Serial Changes in Platelet Activation in Patients After Ischemic Stroke
Role of Pharmacodynamic Modulation

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Background and Purpose—Enhanced platelet activity has previously been reported in the acute phase after ischemic stroke. We tested the hypothesis that activated platelets (expressed by CD62p) are substantially increased in the acute stage after a stroke and decrease thereafter, and that antplatelet therapies can suppress CD62p expression.

Methods—We serially examined platelet CD62p expression using flow cytometry after acute ischemic stroke in 87 consecutive patients. The CD62p expression was also evaluated in 20 healthy volunteers and 33 at-risk control subjects.

Results—CD62p expression was significantly higher in the acute phase after ischemic stroke than in normal and at-risk control subjects (both \( P < 0.0001 \)). CD62p expression decreased to a significantly lower level on day 21, and to a substantially lower level on day 90. CD62p expression was not significantly suppressed by warfarin. However, CD62p expression was significantly suppressed by aspirin treatment (\( P = 0.024 \)) and more substantially suppressed by clopidogrel (\( P < 0.0001 \)) on day 90. Furthermore, only clopidogrel treatment (\( P = 0.0016 \)) was significantly independently associated with decreased CD62p expression on day 90.

Conclusions—Platelet activation was significantly increased in acute ischemic stroke and substantially decreased thereafter. The lesser long-term pharmacodynamic potency of aspirin relative to clopidogrel raises the prospect of the need for more effective antplatelet agents or a synergistic combination therapy for stroke prevention in the future. (Stroke. 2004;35:1683-1687.)

Key Words: stroke, ischemic  platelet activation  antiplatelet therapy

Current studies have demonstrated that increased numbers of activated platelets are found after acute atherothrombotic stroke\(^1,2\) and also in the chronic phase after ischemic stroke.\(^3\) Inhibition of platelet aggregation is believed to be one of the most important strategic managements in secondary prevention after a stroke.\(^4,5\) Surprisingly, the therapeutic decision for antplatelet agents for secondary prevention after a stroke is not based on scientific methods, such as an evaluation of platelet activation, in individual patients.

Many experimental methods such as aggregometry,\(^6\) assessment of plasma levels of products released from platelets,\(^7\) urine excretion of thromboxane metabolites,\(^2,4\) and human platelet antigen Ia/Ib genotyping\(^8\) have been developed to monitor platelet function. However, these methods have recently been challenged for their reliability and usefulness in daily clinical practice.\(^9\) However, measurement of platelet P-selectin (CD62p) expression by flow cytometry appears to be a simple and useful tool to assess platelet activity.\(^10\) Accordingly, we tested the hypothesis that CD62p expression is substantially increased in the acute stage after a stroke and decreases thereafter, and that antplatelet agents can suppress CD62p expression after the acute phase of an ischemic stroke.

Materials and Methods

Study Patients
This study included consecutive patients admitted for acute ischemic stroke to our hospital between October 2002 and April 2003. An estimated sample size of 100 patients was based on the effective size with an \( \alpha = 0.05 \), a power of 80%, a difference in platelet activity between the study and normal volunteers of 0.6%, and a variance of 6.0%, and also assuming a 20% rate of protocol violations and incomplete follow-up. Stroke was defined as sudden onset of loss of global or focal cerebral function that persisted for \( > 24 \) hours. We excluded patients with intracranial hemorrhage, a history of recent surgery or trauma within the preceding 3 months, abnormal liver function, renal insufficiency (creatinine \( > 1.5 \) mg/dL), malignancy, febrile disorders, and acute or chronic inflammatory disease at study entry, as well as those with a history of atrial fibrillation, valvular heart disease, or intracardiac thrombus on echocardiograph, and

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those with an expected survival time of <90 days. Patients were also excluded if fever, infection, or death occurred after the stroke within the study period. Over a period of 7 months, we prospectively recruited 113 consecutive patients with ischemic stroke that had occurred <48 hour before blood sampling. Twenty-six (23.0%) of the 113 patients were subsequently excluded because of fever (15 patients), incomplete follow-up (4 patients), or intracranial hemorrhage (2 patients) that occurred later after the ischemic stroke and that required emergency surgical intervention. Another 5 patients (4.4%) who died within 90 days after the stroke were also excluded. Therefore, the remaining 87 patients constituted the study population.

