Platelet–Leukocyte Interaction and Platelet Activation in Acute Stroke With and Without Preceding Infection

J.A. Zeller, A. Lenz, C.C. Eschenfelder, P. Zunker, G. Deuschl

Objective—Acute coronary syndromes and ischemic cerebral stroke share similarities regarding elevated platelet activation. In coronary syndromes, the importance of inflammation with platelet–leukocyte interaction has been demonstrated. Recent infection is an established risk factor for ischemic stroke; the role of platelet–leukocyte interaction in these patients had not been investigated.

Methods and Results—Using a flow cytometric assay we investigated 58 stroke patients, 21 with and 37 without infection 1 week before acute cerebral ischemia, and compared them to 58 controls with regard to platelet–leukocyte aggregation and platelet activation on admission and on day 7. Patients with previous infection were significantly up-regulated with regard to platelet activation and platelet–leukocyte aggregation compared with patients without infection. On day 7, these increases in the postinfective group had drawn level with the lower values of the other patients. As reported previously, recent infection was associated with a more severe postischemic deficit.

Conclusions—These results suggest an important role of intercellular platelet–leukocyte interaction in the pathophysiology of acute cerebral ischemia which may also contribute to the increased incidence and clinical severity of ischemic stroke following infection. This may lead to therapeutic considerations of blocking intercellular adhesion molecules like P-selectin or the P-selectin glycoprotein ligand. (Arterioscler Thromb Vasc Biol. 2005;25:1519-1523.)

Key Words: ischemic stroke ■ infection ■ platelet activation ■ cell adhesion

Although observations that sudden hemiplegia is often associated with infection date back to early last century, preceding infection was first documented as an independent risk factor for stroke in children and young adults less than twenty years ago.1,2 Later, infection was also shown to precipitate cerebral ischemia in all age groups3–6 with a frequency of 24% to 35% of patients who had an appropriate antecedent illness 1 week before stroke. Onset of infection >1 week before the ischemic event did no longer reach statistical significance.3,5,6

In stroke patients, cytokines, with their ability to recruit leukocytes and to communicate inflammatory signals like tumor necrosis factor (TNF)-α, interleukins (IL)-1, -6 and -8, and the soluble intercellular adhesion molecule-1 (ICAM-1; sICAM-1) have been found to be increased.7–9 sICAM-1 is responsible for adhesion and migration of mononuclear and polymorphonuclear leukocytes and is significantly elevated during infection.10 Large experience from cardiovascular disease showed that platelet–leukocyte interaction was increased in stable and acute coronary events, and after angioplasty,11,12 particularly platelet aggregation with neutrophils.13,14 By interacting, leukocytes can enhance platelet aggregation and thromboxane release,15 and promote further recruitment of activated platelets.16 Vice versa, leukocytes will be modified to higher activity levels by the adhesion of activated platelets.17,18

Isolated platelet activation has already been shown to be increased in stroke patients irrespective of prior infection.19,20 Moreover, quantification of monocyte–platelet aggregates has been suggested to be a more reliable and stable marker of platelet activation than detecting activation-dependent platelet epitopes alone.21

We performed a prospective study in patients with ischemic stroke to investigate whether a history of preceding infection is correlated with the extent of platelet–leukocyte adhesion and platelet activation, whether these parameters change in the early phase of illness, and whether the severity of the clinical presentation is influenced by previous infection.

Methods

Patient Inclusion Criteria

Patients with suspected cerebral ischemia who were symptomatic <24 hours and who gave informed consent were eligible for inclusion into the investigation, 58 patients were studied. All patients had an initial cranial CT (CCT) scan, ECG, doppler and duplex sonography of the brain supplying arteries and routine laboratory testing including full blood count, clotting tests and C-reactive protein (CRP) measurement. In all but 2 patients, echocardiography (transhoral or transesophageal) was performed, 53 patients received follow-up brain imaging with either CCT or MRI. Depending on these findings, the likely stroke etiology was classified according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria.22

E-mail j.zeller@neurologie.uni-kiel.de

Original received February 2, 2005; final version accepted March 30, 2005.
From the Department of Neurology, Christian-Albrechts-University, Kiel, Germany
Correspondence to Dr. Jörn A. Zeller, Klinik für Neurologie, Christian-Albrechts Universität Kiel, Schittenhelmstrasse 10, D-24105 Kiel, Germany.

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000167524.69092.16
Postinfarct stroke was assumed if one of the following conditions was fulfilled: symptoms and signs of upper respiratory, gastrointestinal or urinary tract infection, and fever within the week before admission, elicited by a standardized questionnaire; an appropriate diagnosis had been made by a previous treating medical professional; the body temperature on admission exceeded 38°Celsius and CRP was more than twice the normal range on admission.

