Course of Platelet Activation and Platelet-Leukocyte Interaction in Cerebrovascular Ischemia

Patrik Htun, MD; Suzanne Fateh-Moghadam, MD; Bernd Tomandl, MD; René Handschu, MD; Kathrin Klinger, MD; Konstantinos Stellos, MD; Christoph Garlichs, MD; Werner Daniel, MD; Meinrad Gawaz, MD

Background and Purpose—Platelet activation plays a crucial role in the pathophysiology of cerebral ischemia. The aim of this study was to investigate the contribution of platelet activation and leukocyte-platelet interactions to the disease.

Methods—One hundred thirty-five patients with transient ischemic attack (TIA) or stroke were enrolled in this single-center study. They underwent cranial computer tomography within 24 hours of clinical onset and after 3 months, and systemic venous blood samples were drawn. Platelet activation (CD62P expression), leukocyte activation (L-selectin expression), and the appearance of platelet-specific antigens on leukocytes as an index of platelet-leukocyte aggregation were measured by flow cytometric techniques in the acute state and at 3-month follow-up.

Results—Patients with a completed stroke or TIA had significantly increased circulating platelet-leukocyte aggregates, increased P-selectin expression on platelets, and decreased L-selectin expression in the acute state compared with the control group (healthy volunteers). No differences in regard to the tested activation markers could be detected between patients with stroke or TIA in the acute phase of the disease. However, platelet and leukocyte activations were normalized after 3 months in patients with TIA, whereas leukocyte activation (reduced L-selectin expression) remained in stroke patients.

Conclusions—In patients with TIA and completed stroke, platelet and leukocyte activation is substantially enhanced in the acute phase of the disease. The sustained leukocyte activation observed in stroke but not in TIA patients at 3-month follow up might play a pathophysiological role in the course of the disease. (Stroke. 2006;37:2283-2287.)

Key Words: cerebral ischemia, transient ▪ leukocytes ▪ platelet activation ▪ platelets ▪ stroke

Stroke is the third leading cause of death and an important cause of disability in western Europe and the United States.1 Thrombosis and atherosclerosis are major contributors to the development of ischemic stroke. Platelets play a critical role in triggering arterial thrombosis2 and in promoting atherogenesis.3-7 Furthermore, formation of platelet-leukocyte aggregates and leukocyte activation contribute to vascular repair and microcirculatory disturbances in ischemic tissue. Previous in vitro studies have demonstrated interaction of activated platelets with monocytes and neutrophils.8 Binding of platelets via P-selectin expressed on the surface of activated platelets to the leukocyte counterreceptor P-selectin GP ligand-1 may alter leukocyte recruitment and activation patterns.9 Platelet-neutrophil binding occurs in unstable angina and after myocardial injury and angioplasty.10,11 Elevated levels of platelet P-selectin expression and platelet-leukocyte aggregates have been demonstrated in patients with acute myocardial infarction12,13 and were regarded as an early injury marker.14,15 Platelet and platelet-leukocyte aggregates were also described in patients with stroke.16-18 However, platelet activation, platelet-leukocyte coaggregation, and leukocyte activation in acute ischemic stroke and the chronic phase of the disease have been poorly evaluated to date. Alterations of platelet and leukocyte function in both the acute and chronic phases of cerebrovascular ischemia may cause substantial impairment of the cerebrovascular microcirculation during and after ischemia and thus, may contribute substantially to tissue injury and repair mechanisms.

The aims of this study were to investigate in human cerebrovascular ischemia the degree of platelet activation, platelet-leukocyte interactions, and leukocyte activation in acute ische-
mic stroke and 3 months later. Furthermore, we tested whether there was any difference between patients with transient isch-
emic attack (TIA) or completed stroke and whether the markers may have an impact on disease progression.

