Course of Platelet Activation Markers After Ischemic Stroke

Lars Marquardt, Cand Med; Andreas Ruf, MD; Ulrich Mansmann, PhD; Ralph Winter, MD; Matthias Schuler, MD; Florian Buggle, MD; Horst Mayer, MD; Armin J. Grau, MD

Background and Purpose—The aim of this study was to evaluate the time course of platelet activation after ischemic stroke and to investigate whether platelet activation and inflammation are correlated with each other.

Methods—We serially determined expression of p-selectin (CD62p) and lysosome-associated membrane protein (CD63) by platelets using flow cytometry at 10 time points between days 1 and 90 in patients after ischemic stroke (n=50), in healthy subjects (n=30), and in risk factor control subjects (n=20). Furthermore, we correlated leukocyte count, C-reactive protein, and fibrinogen levels with platelet activation markers.

Results—CD62p and CD63 expression was higher on day 1 after stroke than in both control groups (P<0.005 for both). CD62p expression rapidly declined, whereas CD63 expression remained significantly elevated until day 90. Stroke severity and different medication for secondary stroke prevention did not influence CD62p or CD63 expression. Platelet activation markers and inflammatory parameters were not correlated with each other at any time point after stroke.

Conclusions—The initial increase in both CD62p and CD63 expression by platelets is followed by a differential regulation of both parameters after stroke. The rapid decrease in CD62p expression may be caused by shedding from the cell surface. Its persistent elevation makes CD63 a good candidate for studies on predictors for stroke recurrence. Our findings suggest that the expression of CD62p and CD63 by platelets is regulated independently from inflammatory indexes. (Stroke. 2002;33:2570-2574.)

Key Words: flow cytometry • inflammation • platelets • stroke

Platelet activation is a crucial mechanism in arterial thrombogenesis and therefore in the pathophysiology of ischemic stroke.1–4 Accordingly, antiplatelet therapy plays a central role in secondary prevention of ischemic stroke. Currently, decisions for antiplatelet drugs after stroke are based on clinical grounds but not on platelet activation studies in individual patients because of a lack of clinically applicable methods to monitor platelet function. Artifactual platelet activation in vitro represents the main problem in assessing platelet function. Rapid fixation of blood after venipuncture combined with flow cytometric measurement of activation-dependent platelet neoantigens may be a useful tool to overcome these methodical problems. Two articles recently showed that patients with acute cerebral ischemia have an excess of circulating platelets that express p-selectin (CD62p) and lysosome-associated membrane protein (CD63).5,6 P-selectin is a constituent of the membrane of α-granules and serves as an adhesion receptor mediating the binding of platelets to leukocytes. CD63 is part of platelet lysosomes and may protect the plasma membrane from degradation by lysosomal enzymes. Both p-selectin and CD63 are expressed exclusively on platelet activation.7,8 The time course of these platelet activation markers after stroke has not yet been assessed. Whether their elevation during the subacute phase after stroke could predict recurrent ischemic events and whether they may be helpful in monitoring antiplatelet therapy are of interest. We tested the hypothesis that both platelet activation markers are highly increased in the acute stage after stroke and decline thereafter but remain elevated compared with control groups during the subacute stage. Furthermore, we analyzed whether inflammatory markers [C-reactive protein (CRP), fibrinogen, leukocytes] that were shown to be increased after stroke9–13 are correlated with platelet activation parameters.