Eighteen patients were on 100 mg/d aspirin on clinical presentation (11 without, 7 with previous infection), 1 patient without previous infection was taking 75 mg/d clopidogrel. The control group consisted of volunteers from medical staff, and of patients who were not hospitalized for suspected cerebral ischemia or other vascular disease, and who had not suffered a cerebral ischemia during the last year or were known to have middle- or high-grade stenosis of any brain supplying artery.

The study was approved by the ethical committee of the Christian-Albrechts-University.

Antibodies and Other Reagents
For the flow cytometric assays, we used the following direct fluorescent markers commercially available from Beckman Coulter, Krefeld, Germany: fluorescein isothiocyanate (FITC)-conjugated CD 45 (clone J.33) for leukocyte identification, FITC-conjugated CD 62P (clone CL.BThromb/6) for detection of the P-selectin epitope, and PE-conjugated CD 41 (clone P2) for platelet identification irrespective of their activation status. Buffer consisted of Hank's Balanced Salt Solution with 1% bovine serum albumin added (both Sigma Chemicals). Quality control was assured by daily referencing with Flow-Check Fluorospheres (Beckman Coulter) beads and Dako Fluorospheres beads.

Blood Sampling and Flow Cytometry
Venous blood was drawn from an antecubital vein in a supine position, anticoagulated with 3.8% sodium citrate and processed after 10 minutes of resting time without further manipulation at body temperature during the entire process. Whole blood was diluted 1:10 with warmed buffer and 2 aliquots with an end volume of 50 µL was incubated with CD 41-PE (an activation-independent subunit of the platelet glycoprotein (GP) IIb-IIIa complex) to immunologically identify all platelets. Simultaneously, in a 1-step procedure, the platelet activation sample was additionally stained with anti-CD 62-P (clone CLBThromb/6) for detection of the P-selectin epitope, while the other sample was double-stained with CD 45 to identify leukocytes. After incubation for 5 minutes, the process was stopped using cold buffer, immediately followed by flow cytometry.

First, platelet activation samples were analyzed triggering on FL-2 to select CD 41 positive platelets only, in a different gate platelets were identified by their size and granularity properties using forward/ sideward scatter. 10,000 events that matched both criteria were further analyzed with regard to FITC (P-selectin) positivity, and the mean fluorescence was taken as the numeric value indicating the extent of platelet activation.

The platelet–leukocyte aggregation assay used the CD 45 fluorescence in channel FL-1 as the detection threshold, then forward scatter, sideward scatter, and FL-1 properties of leukocytes were used to discriminate between leukocyte subsets. With simultaneous detection of the CD 41-PE labeled platelet epitope we recorded platelet–leukocyte aggregates and platelet–leukocyte-subset aggregates. For numeric evaluation we quantified the percentage of leukocytes with aggregated platelet in relation to all detected leukocytes and leukocyte subgroups, respectively. In this assay, red cells are not specifically stained, and therefore it is not necessary to lyse red cell. Measurements were performed with an Epics XL cytometer (Beckman Coulter). This assay had been established and in routine use in our laboratory for four years prior to this investigation.

Clinical Assessment Measures
To evaluate clinical severity in our patient cohort, we scored the clinical status on admission by using the National Institutes of Health Stroke Scale (NIHSS). Secondly, an intermediate clinical outcome assessment was made using the Rankin Scale on day 7, or before this point in case the patient was discharged or transferred earlier. Patients who died within 7 days scored a 5 similar to bedridden and totally dependent patients.

Statistic Analysis
Numerical values are expressed in mean±SD. Data analysis on platelet activation, platelet–leukocyte aggregation, cell counts, and CRP was performed using the Student t test, a general linear model to compare the time course within groups between day 1 and day 7. The Mann–Whitney U test was used to compare groups with regard to the NIHSS and the Rankin Scale. Significance was assumed with P<0.05 in appropriate two-tailed tests.

Results
70.7% of the patients suffered ischemia of the anterior, 29.3% of the posterior circulation. A detailed patient description is given in Table 1a. Twenty-one patients (36%) met the criteria for previous infection. The most frequent infection affected the upper respiratory tract (43%) followed by urinary tract infection (24%); in 19% of postinfective patients the source of infection could not be identified. With respect to stroke etiology defined by the TOAST criteria, we saw a moderately higher proportion of postinfective strokes in the cardioembolism group (42% versus 36% in all stroke patients).