Thirty-three subjects matched with respect to age, gender, hypertension, diabetes mellitus, current smoking, and hypercholesterolemia served as risk factor control subjects. We also studied 20 age-matched and gender-matched healthy volunteers. Informed consent was obtained from all study subjects. The study protocol was approved by the Institutional Review Committee on Human Research in our institution.

Neurological Assessment
The physical and social functions of stroke patients were evaluated using the modified Rankin scale score during the acute, convalescence, and chronic phases of stroke.

Imaging Studies and Laboratory Investigations
In addition to a full clinical assessment, ancillary examinations were performed including complete blood counts, biochemical data, a chest radiograph film, routine brain computed tomography (CT) and magnetic resonance imaging (MRI)/MR angiography (MRA), duplex scanning of the carotid arteries, and routine cardiac analysis consisting of 12-lead echocardiogram (EKG) and echocardiography.

Blood Sampling and Assessment of Platelet Activity
In stroke patients, blood samples were obtained within 48 hours of stroke onset and on days 7, 21, and 90 after stroke occurrence. Blood sample was obtained once in normal control and 4 times (days 1, 7, 21, and 90) in at-risk control subjects. Blood samples were obtained in a fasting, nonselective state between 09:00 and 10:00 in the control and study groups to exclude the possible influence of circadian variations. Blood was obtained under minimal tourniquet pressure from the antecubital vein using a sterile 22-gauge needle syringe in a single attempt with the study subjects in a sitting position for at least 5 minutes. Three milliliters of blood was drawn into a Vacutainer containing 3.8% buffered sodium citrate. Mixtures of blood and sodium citrate were centrifuged (model 5400; Kubota Corp) for 15 minutes at 1500 rpm at room temperature. The supernatant platelet-rich plasma was used to assess the platelet activity.

Platelet activity was determined with respect to α-granule degranulation (surface expression of P-selectin or the CD 62p antigen). Details of the procedure have been described previously. In brief, a fluorescein isothiocyanate-conjugated antibody to glycoprotein IIb/IIIa (CD61; Becton Dickinson) was used as an activation-independent marker of platelets. A phycoerythrin (PE)-conjugated anti-CD62 antibody (Becton Dickinson) was used to assess α-granule degranulation. The association of ligands with platelets was determined with a fluorescence-activated cell sorter (FACSCalibur system; Becton Dickinson). The control ligand (IgG-PE conjugate) was used to detect any nonspecific association and to permit the definition of a threshold for activation-dependent binding. The percentage of platelets expressing P-selectin was defined as the fraction exhibiting specific binding (ie, being CD62p-positive) minus that exhibiting nonspecific binding (ie, the percentage defined with the IgG-PE conjugate) from the 10,000 platelets sorted. The assays for P-selectin expression in each blood sample were performed in duplicate, with the mean level reported.

Medications
Aspirin was the first choice for acute stroke patients unless they were allergic or intolerant to aspirin, such as having a history of peptic ulcer or upper gastrointestinal tract bleeding during aspirin therapy. Clopidogrel was used in patients who could not tolerate aspirin therapy. If there were thrombi in the carotid artery confirmed by duplex scanning, heparinization was administered and followed by long-term warfarin therapy. Therefore, 53 patients received aspirin, 26 patients received clopidogrel, and 13 patients received warfarin after the stroke for secondary prevention.

