P-Selectin Expression, but not GPIIb/IIIa Activation, is Enhanced in the Inflammatory Stage of Takayasu’s Arteritis

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Background  Inflammation and thrombosis are closely related processes, but the association between disease activity and thrombogenicity in Takayasu’s arteritis (TA) is poorly understood. To investigate the link between platelet activation and disease activity, flow cytometric analyses of platelet P-selectin and activated GPIIb/IIIa expression were performed in patients with TA.

Methods and Results  Twenty-two patients with TA, classified into active (Group A, n=9) and inactive (Group I, n=13) according to blood-derived inflammatory markers, and 14 healthy age- and gender-matched controls (Group C) were studied. Compared with Group C, the mean fluorescence intensity of P-selectin in response to 0.1–10 μmol/L of ADP was significantly upregulated in Group A, but not in Group I. No differences in platelet GPIIb/IIIa expression in stimulated platelets were seen among the 3 groups. Standard platelet aggregation studies revealed that disease activity did not influence platelet aggregation by ADP.

Conclusions  P-selectin expression, but not activated GPIIb/IIIa, is enhanced in ADP-activated platelets from patients in the inflammatory stage of TA. P-selectin may play a significant role in the inflammatory and thrombotic responses associated with intractable TA, presumably by inducing platelet-leukocyte interactions.

Key Words: P-selectin; Platelets; Takayasu’s arteritis

Takayasu’s arteritis (TA) is a nonspecific vasculitis of unknown etiology that primarily involves the aorta, its branches, and the pulmonary and coronary arteries. Geographic distribution is characteristic, as it mainly affects people in Asian and South American countries and 5,000 patients were listed on the basis of a government-sponsored survey in Japan. TA starts with acute inflammation, thus patients initially present with such symptoms as fever, malaise, and headache. Later, some cases undergo a clinical course of chronic inflammation, whereas others become senescent without signs of inflammation. Those with intractable symptoms have a more serious prognosis and suffer from additional complications such as thrombosis.

Over the past several years, the link between inflammation and thrombosis has attracted renewed interest and it has been postulated that inflammatory cytokines within atheromatous plaque facilitate plaque rupture and acute thrombotic events. Further, an increase in C-reactive protein (CRP) may be a marker of a guarded prognosis in patients with acute coronary syndrome.

Few studies have been conducted in regard to thrombogenicity in TA, of which some have indicated that there is altered coagulation cascade and platelet reactivity in patients with TA. Conventional methods were used in those studies for detecting platelet activation both in vivo (plasma thromboglobulin, platelet factor 4, thromboxane B2 etc) and in vitro (platelet aggregation measured by optical densitometry), but they have limited value because of inherent technical problems. Furthermore, previous aggregation studies only investigated platelet–platelet interactions for the development of thrombosis, whereas recent developments in flow cytometric analysis have enabled the detection of specific markers for platelet activation, including P-selectin (CD62P), as well as the binding of fibrinogen and annexin V. To the best of our knowledge, flow cytometric analyses have not been done using platelets from patients with TA. To investigate the association of platelet activation with disease activity in TA using this advanced method, we determined platelet P-selectin expression and PAC-1 binding, a marker for GPIIb/IIIa activation, in patients with TA and healthy controls.

Methods

Twenty-two female Japanese TA patients and 14 healthy age- and gender-matched controls (Group C: 26–74 years old) were enrolled in the study (Table I). Patients were diagnosed as having TA using criteria reported by the American College of Rheumatology. The patients were classified into 2 groups: 9 had active disease (Group A: 26–71 years old) and 13 were inactive (Group I: 24–69 years old). The ratio of premenopausal to postmenopausal women...
in the 3 groups was not different. Active disease was defined as a persistently elevated erythrocyte sedimentation rate (ESR, >40 mm/h for more than 3 months prior to the study)\(^1\)\(^2\)\(^3\)\(^4\) CRP values were ≥0.3 mg/dl in all active stage patients and <0.3 mg/dl in inactive patients. Acute nonspecific inflammatory diseases, including viral and bacterial infections were ruled out clinically by examination of the medical records. Most patients were being treated with aspirin (40.5–162 mg/day), except for a few who had a history of peptic ulcers or other hemorrhagic diseases, or who declined use of aspirin for gastrointestinal or other symptoms. Instead, 1 patient with active disease took beraprost and 1 patient with inactive disease took cilostazol. No patients received thienopyridines. More than 60% of the patients were treated with a corticosteroid (prednisolone 5–15 mg/day), though 2 with active disease were not treated with the steroids because of side-effects. No statistical differences were noted in the frequency and dosage of aspirin or corticosteroid use between Groups A and I (Table 1). In addition, none of the patients had received immunosuppressant therapy other than the corticosteroid. Five patients with active disease and 7 with inactive disease were diagnosed as having hypertension and took calcium blockers and/or angiotensin converting enzyme inhibitors. One patient each with active or inactive disease had hyperlipidemia although no lipid-lowering drugs were prescribed. One patient with active disease took sultonylurea for diabetes mellitus. All subjects gave informed consent before enrollment.