Subjects and Methods

We investigated 53 patients within 24 hours after acute ischemic stroke, 30 presumably healthy age- and sex-matched control subjects, and 20 risk factor control subjects who were matched with patients in terms of age, sex, hypertension, diabetes mellitus, current smoking, and hyperlipidemia. Three patients died within 90 days after stroke and were excluded from the analyses. The Table gives demographic and clinical data of the groups. Exclusion criteria for all subjects were trauma, surgery, or acute organ ischemia within the preceding 3 months (eg, before the index stroke); severe liver disease; renal failure; cancer; chronic inflammatory diseases; and fever or acute inflammatory or infectious conditions at study entry. Infections frequently occur shortly before...
Demographic and Clinical Characteristics of Patients and Control Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n=50)</th>
<th>Healthy Control Subjects (n=30)</th>
<th>Risk Factor Control Subjects (n=20)</th>
</tr>
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<tbody>
<tr>
<td>Mean±SD age, y</td>
<td>63.5±12.2</td>
<td>61.1±13.8</td>
<td>64.1±12.3</td>
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<tr>
<td>Women, n (%)</td>
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<td>11 (37)</td>
<td>7 (35)</td>
</tr>
<tr>
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<td>24 (48)</td>
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<td>11 (55)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>14 (28)</td>
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<td>4 (20)</td>
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<tr>
<td>Hyperlipidemia, n (%)</td>
<td>18 (36)</td>
<td>...</td>
<td>6 (30)</td>
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<tr>
<td>Current smoking, n (%)</td>
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<td>7 (35)</td>
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<td>8 (40)</td>
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<td>Cardiac diseases,† n (%)</td>
<td>24 (48)</td>
<td>...</td>
<td>9 (45)</td>
</tr>
<tr>
<td>Stroke origin, n (%)</td>
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<tr>
<td>Large-artery atherosclerosis</td>
<td>17 (34)</td>
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<tr>
<td>Cardioembolism</td>
<td>9 (18)</td>
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<tr>
<td>Microangiopathy</td>
<td>6 (12)</td>
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<td>Other or unknown</td>
<td>18 (36)</td>
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<tr>
<td>NIH Stroke Scale, median (range)</td>
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<tr>
<td>Day 1</td>
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<td>Day 14</td>
<td>2 (0–21)</td>
<td>...</td>
<td></td>
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<tr>
<td>Day 90</td>
<td>1 (0–18)</td>
<td>...</td>
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</table>

NIH indicates National Institutes of Health.
*Hypercholesterolemia or hypertriglyceridemia.
†Eg, coronary heart disease, cardiac valve disease.

and after stroke. Therefore, we did not retrospectively exclude patients with an infection after stroke or patients with a history of infection shortly before stroke. However, we systematically assessed symptoms of infection and body temperature during the whole study period to recognize a possible influence of infection on the variables. Eight patients had evidence of an infection during the week before stroke, and 22 patients had elevated body temperature (>37.5°C) and/or symptoms of infection at ≥1 measurement after stroke.

For healthy control subjects, any vascular diseases or risk factors were additional exclusion criteria. Subjects with a history of myocardial infarction or stroke were excluded from the risk factor control group. Vascular risk factors were defined as follows: arterial hypertension, on antihypertensive treatment or blood pressure ≥160/90 mm Hg at 2 readings before stroke or >5 days after stroke; diabetes mellitus, on antidiabetic treatment or elevated hemoglobin A1c or elevated blood glucose at ≥2 readings before stroke or >5 days after stroke; hyperlipidemia, on lipid-lowering medication or total cholesterol >220 mg/dL or triglycerides >180 mg/dL before stroke; and carotid stenosis, diameter reduction of ≥50% of the internal carotid artery on Duplex sonography.

All stroke patients underwent a cranial CT excluding cerebral hemorrhage. The clinical deficit after stroke lasted for at least 24 hours. The origin of cerebral ischemia was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria.14 Stroke severity was assessed with the National Institutes of Health Stroke Scale on days 1, 14, and 90 after ischemia.15 Patients received aspirin (n=24), clopidogrel (n=7), or oral (n=14) or subcutaneous (n=3) anticoagulation or refused any medication (n=2) for secondary prevention after stroke. None of the patients suffered from a new cerebral or cardiac ischemic event during the 90 days of follow-up. The study was approved by the local institutional review board. The participating subjects gave informed consent.