Leukocyte Count and CRP
Stroke patients had significantly elevated leukocyte counts, a higher proportion of neutrophils and higher CRP values compared with controls. Even when only those patients without preceding infection were considered, the increase of white cell count and CRP in the patient group remained significant. The comparison within the subgroups with and without previous infection, respectively, yielded an additional relevant difference regarding leukocyte count and CRP. Detailed results are given in Table 2. These values decreased on day 7 in the postinfecitious patients and did not differ significantly from the level of the remaining patients (white

TABLE 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>All Stroke Patients</th>
<th>Stroke With Infection</th>
<th>Stroke Without Infection</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Demographic data</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>58</td>
<td>21</td>
<td>37</td>
<td>58</td>
</tr>
<tr>
<td>Mean age, years</td>
<td>63.0</td>
<td>66.6</td>
<td>61.0</td>
<td>56.1</td>
</tr>
<tr>
<td>Female</td>
<td>43.1%</td>
<td>42.9%</td>
<td>43.2%</td>
<td>43.1%</td>
</tr>
<tr>
<td><strong>Affected circulation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior circulation</td>
<td>70.7%</td>
<td>75.7%</td>
<td>61.9%</td>
<td>NA</td>
</tr>
<tr>
<td>TOAST</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large artery atherosclerosis</td>
<td>n=21</td>
<td>8 (38%)</td>
<td>13 (62%)</td>
<td>NA</td>
</tr>
<tr>
<td>Cardioembolism</td>
<td>n=12</td>
<td>5 (42%)</td>
<td>7 (58%)</td>
<td>NA</td>
</tr>
<tr>
<td>Small vessel occlusion</td>
<td>n=6</td>
<td>2 (33%)</td>
<td>4 (67%)</td>
<td>NA</td>
</tr>
<tr>
<td>Undefined etiology</td>
<td>n=18</td>
<td>6 (33%)</td>
<td>12 (67%)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>(B) Clinical assessment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIHSS, day 1</td>
<td>5.7</td>
<td>7.9*</td>
<td>4.1</td>
<td>NA</td>
</tr>
<tr>
<td>Rankin Scale, day 7</td>
<td>1.8</td>
<td>2.8*</td>
<td>1.3</td>
<td>NA</td>
</tr>
</tbody>
</table>

*P<0.005 between stroke with and without infection.
cell count 7.4 nL with versus 7.3 nL without infection and CRP 18.8 mg/dL versus 9.8 mg/dL).

**Clinical Course**

Patients over all scored an average NIHSS value of 5.7. Those patients with stroke following infection within 1 week before admission presented with a score of 7.9 as opposed to those patients without previous infection averaging a score of 4.1; this difference was significant. The 7-day outcome status was significantly worse in the postinfectious group compared with the other patients (Table 1b).

**Platelet–Leukocyte Aggregation in Stroke Patients**

The proportion of leukocytes with adherent platelets was significantly higher in stroke patients compared with the patients without previous infection (0.74%; 9.60% versus 6.65%), the percentage of monocyte–platelet aggregates was likewise increased in the postinfectious patients compared with the patients without previous infection (8.51% versus 6.22%). In contrast, lymphocytes with adherent platelets were not increased in the patient group which followed infection when compared with controls (0.39% versus 0.41%; P=0.061) (Table 3).

Patients who had experienced an infection during the week before stroke had a significantly (P=0.003) increased proportion of platelet leukocyte aggregates (7.28%) as compared with those patients without infection (4.96%). This effect was driven by the increased fraction of polymorphonuclear cells aggregated to platelets (9.60% versus 6.65%), the percentage of monocyte–platelet aggregates was likewise increased in the postinfectious patients compared with the patients without previous infection (8.51% versus 6.22%). In contrast, lymphocytes with adherent platelets were not increased in the patient group which followed infection when compared with controls (0.39% versus 0.41%; P=0.80), but significantly reduced in relation to the patients without infection (0.74%; P=0.029). The results are summarized in Table 3.

We found no correlation between the type of infection and the extent of platelet–leukocyte aggregation. We did not find significant differences when comparing platelet–leukocyte aggregation between patients of different stroke etiologies, although, especially the 2 main cohorts of large artery atherosclerosis and cardioembolism were significantly different from the controls.

**Platelet Activation in Stroke Patients**

Similar to the interaction with leukocytes, platelet activation in stroke patients was up-regulated. The P-selectin epitope was significantly more expressed in stroke patients (1.59 versus 1.27; P<0.001) which confirms previous findings in these patients. The analysis for cases with and without previous infection showed an additional effect of the postinfectious group exceeding the platelet activation levels of the remaining patients significantly (1.78 versus 1.49; P=0.001).