Statistical Analysis
Data were expressed as mean±SD. Categorical variables were compared using χ² or Fischer exact test. Univariate analyses were performed using Student t test. Continuous variables among 3 groups were compared using 1-way ANOVA for parametric data and Kruskal–Wallis test for nonparametric data, followed by post hoc multiple comparison procedure. Repeated measures of ANOVA were used for comparison of platelet activity among 4 intervals (<48 hours, and on days 7, 21, and 90) and individual effect of 3 drugs in suppression of platelet activation among 3 intervals (<48 hours, and on days 21 and 90). We used Scheffe multiple comparison to analyze the intradividual course of parameters over time and to compare parameters of different groups. Arcsine transformation of CD62p expression on platelets was used to improve the normality for statistical analysis; P<0.05 was considered statistically significant.

Results
Baseline Characteristics of Study Patients
The baseline characteristics among 3 groups are shown (Tables 1 and 2). The systolic and diastolic blood pressures on presentation were significantly higher in stroke patients than in at-risk control and normal control subjects. The incidence of percutaneous coronary intervention before the study entry was significantly higher in the at-risk control subjects than in stroke patients. White blood cell counts were significantly higher in stroke patients than in the at-risk control and normal control subjects at any time point after ischemic stroke (P<0.005). Platelet counts were significantly lower in stroke patients and at-risk control subjects compared with healthy subjects. However, there was no significant difference in platelet counts between stroke patients and at-risk control subjects at any time point after ischemic stroke.

Serial Changes in Circulating Platelet Activity
Data on platelet expression of P-selectin among the 3 groups and serial changes in platelet activity in stroke patients are shown on Figure 1. The intra-assay variability based on repeated measurements of the same blood sample was low, with mean coefficients of variance of 7.3% (within 48 hours), 6.8% (on day 7), 6.9% (on day 21), and 6.6% (on day 90) in stroke patients; 5.9%, 6.3%, 5.6%, 6.6% in 4 time intervals in at-risk control subjects; and 5.6% in normal subjects, respectively.

CD62p expression was significantly higher in patients during the acute phase of stroke (3.4±2.2%) than in at-risk control subjects (1.6±0.7%) and healthy subjects (1.4±0.6%) (both P<0.0001). There was no significant difference in CD62p expression in the acute stroke period (3.4±2.2% within 48 hours versus 3.2±2.4% on day 7; P=0.86). However, CD62p expression substantially decreased over time after stroke, being 3.4±2.2% in the acute
stage versus 2.6±2.6% on day 21 (P<0.01). It remarkably decreased on day 90 (3.4±2.2% versus 2.1±1.8%; P<0.0001). CD62p expression showed no significant difference between days 21 and 90 after stroke. CD62p expression remained significantly higher in stroke patients on day 21 (P=0.002) and on day 90 (P=0.013) than in normal subjects. Moreover, CD62p expression was also significantly higher in stroke patients on day 21 than in at-risk control subjects (P=0.013). However, this difference was no longer significant between stroke patients and at-risk control subjects on day 90. CD62p expression did not differ between the 2 control groups. Moreover, there was no significant difference in CD62p expression in at-risk control subjects at any time point of study period (P=0.87).

Pharmacodynamic Modulation of CD62p Expression
CD62p expression did not differ among patients treated with aspirin, clopidogrel, or warfarin in the acute phase (<48 hours). However, on day 90, CD62p expression was significantly suppressed by clopidogrel treatment compared with either aspirin or warfarin treatment (P=0.028) (Figure 2). Moreover, repeated measures of ANOVA demonstrated that CD62p expression was most remarkably suppressed by clopidogrel (P<0.0001), followed by aspirin (P=0.024), but was not significantly suppressed by warfarin (P=0.86) on day 90 after stroke.

Two-way ANOVA was used to assess the association between different variables (age, sex, cardiovascular risk