In stroke patients, blood samples were taken within 24 hours and on days 2, 3, 5, 7, 10, 14, 28, 45, and 90 after ischemia. Measurements were performed once in both control groups. In 10 healthy control subjects (5 women), we assessed the intraindividual variability of platelet activation parameters by analyzing 3 different samples taken at least 1 week apart. Serum and citrated plasma samples were collected at each venipuncture and were stored at −86°C until measurements.

Venipuncture of forearm veins was performed with minimal stasis. We used a 2-syringe technique to reject the first few milliliters of the sample. Peripheral venous blood was collected into an aldehyde-based fixation solution that blocks metabolic processes within milliseconds.5,16 This fixation process was shown to preserve CD62p and CD63 for at least 24 hours at room temperature. The fraction of circulating platelets that express the activation markers p-selectin and CD63 was determined by flow cytometry as described previously.5,16 Briefly, the samples were incubated with saturating concentrations of fluorescein isothiocyanate conjugated (FITC)–labeled antibodies (Immunotech) against p-selectin (Clone CLBThromb6) or CD63 (Clone CLBGran12) and with R-phycoerythrin (PE)–labeled antibodies against CD41a (clone P2) for 15 minutes at room temperature in the dark (double immunolabeling). The antibody clone P2 binds to the fibrinogen receptor (glycoprotein IIb/IIIa; CD41/CD61) of platelets independently of the activation state of the receptor. As control experiments, platelets were incubated with FITC-coupled unspecific mouse IgG1 (Immunotech) with the same fluorochrome-to-protein ratio and concentration as the specific IgG. These controls yielded the same fluorescence as platelets whose binding of FITC-labeled specific antibodies was blocked by an excess of unlabeled antibodies of the same clone. After immunolabeling, the samples were analyzed by FACScan (Becton Dickinson). Forward light scatter and expression of CD41a were used to discriminate platelets from other blood cells. Platelet-bound anti-p-selectin or anti-CD63 antibodies were then determined by analyzing 5000 platelets for FITC fluorescence. Results were expressed as percentage of antibody-positive platelets, defined as those with a fluorescence intensity exceeding that of 98% to 99% of the control platelets.

CRP was determined by an ultrasensitive turbidimetric test based on latex beads (Quantex CRP Ultrasensitive, Instrumentation Laboratory). The threshold of detectability was 0.01 mg/dL; interassay and intra-assay variabilities were <3%. Fibrinogen was measured by functional coagulation testing (derived fibrinogen) with Recomb-
plastin (Instrumentation Laboratory) as reagent. The white blood count was determined by Coulter counter analysis.

Data are presented as median and percentiles because not all parameters were normally distributed. We used the Friedman test to analyze the intraindividual course of parameters over time, the Kruskal-Wallis and Mann-Whitney U-test to compare different groups, and the Spearman rank correlation coefficient to correlate various parameters. Analysis of variance (ANOVA) was applied to analyze the influence of various factors on a parameter. To avoid multiple statistical tests, comparisons between groups were limited to days 1, 14, and 90 after stroke and the Spearman rank correlation coefficient to correlate various parameters. Analysis of variance (ANOVA) was applied to analyze the influence of various factors on a parameter. To avoid multiple statistical tests, comparisons between groups were limited to days 1, 14, and 90 after stroke.

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Results

The intra-assay variability based on repeated measurements of the same sample was low (mean coefficients of variation: CD62p, 0.023; CD63, 0.039). Repeated measurements in control subjects showed a low to moderate intraindividual variability over time (mean coefficients of variation, 0.15 for both CD62p and CD63).

The percentage of circulating platelets expressing CD62p was higher in stroke patients on day 1 after ischemia (n=50; median, 2.83%) than in healthy control subjects (n=30; median, 1.38%; P<0.001) and was higher than in subjects with vascular risk factors (n=20; median, 1.41%; P=0.003). CD62p expression declined over time after stroke (P<0.001). The difference between the stroke group and both control groups was no longer significant on days 14 and 90 after stroke. The 2 control groups did not differ in CD62p expression (median, 2.97% for both) and ‡patients and risk factor control subjects (P=0.84). CD63 expression was not different between both control groups (P=0.79) (Figure 2). In 17 patients (34%), CD63 expression did not change by more than ±1% between days 1 and 90; 15 patients (30%) showed an increase and 18 patients (36%) showed a decrease over time. The variability of CD63 expression was particularly high in the acute stage after stroke and lessened later, indicating lower interindividual differences in platelet activation in the subacute stage.

