the other hand, there was no significant correlation between femoral venous platelet P-selectin expression and the severity of MS (p value, not significant; r = 0.128), prevallvuloplasty left atrial pressure (p value, not significant; r = −0.325), or transmitral pressure gradient (p value, not significant; r = −0.085).

**DISCUSSION**

The present study, in which we examined platelet activation in patients with symptomatic rheumatic MS who were undergoing PTMV by measuring platelet P-selectin expression in atrial and peripheral venous blood samples or arterial blood samples, produced four major findings. First, increased regional left atrial platelet P-selectin expression was not reflected in the peripheral venous and right atrial blood samples tested. Second, the increased peripheral venous platelet activity of patients with rheumatic MS was evident both in patients who were in sinus rhythm and those who were experiencing AF. Third, increased regional left atrial platelet P-selectin expression had a significantly direct relationship with the severity of MS. Finally, there was no significant correlation between peripheral venous platelet P-selectin expression and the severity of MS.

**Mechanisms of Direct Relationship Between Increased Regional Left Atrial Platelet P-Selectin Expression and Severity of MS**

To the best of our knowledge, this is the first study to demonstrate a direct relationship between increased regional left atrial platelet activity and severity of MS. Of the 16 patients with rheumatic MS, the fraction of platelets expressing P selectin in the left atrium was significantly higher than that in the right atrium, femoral vein, or femoral artery. In addition, there was no significant correlation between increased left atrial platelet P-selectin expression and left atrial dimension, prevallvuloplasty left atrial pressure, or transmitral pressure gradient. These findings indicated that a local factor (ie, the stenotic mitral valve instead of hemodynamic and echocardiographic factors) had a significant impact on the increased left atrial platelet activity.

There were several mechanisms that contributed...
to this observation. First, previous studies\textsuperscript{12–15} have shown that shear stresses in turbulent flow as a result of stenotic valves induce platelet activation. P selectin is an intrinsic component of platelet alpha-granules, which translocate to the external membrane on platelet activation and are ensconced on platelet surfaces within seconds after activation.\textsuperscript{31} In the absence of further activation, P selectin in platelets is rapidly released from the alpha-granules, becoming soluble P selectin, or these alpha-granules are rapidly shifted to the center of the platelet cytoplasm during internal rearrangements. These two factors might account for the lower fraction of platelets expressing P selectin in the blood drawn from sites distal to the valve, such as the femoral artery and vein, and the lack of correlation between femoral venous platelet P-selectin expression and the severity of MS. Second, previous studies have demonstrated that spontaneous echo contrast, which is an indicator of the stasis of flow and RBC aggregation, is associated with a significantly smaller mitral valve area in patients with MS and normal sinus rhythm\textsuperscript{32} and that the severity of spontaneous echo contrast had a direct correlation to the severity of stenosis in patients with moderate-to-severe MS, such as those in our study.\textsuperscript{33} In addition, Turitto and Weiss\textsuperscript{34} have demonstrated that at a low or high blood flow rate, platelet reactivity, and adhesion increase as the hematocrit value is increased. Therefore, it is reasonable to observe that there is a further activation of the returned circulating platelets with RBC aggregates in the left atrium as a result of stasis and that there is a direct relationship between increased regional left atrial platelet activity and the severity of MS.

\textbf{Platelet Activity in Patients With AF}

In this study, we have demonstrated that the fraction of peripheral venous platelets expressing P selectin in patients with chronic lone AF did not differ from that of healthy volunteers who were in sinus rhythm. Steele and associates\textsuperscript{17} have shown that there was no significant difference in the average half-life of platelet survival when patients with AF were compared with those in normal sinus rhythm. Furthermore, large clinical trials\textsuperscript{35–37} have demonstrated that aspirin was ineffective in prevent-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Correlation between the fraction of left atrial platelets expressing P selectin and the size of the mitral valve area. The increased regional left atrial platelet P-selectin expression had a significantly direct relationship with the severity of MS (i.e., the smaller the mitral valve area, the higher the fraction of left atrial platelets expressing P selectin; p = 0.01). Each point represents one patient with MS.}
\end{figure}
ing thromboembolism in patients with nonvalvular AF. Our study provided one possible basic mechanism to clarify the ineffectiveness of aspirin in preventing thromboembolism in patients with nonvalvular AF.

Study Limitations

There were several limitations in this study. First, although warfarin therapy was discontinued for at least 3 days in patients in groups 1 and 3, we could not completely exclude the potential late effect of warfarin on platelet activity. However, there are controversial results regarding the effect of warfarin on platelet activity.38,39 In addition, the fraction of peripheral venous platelets expressing P selectin in group 1 patients was significantly higher than that in group 2 and 3 patients, and the fraction of peripheral venous platelets expressing P selectin in group 2 patients did not differ from that in group 3 patients. These observations should exclude the potential late effect of warfarin on platelet activity as most of the group 1 and 3 patients had received warfarin therapy before entering the study. Second, platelet activity (i.e., the activation of glycoprotein IIb/IIIa) was not determined in this study; therefore, we could not provide the fibrinogen-binding affinity of our patients in this study. Third, physiologic assays, such as those for determining platelet aggregation or adherence, were not performed in this study. Therefore, the increased platelet P-selectin expression may not necessarily reflect changes in platelet physiologic function. Finally, as the number of patients having a history of systemic arterial thromboembolism was small, it was not our aim to study the difference in platelet activity between patients with and without a history of systemic arterial thromboembolism.

In conclusion, in patients with moderate-to-severe MS, increased regional left atrial platelet P-selectin expression had a significantly direct relationship with the severity of MS, and it was the stenotic mitral valve, instead of hemodynamic and echocardiographic factors, that had a significant impact on the increase in regional left atrial platelet activity. In addition, the increased regional expression of P selectin in left atrial platelets was not reflected in peripheral venous blood samples.

REFERENCES


