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Platelets and acute cerebral infarction

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Abstract
Stroke is worldwide a leading cause of death and disability. Its etiology is regarded as heterogeneous. Platelets are implicated in its pathophysiology, but our understanding of their specific role is incomplete. Only sparse and conflicting information exists about platelet reactivity and activity in acute stroke. Some scientists take the view that platelets activate in conjunction with acute cerebral infarctions. Others put forward evidence corroborating the contrary notion. Increased soluble P-selectin as a sign of platelet and/or endothelial activity seems to be a feature of the disease. The latter point of view is opposed by other researchers. Due to these conflicting opinions, this study is devoted to platelet characteristics in acute cerebral infarctions. We studied subjects ($n = 72$; age 74 ± 10(SD) years; 31 females) having acute stroke. As controls served atrial fibrillation (AF) patients ($n = 58$; age 69 ± 7(SD) years; 12 females) subject to electrical cardioversion, a flow cytometer was put to use for measuring platelet reactivity and activity. After agonist provocation, both platelet bound P-selectin and fibrinogen were employed as estimates of platelet reactivity. Dilutions of a thrombin-receptor-activating peptide (TRAP-6) (74 and 57 µmol/l) (P-selectin and fibrinogen) and ADP (8.5 and 1.7 µmol/l) (fibrinogen only) were put to use as platelet agonists. Membrane-bound P-selectin without agonist stimulation served as a measure of *in vivo* platelet activation. Soluble P-selectin, as determined from a commercial ELISA, was used to assess platelet and/or endothelial activity. In acute stroke neither platelet-bound P-selectin nor fibrinogen after stimulation, i.e. reactivity, differed from AF controls. In contrast, lower platelet activity as judged from surface attached and circulating P-selectin without agonist stimulation proved to be a feature of cerebral infarctions. The p-values were $p < 0.001$ and $p < 0.01$, respectively. It is concluded that acute stroke is not associated with platelet reactivity platelets circulate less activated during the disease. It is evident that the mechanisms reflecting platelet reactivity and activity being investigated in this study play minor roles in stroke pathophysiology. New powerful platelet inhibitory drugs are currently introduced. To avoid major bleeding studies on platelet, behavior in acute stroke are necessary before including these medications in stroke treatment protocols.

Keywords: Flow cytometry, platelet activation, platelet reactivity, platelets, P-selectin

Introduction

Acute cerebral infarctions affect a significant proportion of the elderly population. The diagnosis appears to comprise a collection of syndromes with varying pathophysiologies. Neurologists have known for some time that clinical, imaging, and pathological features identify three prevalent subgroups of the disease [1]. One subtype, large-artery disease, is caused by atherosclerotic plaque lesions in major extracranial vessels. This impairs blood flow to the brain due to obstruction or even complete occlusion of arteries. Small-vessel disease (lacunar infarction) is typically thought to result from small emboli or local thrombosis. Cardioembolic stroke is characterized by thrombus development through sluggish blood flow in the left heart chambers.

P-selectin is located in the α-granule of resting platelets [2] and in the Weibel–Palade bodies of endothelial cells [3]. In response to activating signals, the protein migrates to the platelet surface. The platelets then secrete proteins, such as β-thromboglobulin [4]. The final steps of the platelet activation process turn on surface receptors, such as glycoprotein IIb/IIIa. Fibrinogen binds to the latter receptor allowing platelets to aggregate [5]. The inflammatory response is stimulated as well

and involves substantial platelet–leukocyte cross talk [6]. Initial interactions between platelets and white cells are attributed primarily to the platelet surface-bound adhesion receptor P-selectin [7]. Subsequently, P-selectin is shed and the molecules then circulate. It is hypothesized that soluble P-selectin suppresses platelet–leukocyte interactions [8] and reflects platelet and/or endothelial activity [9]. In health, most circulating P-selectin originates from platelets [10].