**Subjects and Methods**

We enrolled 135 patients within 24 hours after the acute onset of cerebrovascular ischemia. Basal levels of leukocyte activation and formation of platelet-leukocyte coaggregation were analyzed in 40 healthy control subjects (mean age, 38.38±11.7 years; 16 females and 24 males). The demographic and clinical data of patients are summarized in the Table. Patients with major trauma, surgery, severe liver disease, renal failure, cancer, and chronic inflammatory diseases were not enrolled into the study. Patients with an infection after stroke or with a history of infection shortly before stroke were excluded to avoid including the occurrence of platelet activation attributable to parallel infection. The patients were followed up for 5 years in regard to the recurrence of stroke, TIA, and death from all causes, and an individual follow-up was made after 3 months. Computed tomography (CT) of the brain was performed at enrollment within 24 hours after stroke onset to confirm the diagnosis of ischemic stroke. A second cranial CT was performed after 3 months to determine the volume of cerebral infarct size. Infarct size was quantified by measuring the area of hypodensity in each section and multiplying this area by section thickness. Stroke volume was defined as the sum of these measurements. The clinical deficit after insult lasted for at least 24 hours. The origin of cerebral ischemia was classified according to the Trial of Org10172 in Acute Stroke Treatment (TOAST) criteria.20 Stroke severity was assessed with the National Institutes of Health Stroke Scale21 on day 1 and after 3 months. The study was approved by the local institutional review board.

**Immunostaining and Flow Cytometry**

Blood samples were obtained within the first 24 hours after onset of cerebral symptoms and 3 months later. The mean time range from the onset of cerebral symptoms until blood draw was 6.12±0.42 hours. All blood samples were collected at the time of hospital admission before the cranial CT scan was performed and specific therapy (eg, heparin) was initiated. For flow cytometric analysis, 0.5 mL blood was collected into a polypropylene syringe with 1.0 mL of a fixative solution (1:2, vol:vol) containing methacrylate (Cyfix II, a gift of Dr Ruf, Karlsruhe, Germany).22 After 10 minutes of incubation at room temperature, the fixation process was stopped by adding 48.5 mL phosphate-buffered saline. Immunostaining and flow cytometry were performed as previously described.23 Fixed whole blood (35 μL) was incubated with 15 μL phosphate-buffered saline containing saturating concentrations of 3 different monoclonal antibodies for 30 minutes in the dark at room temperature. Thereafter, red blood cells were lysed and fixed with Immunolysate reagent (Coulter Electronics) according to the manufacturer’s protocol and kept on ice before analysis. For flow cytometry analysis, leukocyte subgroups were identified by size and granularity on the forward- versus side-scatter plot. To determine platelet-leukocyte interaction, we evaluated subpopulations of leukocytes for the binding of a specific platelet monoclonal antibody to the von Willebrand factor receptor glycoprotein Ib (anti-CD42b-phycocerythin [PE], Immunotech). Using triple-color whole-blood flow cytometry, we were able to simultaneously analyze the platelet-leukocyte aggregates and the expression of their activation markers. The first antibody, anti-CD42b-PE (clone SZ2, PE labeled), was used to identify the platelets adhering to leukocytes; anti-CD11b (Cy-chrome labeled, α-M-chain of MAC-1, Sigma) and the CD14 antibody (quantum red labeled; endotoxin receptor, Immunotech) were used to recognize specific epitopes on leukocytes or monocytes, respectively. The third antibody, anti-CD62P-FITC (fluorescein isothiocyanate labeled, P-selectin, Immunotech) and anti-CD62L-FITC (clone Dreg 56) served as markers for the activation status of the platelets (CD62P) or leukocytes (CD62L or L-selectin), respectively.

For flow cytometry, a FACSCalibur apparatus (Becton-Dickinson) equipped with a 488-nm argon laser at 500 mW was used. To establish reference values, control samples obtained from healthy individuals were always run and processed simultaneously with patient samples. The mean intensity of immunofluorescence was used as index for antigen surface exposure.

**Statistical Analysis**

For statistical analysis, we used the nonparametric Mann-Whitney U test for comparisons between groups. A value of $P<0.05$ was regarded as significant (SPSS, Windows version 10.0). Results of flow cytometric parameters are reported as median (interquartile range) unless otherwise indicated. Time courses were tested with Friedman test followed by Wilcoxon rank-sum test. To corroborate these analyzes, we also performed ANOVA for repeated measures, which always confirmed the results of the nonparametric tests.