| TABLE 1. Baseline Characteristics of Stroke in At-Risk and Normal Control Groups |
|--------------------------|-------------------|-----------------|------------------|-----------------|
| Variables                | Study Patients    | At-Risk Control | Normal Control   | P               |
| Age (y) (mean±SD)        | 66.3±10.9         | 66.1±8.6        | 63.2±6.9         | 0.44            |
| Male % (n)               | 58.6 (51)         | 54.5 (18)       | 55.0 (11)        | 0.90            |
| Hypertension % (n)       | 77.0 (67)         | 72.7 (24)       | —                | 0.62            |
| Diabetes mellitus % (n)  | 43.7 (38)         | 39.4 (13)       | —                | 0.76            |
| Current smoking % (n)    | 25.3 (22)         | 27.3 (9)        | —                | 0.82            |
| Hypercholesterolemia % (n)| 54.0 (47)     | 51.5 (17)       | —                | 0.81            |
| Previous stroke by history % (n) | 27.6 (24) | —                | —                | —               |
| Previous stroke by MRI % (n) | 36.8% (32)   | —                | —                | —               |
| Coronary angioplasty % (n) | 21.8% (19)    | 51.5% (17)      | —                | 0.0015          |
| Systolic BP (mm Hg)      | 164.0±27.3*       | 138.2±13.5†     | 128.5±11.6†      | <0.0001         |
| Diastolic BP (mm Hg)     | 89.4±19.5*        | 75.2±8.6†       | 76.0±7.2†        | <0.0001         |
| Body temperature (°C)    | 36.6±0.5          | 36.4±0.5        | 36.7±0.4         | 0.09            |

Modified Rankin scale score

| Score on presentation ≥3, % (n) | 85.1 (74) | — | — | — |
| Score on day 90 ≥3, % (n)      | 21.8 (19) | — | — | — |
| Creatinine (mg/dL)             | 1.1±0.7   | 1.1±0.3 | 1.0±0.2 | 0.77 |
| Calcium (mg/dL)                | 8.8±0.4  | 9.0±0.3 | 9.1±0.3 | 0.16 |
| Hs-CRP (mg/L)                  | 6.0±6.7* | 2.2±2.4† | 1.4±1.3† | <0.0001 |

* and † indicate significant difference (at 0.05 level) by Tukey multiple comparison procedure.

| TABLE 2. Serial Changes of WBC and Platelets in Patients After Ischemic Stroke |
|------------------------|-------------------|-----------------|-----------------|-----------------|
| Variables              | <48 Hours         | Day 7           | Day 21          | Day 90          | P               |
| WBC (×10^3/mL)         |                   |                 |                 |                 |                 |
| Study patients (n=87)  | 7.5±2.0*          | 7.3±4.0†        | 6.9±2.8‡        | 7.1±3.3‖        | 0.324           |
| At-risk control (n=33) | 5.9±1.3†          | 5.7±1.8|         | 5.6±2.8∥        | 6.0±1.2∥        | 0.421           |
| Normal control (n=20)  | 5.4±0.8†          |                 |                 |                 |                 |
| Platelet (×10^3/mL)    |                   |                 |                 |                 |                 |
| Study patients (n=87)  | 21.1±5.5‡         | 22.8±5.8        | 22.1±3.8        | 21.8±4.3        | 0.621           |
| At-risk control (n=33) | 22.3±6.0§         | 23.2±4.9        | 22.1±8.0        | 22.6±7.0        | 0.648           |
| Normal control (n=20)  | 24.5±4.8§         |                 |                 |                 |                 |

Data are expressed as mean±SD of patients. WBC indicates white blood cells.

Symbols (*, †, ‡, §, ‖) indicate significant difference (at 0.05 level) by Tukey multiple comparison procedure.

* versus †, P<0.0001.
‡ versus §, P=0.019.
§ versus ‖, P<0.005 at any time point after ischemic stroke.
factors, previous stroke and aspirin, warfarin or clopidogrel treatment) and platelet activity. On day 90, only clopidogrel treatment (1.4 ± 0.8 versus 2.4 ± 1.9; \( P = 0.0016 \)) was significantly independently associated with decreased CD62p expression.

**Discussion**

The present study, in which we examined circulating platelet activation expressed by CD62p in patients after acute ischemic stroke, produced 3 major findings. First, CD62p expression was significantly higher in acute stroke patients compared with at-risk control subjects and healthy volunteers. Second, CD62p expression substantially decreased over time after the stroke. However, CD62p expression remained significantly higher in stroke patients in the convalescence stage than in both control groups and in the chronic stage compared with healthy subjects. Finally, after 3 months of follow-up, clopidogrel treatment was associated with significantly decreased CD62p expression.