For an additional experiment, 4 healthy female volunteers (24–30 years, mean 26±3) were recruited, after giving informed consent, to investigate the effect of aspirin on the expression of platelet markers. Blood samples were collected before and after aspirin ingestion (81 mg/day, once after breakfast) for 7 days.

These investigations conformed to the principles outlined in the Declaration of Helsinki.

Sample Preparation

Venipuncture was performed in the antecubital vein and blood was drawn directly into a syringe containing 3.8% sodium citrate (9:1 vol/vol) using a 21-gauge needle. The first 3 ml of the sample was discarded using a 2-syringe technique, in order to reduce platelet activation to a minimum\(^5\)\(^6\). Immediately after sampling, each aliquot of blood was diluted 1:9 in HEPES buffer (in mmol/L: 10 HEPES, 136 NaCl, 2.7 KCl, 1 MgCl\(_2\), 0.47 NaH\(_2\)PO\(_4\), 11.62 NaHCO\(_3\), 0.35% bovine serum albumin, pH 7.4). After stimulation with selected concentrations of ADP for 2 min, each aliquot was incubated for 20 min at room temperature with fluorescein-isothiocyanate (FITC)-conjugated PAC-1, phycoerythrin (PE)-conjugated anti-CD61 (GPIIIa) antibody.20 Because CD61 is expressed in both resting and activated platelets,\(^7\) anti-CD61 antibody was used to identify platelet populations in the whole blood samples. The samples were kept at 4°C for at least 2 h after fixation in cold 1% paraformaldehyde. Platelets treated with ARG-GLY-ASP-SER (RGDS), anti-CD61 PerCP, and mouse IgG1-PE were used as negative controls.

Flow Cytometry

Within 24 h of collection, the samples were subjected to analysis of platelet activation using a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA). Platelet populations were identified by their characteristic light-scatter profiles and gated platelets were 95.9±4.3% (mean±SD, n=22) positive for CD61. To quantify the degree of expression in platelets stimulated with ADP and in those that were not stimulated, 5,000 platelets were examined for FITC and PE fluorescence to calculate the mean fluorescence intensity (MFI) for both PAC-1 and anti-CD62P antibody.

Platelet Aggregation

Platelet aggregation in response to ADP was compared between Group A and Group I using a standard Born's method.\(^8\) Fresh citrated whole blood samples were immediately centrifuged at 200 g (KS-5200C, Kubota, Tokyo, Japan) for 15 min at room temperature. The supernatant

### Table 1 Baseline Clinical Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th>Medication</th>
<th>Hypertension (+)</th>
<th>Steroid</th>
<th>Aspirin</th>
<th>Hb (g/dl)</th>
<th>ESR (mm/h)</th>
<th>CRP (mg/dl)</th>
<th>Fbg (mg/dl)</th>
<th>WBC (× 10(^3)/μl)</th>
<th>Ph (mmol/L)</th>
<th>NaHCO(_3) (mEq/l)</th>
<th>Albumin (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Group C)</td>
<td>7/13</td>
<td>5/9</td>
<td>9/11</td>
<td>13.1 ± 1.3</td>
<td>17.3 ± 6.7</td>
<td>0.14 ± 0.7</td>
<td>271 ± 72</td>
<td>7.7 ± 1.1</td>
<td>22.9 ± 5.5</td>
<td>13.0 ± 0.8</td>
<td>1.3 ± 0.96</td>
</tr>
<tr>
<td>Takayasu's arteritis</td>
<td>6/9</td>
<td>7/9</td>
<td>9/13</td>
<td>11.7 ± 1.7</td>
<td>13.8 ± 6.9</td>
<td>0.11 ± 0.6</td>
<td>296 ± 46</td>
<td>7.8 ± 1.0</td>
<td>25.7 ± 6.0</td>
<td>12.7 ± 1.4</td>
<td>1.2 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SD. Statistical analysis was done using 1-way analysis of variance with a Scheffe's test for multiple comparisons, or a non-paired t-test (in the case of Group I vs Group A). Fisher's exact probability test was used to test for the difference in the number of patients having hypertension or using aspirin and steroid. The chisquare test was used to test for the difference in the menstruation status among the 3 groups.

NS, not significant; Hb, hemoglobin; WBC, white blood cell count; Plt, platelet; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Fbg, fibrinogen.
(platelet-rich plasma (PRP)) was transferred by plastic pipette to a polyethylene tube, which was then tightly capped. Platelet-poor plasma (PPP) was obtained by centrifuging the leftover sample at 2,000 g for 15 min. The platelet concentration in PRP was adjusted to 250,000 /μl by adding PPP from each subject. Platelet aggregation was determined by optical densitometry (PAM-8T, Mebanix, Tokyo, Japan).