**Results**

One hundred thirty-five patients were included in this study. The baseline characteristics are presented in the Table. The mean age...
of all identified patients was 63.7 years (SD, ±13.9), and 67 patients (49.6%) were female. Seventy-three patients had a TIA (group I) and 62 patients had a completed stroke (group II). The National Institutes of Health Stroke Scale score for the stroke group at admission was 7 (4 to 10) and after 3 months was 2 (1 to 5); for the TIA group, respective scores at admission and after 3 months were 3 (2 to 6) and 0 (0 to 2).

The frequencies of risk factors for each subtype are also presented in the Table. The incidence of hypertension, diabetes mellitus, smoking, or cardiac disease did not vary among the 2 subtypes. The proportion of cardioembolic and atherothrombotic etiologies of the cerebrovascular insult were similar in both subgroups. Only the number of leukocytes and platelets were significantly higher in the stroke group ($P=0.001$ for leukocytes and $P=0.024$ for platelets) when compared with the TIA group. During the 3-month follow-up period, 8 patients died of cardiovascular causes. Three patients were lost to follow-up at 3 months; therefore, the follow-up results include data for 124 patients.

Platelet activation as assessed by degranulation of P-selectin was significantly ($P<0.05$) increased in patients with acute stroke (proportion of CD62P-positive platelets was 6.4 [5.8% to 8.3%]) and in the acute TIA group (7.5% [5.2% to 10.7%]) compared with the control group (5.4% [4.4% to 6.7%]; Figure 1). There was no difference between patients with TIA or completed stroke either in the acute phase or 3 months later.

The expression of CD62P on platelet-leukocyte aggregates (mean intensity of immunofluorescence) was significantly increased in the acute phase of stroke (13.9 [11.6 to 19.2]) and TIA (13.8 [10.7 to 16.6]) compared with the control group (9.5 [7.6 to 14.3]; Figure 2).

The expression of CD42b on platelet-leukocyte aggregates (mean intensity of immunofluorescence) was also significantly increased in the acute stage of stroke (17.3 [13.6 to 23.3]; $P<0.001$) and in the acute TIA group (15.2 [12.9 to 24.1]; $P=0.003$) compared with the control group (12.8 [9.2 to 18.7]). There was no significant difference between patients with TIA or stroke either in the acute phase or 3 months later. After 3 months, CD42b expression returned to a normal level, comparable to that in the control group. The difference between the acute phase and after 3 months was significant ($P<0.01$) for both groups (TIA and stroke).

L-selectin expression, a marker of leukocyte activation, was decreased in patients with acute stroke (mean fluorescence intensity: 20.9 [15.7 to 36.2]) or TIA (19.7 [15.7 to 42.3]) compared with control subjects (61.5 [20.1 to 144]; Figure 3). L-selectin expression remained attenuated in patients with stroke after 3 months (36.8 [17.8 to 108.0]). However, in patients with TIA, leukocyte activation returned to normal levels within 3 months (70.1 [18.6 to 97.6]) versus control 61.5 [20.1 to 144]; $P<0.05$; Figure 3). We could not show a significant correlation between recurrent stroke in a time period of 5 years and a sustained attenuated L-selectin expression after 3 months of follow up. Eighteen (29%) of patients with TIA but remained decreased in patients with completed stroke (36.8 [17.8 to 108.0]; TIA, 70.1 [18.6 to 97.6]). *Statistical significance compared with controls ($P<0.05$).
patients in the stroke group experienced a recurrent stroke, and 11 (15%) patients in the TIA group had a new stroke.

There was no significant correlation between infarct size (stroke volume), assessed by volumetric measurement of the hypodense infarct area on the CT scans after 3 months, and the extent of platelet activation, formation of platelet-leukocyte aggregates, and leukocyte activation: for CD62P-positive platelets, $r = -0.137$, $P = 0.258$; for platelet-leukocyte aggregates, $r = -0.049$, $P = 0.639$; and for L-selectin, $r = 0.129$, $P = 0.176$.