Other vascular risk factors like hypertension, hypercholesterolemia, nicotine use or diabetes did not influence the amount of platelet activation or platelet–leukocyte interaction in our patient group.

**Time Course of Platelet Function Parameters**

In 31 of the 58 patients, we had the opportunity to re-evaluate platelet–leukocyte aggregation and platelet activation 7 days after onset of stroke. Two of these patients had a newly acquired infection and were excluded from follow-up analysis.

Nine patients who were taking aspirin on first presentation had been discontinued from this drug and received intravenous heparin instead, as did 10 other patients. Ten patients had been started on aspirin since day 1, thus changing the proportion of patients on aspirin from 31% to 34%.

These remaining 29 patients showed moderately reduced values in platelet–leukocyte aggregation (4.65% versus 5.81%) and unchanged levels of platelet activation (1.59 versus 1.58) 7 days after stroke onset. If the 7 patients with preceding infection were analyzed separately, a significant decrease could be demonstrated in both platelet activation (1.77 to 1.58 on day 7) and platelet–leukocyte aggregation (7.9% on day 1 to 4.3% on day 7; P<0.01; significance also applied to the reduction in the subgroups of polymorph–platelet-aggregates and monocyte–platelet-aggregates; other patients 5.2% to 4.7% on day 7).

**TABLE 3. P-Selectin Expression and Platelet–Leukocyte Aggregation**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>All Stroke</th>
<th>Stroke With Infection</th>
<th>Stroke Without Infection</th>
<th>P, Control vs All Stroke</th>
<th>P, Control vs No Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-selectin expression</td>
<td>1.27±0.18</td>
<td>1.59±0.35</td>
<td>1.78±0.28</td>
<td>1.49±0.36</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Platelet–leukocyte-aggregation, %</td>
<td>3.50±1.71</td>
<td>5.80±2.85</td>
<td>7.28±2.76</td>
<td>4.96±2.58</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>Polymorph–platelet-aggregation, %</td>
<td>4.46±3.10</td>
<td>7.71±4.24</td>
<td>9.60±4.49</td>
<td>6.65±3.75</td>
<td>&lt;0.001</td>
<td>0.015</td>
</tr>
<tr>
<td>Monocyte–platelet-aggregation, %</td>
<td>5.07±3.15</td>
<td>7.05±3.57</td>
<td>8.51±3.64</td>
<td>6.22±3.31</td>
<td>0.002</td>
<td>0.023</td>
</tr>
<tr>
<td>Lymphocyte–platelet-aggregation, %</td>
<td>0.41±0.53</td>
<td>0.61±0.63</td>
<td>0.39±0.47</td>
<td>0.74±0.68</td>
<td>0.061</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Values given in mean and SD.
Discussion

This is the first report demonstrating that platelet activation and platelet–leukocyte interaction is exceedingly up-regulated in postinfectious stroke patients. Mostly polymorphonuclear cells and monocytes contribute to the interaction with platelets beyond the general elevation of this parameter seen in the entire stroke cohort. This mechanism is likely to play a role in the increased incidence of stroke after infection and to contribute to the increased stroke severity in patients with previous infections that was also confirmed in our study.

In our patient group we can support earlier findings of an increased stroke incidence following infection which exceeds the general age-matched stroke incidence by 2- to 3-fold. It has been shown that infection leading to a poorer outcome is not necessarily related to fever and all types of infection contribute to the effect.1,5–7,25 Bacterial and viral infection are increasing the risk of stroke to a similar extent.3 The mechanism of action of how infection propagates stroke has not been satisfactorily established. Recently, several bacteria, especially Helicobacter pylori and Streptococcus sanguis have been found to propagate platelet activation and aggregation.26,27 A main mechanism seems to be the interaction of bacteria-bound von Willebrand factor (vWF) with the GPIb receptor. Although this mechanism has not yet been shown for a broad spectrum of pathogens, a similar principle could be a likely explanation for increased incidence and severity of stroke in postinfective patients. An increase of tissue factor expression from endothelial cells and significantly enhanced platelet adhesion, thus leading to a procoagulatory state, has been described.28 Similarly, high levels of antidiolipin IgG antibodies may contribute, which are particularly more frequent in young patients with cerebral ischemia.29,30 A decrease of activated protein C and tissue plasminogen activator/plasminogen activator inhibitor activity index seems also to be relevant.31 Cytokines play a potential role as they recruit leukocytes and communicate inflammatory signals like TNF-α, IL-1, -6 and -8.7,8 The soluble ICAM-1 after stroke,9 which is responsible for adhesion and migration of mononuclear and polymorphonuclear leukocytes is also significantly elevated during infection.9 Particularly these observations led us to evaluate the platelet–leukocyte interaction as a common pathway of parainfectious alterations in the signaling and coagulation system before stroke.

