Platelet P-Selectin Expression Is Associated With Atherosclerotic Wall Thickness in Carotid Artery in Humans

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Background—Recent genetic animal models reveal important roles of platelet P-selectin on progression of atherosclerosis. In the present study, we examine the relation between platelet P-selectin expression and atherosclerotic parameters in 517 subjects.

Methods and Results—Unrelated subjects (n=517; 235 male and 282 female), including 187 with type 2 diabetes, 184 with hypertension, and 366 with hyperlipidemia, were enrolled in the study. P-selectin expression was determined by whole-blood flow cytometry. Arterial stiffness (stiffness index) and arterial wall thickness (intima-media thickness [IMT]) were determined by carotid ultrasound. P-selectin expression was significantly and positively correlated with carotid IMT and stiffness index. Multiple regression analyses showed that the association of the percentage of P-selectin–positive platelets with carotid IMT was independent of other clinical factors. Moreover, the percentage of P-selectin–positive platelets was higher in subjects with carotid plaque and was an independent factor associated with occurrence of carotid plaque analyzed by multiple logistic regression analysis. Finally, the percentage of P-selectin–positive platelets was positively associated with age, body mass index, systolic and diastolic blood pressure, and HbA1c and inversely associated with HDL cholesterol.

Conclusions—Platelet P-selectin is independently associated with atherosclerotic arterial wall changes in human subjects. (Circulation. 2003;108:524-529.)

Key Words: atherosclerosis ■ cell adhesion molecules ■ diabetes mellitus ■ platelets

It was shown that activated platelets were found in the circulating blood of patients with coronary artery diseases. Activated platelets can bind and form aggregates with leukocytes, preferentially monocytes, and the aggregates are shown to be increased in patients with coronary artery diseases. P-selectin (or GMP-140, PADGEM, CD62P), a member of the selectin family, is a 140-kDa glycoprotein that is a component of the α-granule membrane of resting platelets and the Weibel-Palade bodies of endothelial cells. P-selectin mediates rolling of monocytes on activated endothelium, the first step in the cell adhesion cascade. In platelets, after activation induced by agonists such as thrombin or histamine, P-selectin is translocated from α-granule membrane into the surface of plasmatic membrane and mediates adhesion of platelet to neutrophils and monocytes. P-selectin expressed on activated platelets was shown to increase monocyct cell adhesion to endothelial cells. Activated platelets also release proinflammatory cytokines and growth regulatory molecules, resulting in endothelial activation and smooth muscle cell proliferation. These observations suggest that P-selectin expressed on activated platelets may be involved in the initial process of atherosclerotic lesions in vivo. Targeted disruption of the P-selectin gene in the mouse results in marked inhibition of leukocyte rolling along a stimulated vessel wall and in reduction of atherosclerotic lesion formation in apolipoprotein (apo) E-deficient mice, even though these observations do not separate the role of platelet P-selectin from that of the endothelial one.

Recently, evidence has been shown that P-selectin expressed on activated platelet contributes to progression of atherosclerosis in apoE-deficient mice.

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ported that circulating activated platelets and platelet-leu-
koocyte/monocyte aggregates promote formation of atheroscle-
rotic lesions. They showed that the role of activated platelets
in atherosclerosis is attributed to platelet P-selectin
–mediated
delivery of platelet-derived proinflammatory factors to mono-
cytes/leukocytes and the vessel wall. Burger and Wagner 17
also performed bone marrow transplant experiments using
apoE-deficient and apoE/P-selectin double-deficient mice to
show the contribution of platelet P-selectin to the develop-
ment of atherosclerotic lesion. However, no human studies
have been reported examining the relation of platelet
P-selectin with quantitatively determined atherosclerosis. In
the present study, we examined the relation between platelet
P-selectin and morphological and functional parameters of
atherosclerosis in 517 Japanese subjects. We found that
platelet P-selectin expression was significantly and positively
correlated with arterial wall thickness and stiffness of carotid
arteries, with their associations independent of other clinical
factors. Moreover, platelet P-selectin was found to be an
independent factor associated with occurrence of carotid
plaque.