Age, sex, vascular risk factors, and history of infection before stroke did not influence the results for CD63. Stroke severity was not correlated with CD63 expression at any time point (R<0.20; P>0.1). CD63 expression on day 1 was influenced by stroke origin (P=0.045, ANOVA), with cardioembolism being associated with the highest values (median, 5.27%) and all other groups showing similar rates of CD63-expressing platelets. CD62p expression was not influenced by any of the above-mentioned factors (ANOVA). Patients with ≥2 risk factors (n=30) and patients with ≤1 risk factor (n=20) for stroke were not different in terms of CD62p and CD63 expression on days 14 and 90 after stroke. On day 1, patients with ≥2 risk factors had less CD62p expression (median, 2.3% versus 3.2%; P=0.004), whereas there was no difference with respect to CD63. In a regression analysis, CD63 expression (P<0.001) but not CD62p expression was increased when the body temperature was elevated on the day of venipuncture after stroke (>37.5°C, with or without signs or symptoms of infection). CD63 expression remained increased in stroke patients compared with both control groups on days 1 (median, 2.64%), 14 (median, 2.97%), and 90 (median, 2.97%) (P<0.001 for all) when patients with recent infection and all measurements on days with elevated body temperature after stroke were excluded from the analyses. On day 90 after stroke, CD62p and CD63 expression did not differ between patients treated with aspirin (n=24; CD62p, 1.18%; CD63, 3.06% [medians]), clopidogrel (n=7; CD62p, 1.87%; CD63, 2.31%), or anticoagulants (n=17; CD62p, 1.23%; CD63, 2.92%) (P>0.5 for all).

There was no significant correlation between both platelet activation parameters on one hand and CRP, fibrinogen, and
leukocyte count on the other hand in either healthy or risk factor control subjects or on days 1, 14, or 90 after stroke ($R<0.28, P>0.1$ for all).

**Discussion**

There is a well-recognized need for clinically applicable methods to monitor platelet function in the treatment and prevention of ischemic stroke. Platelet activation in the acute and chronic stages after stroke has been demonstrated with methods such as aggregometry, assessment of plasma levels of platelet release products, and urinary excretion of thromboxane metabolites. None of these methods proved to be a useful tool in daily clinical practice partly because of insufficient reproducibility of results and partly because of the expense of time and equipment required. The main methodical problem is artificial in vitro activation of platelets during blood sampling and processing. Furthermore, measurements of release products may have a low sensitivity because of dilution effects in plasma. Flow cytometric analysis of platelet function has the advantage that it can use fixation procedures that block metabolic processes in cells within milliseconds but leave activation-dependent neoantigens on the cell surface preserved. Therefore, the possibility of artifactual cell activation is reduced to the venipuncture in this technique. We found good reproducibility of the assessment of both platelet activation markers with a low intratest variability and a low to moderate intertest variability in healthy subjects. Therefore, an important prerequisite for clinical application is fulfilled by this method.

To the best of our knowledge, this is the first study to closely analyze the time course of activation-dependent neoantigens on platelet surface after ischemic stroke. In accordance with previous reports, we found a significant increase in CD62p and CD63 expression within 24 hours after cerebral ischemia. Our study adds to previous knowledge by showing that CD62p expression declines during the first weeks after stroke, whereas CD63 expression remains increased for at least 3 months after stroke. Therefore, CD63 could be an interesting parameter for future prospective studies that evaluate the role of platelet activation markers as potential predictors for first or recurrent ischemic events. In contrast to our results, Yamazaki et al. showed increased p-selectin expression >1 month after stroke; however, a serial follow-up was not performed in their study.