Isolated platelet activation and leukocyte interaction have been shown to be increased in stroke patients.19,20 Large experience from cardiovascular research showed the interaction between platelet and leukocytes and particularly platelet aggregation with polymorphonuclear leukocytes to be increased in stable and acute coronary disease and after angioplasty.11–14 The intercellular communication is established between surface glycoproteins on the leukocyte, namely the high-affinity binding to the P-selectin glycoprotein ligand 1 (PSGL-1).14,15,32 This glycoprotein is mainly expressed on polymorphonuclear leukocytes and monocytes which explains the minor affinity between lymphocytes and platelets in our study. By interacting with platelets, leukocytes can enhance platelet aggregation and thromboxane release15 and, thus, promote further recruitment of activated platelets.16 Vice versa leukocytes will be activated resulting in up-regulation of inflammatory responses by the adhesion of activated platelets.17,18,34 Local inflammation at the stroke site contributes to the poorer clinical outcome. In cases of cerebral ischemia the migration of leukocytes into the damaged brain cells are mediated through the up-regulation with adhesion molecules like P-selectin. This does also support the process of regular apoptosis and facilitates the death of neurons which could potentially be salvaged.35

This effect could partly explain why infection-triggered stroke is more severe than stroke without infection.7 Pathological high white cell count on admission in stroke patients is a predictor of poor outcome,36 as we confirmed in our investigation. Moreover, high leukocyte counts with mainly polymorphonuclear cells are independently associated with first ischemic stroke and stroke recurrence.37

It must be emphasized that infection precipitates the evolution of ischemic stroke not only via humoral messaging but also uses a cellular pathway with platelets and leukocytes involved. In our study, this is reflected by the elevated values of platelet leukocyte aggregates which normalize during the first week, thereby further indicating the potential role of this cellular cross-talk in the development of stroke rather than merely being a consequence. This implies possible specific therapeutic approaches. By blocking the linkage molecules between platelets and leukocytes, namely the P-selectin glycoprotein on the platelet surface, and the PSGL-1 receptor on leukocytes with monoclonal antibodies, it may be possible to interfere with a crucial step in the cascade leading to inflammatory messaging and finally brain cell death. Faraday and coworkers showed in an in vitro model that it is possible to reduce induced platelet aggregation by blocking the P-selectin and PSGL-1 site.38 In a canine model of coronary artery occlusion, the administration of recombinant PSGL-Ig significantly reduced myocardial neutrophil infiltration and thus ischemic injury.39 Likewise in patients with acute myocardial infarction, blocking of platelet leukocyte aggregation with monoclonal antibodies at the P-selectin site led to a reduced induction of the proinflammatory IL 1-β, IL 8 and monocyte chemoattractant protein in leukocytes.34

The detection of increased platelet activation and intercellular communication shortly after ischemic stroke does not necessarily prove a causative role in the development of the disease. The entire patient group exhibits increased platelet–leukocyte aggregation; however, the fact that postinfective stroke patients show high levels of platelet–leukocyte interaction in the acute phase, which decreases to levels of the patients without infection within a few days, favors the increased platelet–leukocyte cross-talk to cause stroke, possibly by facilitating thrombogenesis. The statistical power of these observations needs to be improved by a larger number of patients in further investigations.

One additional patient out of 29 had received additional aspirin at the follow-up investigation which may have interfered with platelet activation and leukocyte interaction. However, this represents a minor change from 31% to 34% of patients on aspirin, and the substance was repeatedly shown not to influence platelet activation.39,40

On the basis of our findings, we propose that platelet–leukocyte interaction represents an important mechanism for the development of increased thrombogenesis leading to stroke and to a potentially cytotoxic inflammatory reaction at the injured brain cells after acute ischemic stroke has occurred. This reaction is likely to negatively influence the clinical outcome.
The poorer neurological condition in postinfectious patients may already be evidence of such a process. Similar to first trials in myocardial ischemia, interfering with the intercellular communication through monoclonal antibodies could be a new promising therapeutic approach in ischemic cerebral stroke.

References


Platelet-Leukocyte Interaction and Platelet Activation in Acute Stroke With and Without Preceding Infection

J.A. Zeller, A. Lenz, C.C. Eschenfelder, P. Zunker and G. Deuschl

Arterioscler Thromb Vasc Biol. 2005;25:1519-1523; originally published online April 21, 2005; doi: 10.1161/01.ATV.0000167524.69092.16

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/7/1519

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/