**Discussion**

The major findings of the present study are as follows: (1) Platelet activation (P-selectin expression) and platelet-leukocyte aggregation are enhanced in acute ischemic stroke and normalize within 3 months. There are no differences in the degree of platelet activation or formation of platelet-leukocyte aggregates between patients with TIA and those with completed stroke. (2) Leukocyte activation as assessed by decreased L-selectin expression is also enhanced in patients with TIA and stroke. Whereas L-selectin expression normalizes in patients with TIA, leukocyte activation remains sustained after 3 months in patients with complete stroke. The findings imply that a sustained activation of circulating leukocytes after completed acute stroke may play a role in the pathophysiology of stroke.

Systemic inflammation has been implicated to play an important role during both the acute and chronic phase of ischemic stroke. Persistent systemic inflammation is associated with increased risk for recurrent vascular events in stroke patients. Platelets play a critical role in acute and chronic inflammation. Hence, it is not surprising that leukocyte number was also elevated in our stroke group compared with the TIA group. Because platelets and especially activated platelets act as prominent players in the disease process by acting as a bridge between atherosclerosis and atherothrombosis in forming platelet-leukocyte aggregates, they are raised in the same way as leukocytes.

Enhanced platelet activation has been described in patients with TIA and stroke. Marquardt et al. found elevated P-selectin expression in the acute state of cerebrovascular ischemia and a rapid decrease of CD62P expression after the onset of stroke. In contrast, McCabe et al. reported a persistent elevation of platelet-associated CD62P levels 3 months after stroke onset. In these studies, platelet activation was not correlated with the extent of the ischemic insult. In the present study, we confirmed the previous report by Marquardt et al. that platelet expression of P-selectin is enhanced in TIA and stroke and normalizes in the months after the acute event. We also did not find a correlation between the degree of platelet activation and stroke volume as assessed by CT scan.

Activated platelets degranulate and adhere to leukocytes, thus forming platelet-leukocyte coaggregates. Enhanced platelet activation and formation of platelet-leukocyte aggregates have been previously described in acute coronary syndromes and acute ischemic stroke. Platelet interaction with leukocytes via P-selectin/P-selectin GP ligand-1 induces leukocyte activation and release of inflammatory cytokines. Our findings show an increased level of circulating platelet-leukocyte aggregates and leukocyte activation in patients with both TIA and stroke. Whereas platelet-leukocyte coaggregates normalized within 3 months after onset of cerebral ischemia in patients with both TIA and stroke, leukocyte activation persisted in patients with stroke but not with TIA. Thus, it is tempting to speculate that the persistent activation of circulating leukocytes plays a pathophysiological role in the acute and chronic phases of ischemic stroke. Sustained leukocyte activation in the chronic phase of cerebrovascular ischemia may cause substantial impairment of the cerebrovascular microcirculation and thus, may contribute substantially to brain recovery and repair mechanisms.

Markers of inflammation, including fibrinogen, C-reactive protein, and leukocyte count, are increased after ischemic stroke and are independently associated with recurrent ischemic events. Therefore, persistent circulation of activated leukocytes in the chronic time course after onset of ischemic stroke may describe an enhanced risk of recurrent ischemic events and thus, poor prognosis of the disease. Because platelet activation and platelet-leukocyte aggregation returned to normal values during the 3-month follow-up in patients with stroke, it seems unlikely that platelet activation and platelet-leukocyte interaction are the cause of persistent leukocyte activation and therefore systemic inflammation in stroke patients. At present, we are unable to provide clinical follow-up data with respect to recurrent ischemia in stroke patients with persistent leukocyte activation.

In conclusion, the present study demonstrated increased platelet activation, platelet-leukocyte interaction, and leukocyte activation in the acute state of TIA and stroke. Whether the persistent leukocyte activation in patients with stroke but not TIA is attributable to ongoing repair mechanisms or is a predictor of recurrent ischemic events has to be investigated in future studies.

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**Disclosures**

None.

**References**

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