The differential expression pattern of CD62p and CD63 over time is an interesting finding that may be explained as follows. A recent report has shown that increased expression of CD62p or CD63 after stroke was not associated with an increased proportion of circulating platelets with shape change. The shape change is a very early and reversible event during platelet activation and a very sensitive marker of instantaneous platelet activation. This indicates that platelets after stroke are not particularly activated during their passage in peripheral venous blood. Thus, CD62p and CD63 are memory markers of platelet activation, and the increased expression of secretion-dependent antigens after stroke indicates that platelets had previously undergone activation. In a baboon model, degranulated platelets rapidly lost surface CD62p but continued to circulate and function. A similar mechanism may be operative in humans, and the degranulated platelets may not lose CD63, which could explain the decline in CD62p expression parallel to continuously elevated CD63 expression after acute stroke. Platelets possess a lifespan of about 7 to 10 days. Therefore, increased CD63 expression in subacute stroke indicates continuously ongoing platelet activation. Altogether, in the subacute stage after ischemic stroke, CD63 is a more sensitive marker of platelet activation than CD62p, most likely because of shedding of CD62p.

Single vascular risk factors and clinical stroke severity did not significantly modify CD62p and CD63 expression, and stroke patients had persistently higher CD63 expression than subjects with vascular risk factors. The simultaneous presence of multiple risk factors was not associated with higher platelet activation. This indicates that platelet activation markers may confer relevant information independently from and in addition to clinical risk factor assessment. Our findings do not support the previous observation that atherothrombotic stroke is associated with a particularly high platelet activation. However, our study was not designed to investigate the influence of different stroke origins on platelet activation, and results have to be viewed with caution because of the small numbers of subjects in subgroups. Fever after stroke, caused primarily by acute infection, was associated with particularly high CD63 expression, a finding that may indicate an increased risk for recurrent ischemia during febrile episodes. However, the occurrence of fever did not explain the entire difference between patients and control groups because patients still had higher CD63 expression when all measurements at times of increased body temperature were excluded. We did not find a significant influence of medication for secondary prevention on platelet activation markers. The fact that medication with platelet inhibitors was not associated with lower antigen expression by platelets is in line with previous results showing that aspirin does not modify the excretion of α-granules.

Several studies have shown that fibrinogen, CRP, and leukocyte count are increased after ischemic stroke. Furthermore, all 3 parameters were shown to be independently associated with the risk of first-ever and recurrent vascular events, although the specificity of the link with vascular events was questioned recently. There is an intense crosstalk between platelets and cellular and plasmatic components of the inflammatory system. Regulation of the effector function of platelets and leukocytes and hence thrombotic and inflammatory processes are interrelated by several mediator systems such as eicosanoids, chemokines, and adhesion molecules. Although multiple pathways link platelet function with inflammation in general, the correlation between the platelet activation parameters assessed here and inflammatory indexes has not been analyzed before. Neither in control subjects nor in patients after stroke did we find any association between platelet neoantigens and inflammatory parameters, suggesting that both groups of parameters are independently regulated. The role of inflammatory parameters, mainly CRP, as an important predictive index for recurrent ischemic events after stroke was recently established. An interesting question is whether the ex-
pression of platelet neoantigens, mainly of CD63, may provide additional information on future vascular events after stroke and thus may guide therapeutic decisions. This question needs to be answered in larger prospective studies.

In conclusion, increased platelet expression of both CD62p and CD63 acutely after stroke is followed by a rapid decline in CD62p expression but a persistent increase in CD63 expression under secondary preventive treatment. Whether CD63 is a predictor for recurrent ischemic events has to be investigated in future studies. Expression of CD62p and CD63 was not correlated with leukocyte counts, fibrinogen, or CRP, indicating that the platelet activation parameters are regulated independently from inflammatory indexes.

References
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