Numerous laboratory procedures have been proposed to determine platelet reactivity. To quantify the platelet function, one usually measures platelet aggregation after *in vitro* agonist provocation [11]. Frequently, surface expression of P-selectin and fibrinogen following stimulation, as determined by flow cytometry, is used as a platelet reactivity measure [12, 13]. The latter determination reflects activated glycoprotein IIb/IIIa [14]. Surface-bound P-selectin *in vivo* is a sign of platelet activation and α-granule release. Circulating platelet secretion products, such as β-thromboglobulin [4] and soluble P-selectin [12], are frequently utilized as indicators of platelet activity. However, these determinations require sample centrifugation and manipulation, rendering them susceptible to *in vitro* artifacts.

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Platelets are deemed to be important in the pathophysiology of ischemic stroke. It is unclear whether platelet alterations constitute a cause of or a sequel to the disease. Limited work analyzes platelet reactivity in acute cerebral infarction, i.e., the effects of agonists *ex vivo* upon platelets. Early aggregation studies show augmented platelet reactivity in acute stroke [15]. Other scientists have demonstrated an absence of alterations or even a decline in platelet reactivity in conjunction with acute cerebral infarction [6]. Obviously, increased platelet size signifying greater reactivity, is a feature of stroke sufferers [16]. Science devoted to platelet activity in stroke is equivocal as well. Some authors substantiate increased platelet activation both in the acute event and during recovery [17–19]. Both platelet–granulocyte aggregates and platelet-derived microparticles appear to increase quantitatively [20, 21], suggesting increased platelet activity. Some researchers argue that platelet activity increases only in the acute phase of stroke [22]. Others, however, have reservations about the existence of increased platelet activation associated with cerebral infarctions [23]. In the acute event, platelet-bound P-selectin is increased but platelet activity markers decreased during recovery [24]. Conflicting data further exists with respect to circulating P-selectin. In acute stroke, concentrations of the protein appear to increase [6]. Still, other scientists have been unable to demonstrate soluble P-selectin alterations in stroke [25]. Even fewer studies have been devoted to platelet alterations in different stroke subsets. Platelet size is increased in large artery disease, indicating elevated platelet reactivity [26]. In contrast, other authors claim that the mean platelet volume is unrelated to stroke severity and subtype [27]. From the literature it also appears that lacunar strokes are associated with increased platelet activity [25] and that circulating P-selectin increases in large-vessel infarctions [28].

Aspirin, the archetypal platelet inhibitory drug, is frequently used for trials in stroke prevention. It impedes the platelet cyclooxygenase enzyme, thereby inhibiting thromboxane A_2, a potent cause of platelet aggregation and activation. Apparently, surface-bound P-selectin is unaffected by aspirin, whether after agonist provocation or in non-stimulated samples [29]. Based on extensive clinical trials resulting in worldwide treatment recommendations, it is accepted that aspirin reduces the risk of stroke relapse substantially [30]. However, it is unclear if the benefit is due to prevention of recurrent events originating from outside the central nervous system, or due to anti-inflammatory properties of the drug [31].

It is generally agreed that cerebral infarctions are associated with inflammatory reactions. The platelet–leukocyte crosstalk is important in stroke pathogenesis [19, 22, 32]. The mechanisms contributing to the inflammatory response have not been extensively characterized. It remains uncertain whether inflammation precedes or follows the brain injury. In coronary heart disease, inflammatory reactions neither affect the long-term outcome [33] nor is it clear if inflammation has similar impact on long-term prognosis in stroke. Recent reports have offered little evidence of an association between the inflammatory responses and recurrent vascular events [34].

The differing opinions with respect to platelet reactivity and activity in conjunction with acute cerebral infarctions

prompted us to perform this study. We hypothesized that platelet characteristics of stroke sufferers deviate from those detected in suitable control groups. The expressions of platelet surface activity markers, both with and without agonist stimulation, were quantified. In addition, determination of circulating P-selectin and various parameters believed to reflect the inflammatory response was carried out.