Methods

Study Subjects

This study was approved by the Ethical Committee at Osaka City
University Graduate School of Medicine (approval No. 307), and
informed consent was obtained from all subjects enrolled in the
study. Consecutive subjects (n=517; 235 male and 282 female) who
participated in a medical check program performed at the Diabetes
Center in Osaka City University Hospital (Osaka, Japan) and the
Osaka Health Promotion Center (Osaka, Japan) were enrolled in the
study. Clinical characteristics are summarized in Table 1. One
hundred eighty-seven subjects were diagnosed as having type 2
diabetes, as defined by criteria proposed by American Diabetes
Association.18 Hypertension was defined as a blood pressure higher
than 140/90 mm Hg or the use of known agents for the treatment of
hypertension. Hypercholesterolemia was defined as total cholesterol
higher than 5.2 mmol/L or the use of any drugs for the treatment.

Measurement of P-Selectin Expressed on
Platelet Surface

Platelet surface P-selectin was analyzed by whole-blood flow cy-
tometry (EPICS ELITE EST, Coulter) essentially as described
previously.19 In brief, peripheral blood samples were collected in
0.313% sodium citrate and were fixed in 1.0% formaldehyde/PBS
for 30 minutes at 4°C. After the cells were washed twice with FACS
buffer (0.2% BSA, 0.1% sodium azide/PBS), the cells were double-
stained with FITC-labeled IgG or anti–P-selectin and PE-labeled
anti-CD41 antibody as a platelet identifier. Typical flow cytometric
plots for P-selectin staining are shown in Figure 1. P-selectin
expression was expressed as the percentage of the platelets positive
for the molecule. All antibodies were purchased from Beckman
Coulter, Inc.

Ultrasonography

Ultrasonographic scanning of the carotid artery was performed by an
ultrasonic phase-locked echotracking system, which was equipped
with a high-resolution real-time 10-MHz linear scanner (SSD 650
CL., Aloka Co Ltd). The site of the most advanced atherosclerotic
lesion was examined in the longitudinal and transverse projections to

<table>
<thead>
<tr>
<th>No.</th>
<th>517</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56.8±12.0</td>
</tr>
<tr>
<td>Gender, male/female</td>
<td>235/282</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.1±3.2</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>124.9±16.4</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>76.5±10.2</td>
</tr>
<tr>
<td>Smoking index, cigarette-years</td>
<td>256±424</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/L</td>
<td>6.4±1.8</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.7±1.4</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mmol/L</td>
<td>4.04±1.04</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.57±0.43</td>
</tr>
<tr>
<td>Diabetes</td>
<td>187 (36.1%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>184 (35.6%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>366 (70.8%)</td>
</tr>
</tbody>
</table>

Data are mean±SD.
record the maximum intima-media thickness (IMT).

Carotid plaque was evaluated as previously described. The stiffness index $\text{stiffness} = \ln\left(\frac{P_s}{P_d}\right) - \frac{D_d(D_s - D_d)}{D_s}$, where $P_s$ and $P_d$ were the systolic and diastolic blood pressure and $D_s$ and $D_d$ were the systolic and diastolic inner diameters of the artery.

Biochemical Analyses

Serum levels of creatinine, total cholesterol, and HDL cholesterol were measured by enzymatic methods adapted to an autoanalyzer (Hitachi 7470; Hitachi). Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol. Plasma glucose levels were measured by the glucose oxidation method and hemoglobin A1c (HbA1c) by high-pressure liquid chromatography (normal range, 4.0% to 5.8%).

Statistical Analyses

Statistical analyses were performed with the StatView V software (SAS Institute). Because distribution of P-selectin expression was skewed, nonparametric analysis (Mann-Whitney $U$ test) was performed for comparisons between groups. To evaluate the relation between P-selectin and other factors, simple or multiple regression analyses were performed. Predictive variables including P-selectin for the occurrence of carotid plaques were analyzed by logistic regression analysis. Logarithm transformation of P-selectin positivity (%) $\ln(\text{P-selectin})$ was performed to achieve normal distribution and was used for regression analyses. $P<0.05$ was considered significant.