**Kinetic Changes in CD62p Expression After Acute Ischemic Stroke**

Recently, Marquardt et al. demonstrated that CD62p expression was higher on day 1 after a stroke than in normal and at-risk control subjects, and that the expression decreased rapidly thereafter. They suggested that such a rapid and dramatic decrease in CD62p expression was attributed to the shedding of CD62p from cell surfaces. In our study, we also found the same kinetic changes in CD62p expression. P-selectin, which is a platelet \( \alpha \)-granule protein, serves as an adhesion receptor involved in the binding of platelets to leukocytes during the inflammatory reaction. We propose that in addition to shedding of CD62p from cell surfaces, diminution of the underlying inflammatory process after stroke also contributes, in part, to decreased CD62p expression in the convalescence and chronic phases after stroke. Given that a substantial percentage of our patients had pre-existing cardiovascular or cerebrovascular disease, it is not surprising to observe enhanced platelet activity in the chronic phase after a stroke caused by shear stresses as a result of narrowing of the vascular trees in our patients. Our results are concordant with recent studies, which demonstrated that persistently increased platelet activation in stroke patients could, in part, be the result of the chronic inflammatory process, vascular risk factors, diffuse atherosclerotic lesions, or the extent of vascular damage caused by a stroke.

**Pharmacodynamic Modulation of CD62p Expression**

A previous study demonstrated that there was no correlation between platelet activity and the coagulation status. Despite similar international normalized ratio, platelet reactivity showed great individual variation. Therefore, there are still controversial results regarding the effect of warfarin on platelet activity. In our study, CD62p expression with warfarin therapy did not differ between the acute and chronic stages after a stroke. Our result corroborates the findings of recent studies that observed that warfarin therapy did not affect CD62p expression. Although our study was not designed to test whether persistent CD62p expression is a risk factor for recurrent ischemic events, our finding should raise suspicion about whether anticoagulants are effective in stroke prevention in patients with ischemic events other than cardioembolic events.

Antiplatelet therapy has been a mainstay of primary and secondary stroke prevention. Despite numerous large clinical trials showing a risk reduction for cardiovascular and cerebrovascular events in patients using aspirin, some investigators found that aspirin substantially suppressed platelet activation, whereas other investigators found partially suppressed or unchanged CD62p expression after treatment with aspirin. In our study, although aspirin therapy was used in the majority (57.5%) of our patients, aspirin therapy
was not significantly independently associated with decreased CD62p expression on day 90 after stroke using 2-way ANOVA. Perhaps our findings may help explain why aspirin is only partially effective in preventing thrombotic events and degranulation of α-granules. However, we found that clopidogrel therapy was significantly independently associated with decreased CD62p expression on day 90 after stroke. Our results indicate that clopidogrel has clear advantages over aspirin in terms of suppression of CD62p expression; therefore, these results further confirm recent other observations.15,20

Study Limitations
There are several limitations to this study. First, although we demonstrated that aspirin therapy is not associated with decreased CD62p expression on day 90 after the stroke using 2-way ANOVA, we could not completely exclude the role of aspirin in primary and secondary prevention of ischemic stroke. Second, physiological 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 be reflected in changes in platelet physiological function. Finally, we did not evaluate the association between the extent of brain infarction, clinical outcomes, and the fraction of circulating platelets expressing P-selectin. Neurological scores might have correlated better with the site of brain infarct rather than the extent of brain infarction.

In conclusion, platelet activation, as reflected by measurements of CD62p expression of circulating platelets, is present in the acute phase of stroke and substantially decreases over time after a stroke. In addition, CD62p expression remained significantly higher among stroke patients in the convalescence and chronic phases as compared with healthy volunteers. As compared with aspirin, clopidogrel treatment was associated with significantly decreased CD62p expression in the convalescence and chronic phases of ischemic stroke.

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