Statistical Methods
Statistical analysis was done by 2- or 1-way analysis of variance for repeated measures, with Scheffe’s test for multiple comparisons, unless indicated otherwise; p<0.05 was considered significant.

Materials
PAC-1, FITC and anti-CD61 antibody PerCP were purchased from Becton Dickinson (San Jose, CA, USA). Anti-CD62P PE and mouse IgG1-PE were from Immunotech (Marseille, France). RGDS, ADP, and HEPES were from Sigma (St Louis, MO, USA). Paraformaldehyde was obtained from Wako Pure Chemical, Osaka, Japan.

Results
Whole Blood Flow Cytometry
In the resting state, there were no differences in MFI for both P-selectin expression and PAC-1 binding among the 3 groups. Following stimulation, the expression of P-selectin was significantly upregulated in Group A to 0.1–10 μmol/L.
ADP as compared with Groups C and I (Fig 1). Further, platelets from Group I patients did not show enhanced expression when compared with those from the controls. PAC-1 binding in response to ADP was similar among the 3 groups (Fig 2). A representative profile is shown in Fig 3.

The additional experiment to the main study revealed that taking aspirin for 7 days did not cause significant differences in MFI for P-selectin expression or PAC-1 binding in female volunteers (Fig 4).

**Platelet Aggregation**

Results of platelet aggregation using ADP as agonist are shown in Table 2. Platelet aggregation in response to 10 μmol/L ADP was attenuated in Groups A and I as compared with Group C, despite no differences among the 3 groups in response to lower concentrations of ADP. No statistical differences in platelet aggregation were noted between Groups A and I.

**Discussion**

The salient feature of this study is that P-selectin expression, but not PAC-1 binding, was augmented in stimulated platelets in patients with active TA. The present results suggest that platelet–leukocyte interactions, rather than platelet–platelet, may be relevant to thrombotic complications in patients with active disease who receive conventional medication, including corticosteroid and aspirin.

P-selectin is a member of the selectin family and resides in platelets and endothelial cells. It mediates the adhesion of activated platelets to neutrophils and monocytes via a specific interaction with P-selectin glycoprotein-1 (PSGL-1) and may trigger multiple intracellular events within leukocytes to promote vascular inflammation and facilitate atherosclerosis and thrombosis. Clinically, an increased expression of platelet P-selectin has been shown in patients with atherothrombotic diseases, including those in the acute phase of ischemic stroke and with acute coronary syndromes. Clinically, an increased expression of activated platelets to neutrophils and monocytes via a specific interaction with P-selectin glycoprotein-1 and may trigger multiple intracellular events within leukocytes to promote vascular inflammation and facilitate atherosclerosis and thrombosis.

Another study has shown that PAC-1 binding was not different among active TA patients, inactive TA patients, and control subjects when the platelets were stimulated with ADP. Although the majority of patients were being treated with aspirin, aspirin intake for 7 days did not affect either PAC-1 binding or P-selectin expression in healthy volunteers (Fig 4), as also reported previously. In contrast, platelet aggregation, another marker for platelet to platelet interaction, was attenuated in response to ADP (10 μmol/L) in both active and inactive patients as compared with controls (Table 2). It is well-established that aspirin, a cyclooxygenase inhibitor, inhibits thromboxane A2 synthesis and secondary aggregation responses triggered by a high concentration of ADP. Thus, it seems likely that differential effects of aspirin in the 2 assays are responsible for the apparent discrepancy of the results. Interestingly, disease activity made no difference to platelet aggregation in response to ADP. We speculate that a conventional regimen consisting of corticosteroid and aspirin for the treatment of TA is adequate for controlling the platelet–platelet response, but cannot overcome platelet–leukocyte interactions, which may be activated by vascular inflammation. If this is true, inhibition of platelet P-selectin may be an important therapeutic approach for TA to suppress inflammation and thrombosis.
Study Limitations
First, this was a cross-sectional study of a relatively small number of patients, with some differences in clinical background. Second, because of the low prevalence of TA, our findings are not supported clinically, because large clinical studies have not been performed to elucidate the effects of disease activity on prognosis or efficacy of antithrombotic agents against thrombotic complications. Further, basic studies on the role of platelet-leukocyte interaction are also lacking. Measuring platelet-leukocyte aggregates could provide supportive information for our findings. Third, the majority of the subjects were postmenopausal women and mean duration of illness exceeded 25 years. On the other hand, the subjects of our additional study (Fig 4) were younger than the subjects of the main study. In addition, all the study subjects were female although 10% of the patients with TA in Japan are males! These age and gender bias could influence the results.

In conclusion, P-selectin expression, but not activated GP Ib/IIIa, was augmented in ADP-activated platelets in patients in the active stage of TA with conventional therapy. Platelet P-selectin may play an important role in the inflammatory and thrombotic disease process associated with intractable TA by inducing platelet-leukocyte interactions.

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References