Results

We first examined the association of the percentage of P-selectin–positive platelets with the atherosclerotic parameters, arterial wall thickness and stiffness (Figure 2). The $\ln(\text{P-selectin})$ significantly and positively correlated with IMT and stiffness of the carotid artery (Figure 2A). When subjects were divided into tertiles based on levels of P-selectin positivity (low, 0 to 0.60%, median 0.31, $n=179$; middle, 0.61 to 1.20%, median 0.88, $n=168$; high, 1.21 to 8.30, median 1.92, $n=170$), IMT in high group was significantly larger than in the low or middle group (Figure 2B). The high group also showed significantly higher stiffness levels than the low or middle group (Figure 2B). Moreover, the percentage of P-selectin–positive platelets was significantly

### TABLE 2. Simple Regression Analyses of the Associations Between the Atherosclerotic and Clinical Parameters

<table>
<thead>
<tr>
<th></th>
<th>IMT</th>
<th>Stiffness β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.457*</td>
<td>0.510*</td>
</tr>
<tr>
<td>Body mass index</td>
<td>0.172*</td>
<td>0.207*</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.289*</td>
<td>0.337*</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.211*</td>
<td>0.288*</td>
</tr>
<tr>
<td>Smoking index, cigarette-years</td>
<td>0.159*</td>
<td>-0.029</td>
</tr>
<tr>
<td>Fasting plasma glucose</td>
<td>0.329*</td>
<td>-0.029</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.457*</td>
<td>0.190*</td>
</tr>
<tr>
<td>Non-HDL cholesterol</td>
<td>0.159*</td>
<td>0.101†</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>-0.030</td>
<td>-0.028</td>
</tr>
<tr>
<td>Platelet counts</td>
<td>0.031</td>
<td>-0.084</td>
</tr>
<tr>
<td>Log(P-selectin)</td>
<td>0.339*</td>
<td>0.205*</td>
</tr>
</tbody>
</table>

*R values are shown.

$^*P<0.01$; $†P<0.05$.

### TABLE 3. Multiple Regression Analyses of Clinical Factors Affecting Atherosclerotic Parameters

<table>
<thead>
<tr>
<th></th>
<th>IMT Model 1</th>
<th>Stiffness β Model 1</th>
<th>Stiffness β Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.312*</td>
<td>0.410*</td>
<td>...</td>
</tr>
<tr>
<td>Gender (male 0, female 1)</td>
<td>-0.099†</td>
<td>0.103†</td>
<td>0.201*</td>
</tr>
<tr>
<td>Body mass index</td>
<td>0.022</td>
<td>0.093†</td>
<td>0.113†</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.073</td>
<td>0.213*</td>
<td>0.268*</td>
</tr>
<tr>
<td>Smoking index</td>
<td>0.062</td>
<td>-0.014</td>
<td>0.005</td>
</tr>
<tr>
<td>Non-HDL cholesterol</td>
<td>0.079</td>
<td>-0.053</td>
<td>-0.007</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.075</td>
<td>-0.019</td>
<td>0.020</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.228*</td>
<td>0.016</td>
<td>0.147†</td>
</tr>
<tr>
<td>Log(P-selectin)</td>
<td>0.182*</td>
<td>0.070</td>
<td>0.115†</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.365*</td>
<td>0.325*</td>
<td>0.200*</td>
</tr>
</tbody>
</table>

All data are β values.

$^*P<0.01$; $†P<0.05$. 

Figure 2. A, Correlation between logarithm-transformed platelet P-selectin positivity and atherosclerotic parameters (intima-media thickness and stiffness index β). P-selectin expression was determined by whole-blood flow cytometry and IMT and stiffness index β (stiffness β) by ultrasound at right carotid artery ($n=517$). B, Differences in IMT or stiffness β among tertile groups based on platelet P-selectin positivity. $^*P<0.05$ vs low or middle group (multiple comparisons, Scheffé type).
higher ($P<0.0001$) in subjects with carotid plaque (median 1.72) than those without it (median 0.80). Table 2 shows the summary of simple regression analyses of the associations between the atherosclerotic and clinical parameters. Besides platelet P-selectin expression, age, body mass index, systolic and diastolic blood pressure, smoking index, fasting plasma glucose, HbA1c, and non-HDL cholesterol were positively correlated with IMT. Associated with stiffness $\beta$ were age, body mass index, systolic and diastolic blood pressure, HbA1c, and non-HDL cholesterol along with platelet P-selectin expression.

To examine if the association of P-selectin expression with atherosclerotic parameters (IMT and stiffness $\beta$) is independent of the other clinical parameters, multiple regression analyses were performed (Table 3). In a model including age, sex, body mass index, systolic blood pressure, smoking index, non-HDL cholesterol, HDL cholesterol, HbA1c, and log(P-selectin) as variables (model 1, $R^2=0.365$, $P<0.0001$), age, gender, HbA1c, and log(P-selectin) were significantly and independently associated with IMT. In a model only including log(P-selectin) and other significant influence factors (age, body mass index, systolic blood pressure, smoking index, non-HDL cholesterol, HbA1c; $R^2=0.356$, $P<0.0001$), log(P-selectin) was still independently associated with carotid IMT ($\beta=0.177$, $P<0.0001$). In model 1, age, gender, body mass index, and systolic blood pressure were significantly and independently associated with stiffness $\beta$ in carotid artery. When age was excluded from this model (model 2), the association between stiffness $\beta$ and log(P-selectin) was significant, suggesting close relation among P-selectin, arterial stiffness, and aging. In our study population, carotid IMT and stiffness $\beta$ showed weak interrelation ($r=0.298$, $P=0.0001$), with distinct clinical factors independently associated.

We next performed multiple logistic regression analyses of factors associated with the presence or absence of carotid plaque (Table 4). In model A, including age, gender, smoking index, systolic blood pressure, HbA1c, non-HDL cholesterol, HDL cholesterol, and log(P-selectin) as independent variables, association between the occurrence of carotid plaque and log(P-selectin) was observed with an adjusted OR of 3.371 (95% CI, 1.302 to 8.733; $P=0.012$). Model B [age, gender, smoking index, presence of diabetes mellitus, hypertension, or hyperlipidemia, and log(P-selectin) as independent variables] also had an adjusted OR of 3.310 (95% CI, 1.292 to 8.478; $P=0.013$) for the association between carotid plaque and log(P-selectin), suggesting that the P-selectin expression on platelet surface is an independent factor associated with the occurrence of carotid plaque.

Finally, we examined factors associated with the percentage of P-selectin–positive platelets. The percentage of P-selectin–positive platelets was not significantly different ($P=0.3257$) between male (median 0.90%) and female (median 0.81%) subjects. Diabetic subjects (median 1.42%) showed significantly ($P<0.0001$) higher P-selectin–positive platelets than nondiabetic subjects (median 0.70%). Diabetic subjects (median 1.42%) showed significantly ($P<0.0001$) higher P-selectin–positive platelets than nondiabetic subjects (median 0.70%). P-selectin–positive platelets were also significantly ($P<0.0001$) higher in subjects with hypertension (median 1.01%) than without hypertension (median 0.71%). There was no significant difference between subjects with (median 0.80) and without hyperlipidemia (median 0.82). Table 5 shows the results of simple regression analyses between log(P-selectin) and clinical factors in all subjects. Log(P-selectin) expression positively correlated with age, body mass index, systolic and diastolic blood pressure, fasting plasma glucose, and HbA1c. It also correlated negatively with HDL cholesterol. Among these factors, multiple regression analyses showed that age, HDL cholesterol, and HbA1c were independently associated with log(P-selectin) (data not shown).

**Discussion**

It is becoming clear that P-selectin is essential for progression of atherosclerosis. P-selectin deficiency reduces atheroscler-
rotic lesion formation in mice.\textsuperscript{14,15,24,25} Deficiency of P-selectin inhibits neointimal formation after arterial injury.\textsuperscript{26,27} Besides the role of endothelial P-selectin in monocyte recruitment to the atherosclerotic lesion, recent observations unveil involvement of platelet P-selectin on progression of atherosclerosis.\textsuperscript{36,37} In this study, we now showed in human studies (n = 517) that the platelet P-selectin was significantly and positively correlated with morphological parameters of atherosclerosis, IMT, that are quantitatively determined, with their associations independent of other clinical factors. We also showed that platelet P-selectin was found to be an independent factor associated with occurrence of carotid plaque.

Previous reports revealed that in patients with coronary artery diseases, significant populations of platelets were activated.\textsuperscript{1–3} Only limited reports showed the association between platelet activation and quantitatively determined atherosclerotic parameters.\textsuperscript{28,29} Salonen and Salonen\textsuperscript{28} revealed that ADP-induced platelet aggregability was significantly associated with 2-year increase of carotid IMT in 126 Eastern Finnish middle-aged men in a multivariate regression model. Kawamori et al\textsuperscript{30} showed in 27 type 2 diabetic patients that plasma β-thromboglobulin and platelet factor 4 were significantly higher in patients with carotid atherosclerosis. Platelet function tests used in these studies were platelet aggregation and the measurement of plasma platelet-specific proteins, which have some problems, including low sensitivity and poor specificity. Recently, increasing numbers of published studies have attempted to use whole-blood flow cytometry as a platelet function test in clinical settings.\textsuperscript{19} Whole-blood flow cytometry has the advantage of directly analyzing individual platelets with a high degree of sensitivity in their native milieu and with minimal artificial platelet activation.\textsuperscript{19} Using advantage of this whole-blood flow cytometry technique, we demonstrated that the P-selectin–positive platelets were significantly associated with atherosclerotic wall thickness that was quantitatively determined by carotid ultrasound. It has been shown that wall thickness of carotid artery is greater in patients with diabetes mellitus than nondiabetic subjects.\textsuperscript{30,31} In our study, multiple regression analyses showed that association of P-selectin with arterial wall thickness was independent of HbA1c or presence of diabetes. We also demonstrated that the percentage of P-selectin–positive platelets was found to be associated with carotid plaque formation, which was independent of other major risk factors. As shown in our recent reports,\textsuperscript{23} age and HbA1c (or presence of diabetes) were the other factors significantly associated with plaque occurrence in carotid artery. Taken together, our human study is in good agreement with experimental observations in genetically modified mice study, with platelet P-selectin expression closely associated with atherosclerosis.

The stiffness index $\beta$, representing the arterial wall stiffness, was shown to increase with age, hypertension, diabetes, insulin resistance, and the severity of coronary atherosclerosis.\textsuperscript{22,23,32,33} In our present study, the platelet P-selectin expression was also associated with carotid arterial stiffness, suggesting a potential role of platelet P-selectin on functional alteration of arterial wall. It is well-known that activated platelets secrete numbers of vasoactive substances, including thromboxane A2, which may result in increase in vascular tonus and arterial stiffness. Additional basic and clinical studies are necessary to elucidate the role of platelet P-selectin on altered arterial function.

Increased percentage of P-selectin–positive platelets has been reported in patients with diabetes.\textsuperscript{34,35} Hypercholesterolemia,\textsuperscript{36,37} hypertension,\textsuperscript{38} and smoking.\textsuperscript{39} Some reports showed no relation of platelet P-selectin with plasma glucose\textsuperscript{40} and hypertension.\textsuperscript{35} These inconsistent results may be attributable to relatively small numbers of subjects (less than 50) enrolled in the studies. We could show using much larger numbers of subjects (n = 517) that age, hypertension, diabetes, obesity, and HDL cholesterol were the candidate factors determining platelet P-selectin expression. Finally, our present study does not negate the possibility that the increase in circulating P-selectin–positive platelets is the secondary result of increased atherosclerosis (ie, that circulating platelets may become activated as a result of the vessel wall disease).

In summary, we showed for the first time in human studies that platelet P-selectin was significantly and positively correlated with morphological and functional parameters of atherosclerosis that are quantitatively determined. Our data also showed that platelet P-selectin was found to be an independent factor associated with occurrence of carotid plaque.

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