Methods
Study subjects
When laboratory resources were available, we prospectively enrolled individuals ($n = 72$; age 74 ± 10(SD) years; 31 females) with manifest acute cerebral infarction(s). All the patients were admitted to a specialized stroke unit. Acute stroke was defined as a sudden loss of focal cerebral function persisting for more than 24 hours. Stroke subjects underwent a CT brain scan. Intracerebral and subarachnoid hemorrhages were excluded from the study. Comatose patients and individuals suffering from very severe disease were also excluded. Otherwise, we did not apply any specific exclusion criteria. Stroke etiology was classified according to predefined criteria into large- and small-artery disease and cerebral damage due to cardioembolism [1]. Individuals with atrial fibrillation (AF), ($n = 58$; age 69 ± 7(SD) years; 12 females) who had undergone electrical cardioversion and apparently healthy elderly subjects ($n = 24$; age 66 ± 8(SD) years; 11 females), served as controls. Table I summarizes clinical and demographic information at hospital admission.

Table I. Demographic data for the two study groups with cerebral infarctions and AF at hospital admission.

	Acute stroke	AF	*p*-Value
Subjects (*n*)	72	58	
Age (years)[a]	74 ± 10	69 ± 7	<0.001
Body weight (kg)[a]	79 ± 15	85 ± 17	NS
Sex (m/f)	41/31	46/12	<0.05
Sampling time after the acute stroke (days)[a]	2.3 ± 1.8		
Cardioembolic disease (*n*)	16		
Large artery disease (*n*)	20		
Small artery disease (*n*)	36		
Restroke (*n*)	19		
A_2-blockers (*n*)	4	12	NS
ACE-inhibitors (*n*)	18	16	NS
Aspirin (*n*)	30	7	<0.001
β-blockers (*n*)	21	48	<0.01
Ca^{2+}-blockers (*n*)	16	4	<0.05
Diuretics (*n*)	14	22	NS
Statins (*n*)	15	17	NS
Vitamin K antagonists (*n*)	6	48	<0.001
Current smokers (*n*)	10	3	NS
Diabetes (*n*)	11	6	NS
Hypertension (*n*)	32	25	NS
Previous myocardial infarction (*n*)	7	5	NS
Blood pressure (diastolic) (mm/Hg)[a]	87 ± 19	79 ± 10	<0.01
Blood pressure (systolic) (mm/Hg)[a]	158 ± 31	132 ± 18	<0.001
Pulse (beats/minute)[a]	76 ± 17	83 ± 16	<0.05

Notes: [a]Mean $\pm$ SD and NS, not significant.

Continuous variables are reported as mean $\pm$ SD. The chi-squared test and the independent Student's *t*-test were employed for statistical evaluation. Two-sided *p*-values ($p < 0.05$) were regarded as indicating the significance. All patients and/or responsible parties gave informed consent to participate. The ethics committee of the nearby university hospital approved the study protocol.

Analytical procedures

At recruitment, approximately 20 ml of venous blood was sampled. Blood for flow cytometer analyses was collected in citrate. The samples were processed within a maximum of 1.5 hours after venipuncture. Both surface-bound P-selectin and fibrinogen were determined using a Beckman Coulter EPICS XL-MCL flow cytometer (Beckman Coulter Inc., Brea, CA, USA). Previous communications from our laboratory describe the procedures in detail [13, 14]. In brief, a phycoerythrin-conjugated monoclonal antibody against glycoprotein Ib (Dako AS, Denmark) recognized platelets. A monoclonal fluorescein isothiocyanate-conjugated monoclonal antibody (Immunotech, France) distinguished the surface-bound P-selectin. A chicken polyclonal antibody identified the membrane-attached fibrinogen. Subsequently, P-selectin trans-locations and membrane-bound fibrinogen *in vitro* after agonist provocation, i.e. platelet reactivity, were determined. Dilutions of a thrombin-receptor-activating peptide (TRAP-6) (74 and 57 μmol/l) (Biotechnology Centre of Oslo, Norway) and ADP (8.5 and 1.7 μmol/l) were employed as platelet agonists. The proportions of platelets (%) displaying more surface-bound P-selectin and fibrinogen, respectively, vs. a non-stimulated control were used as experimental parameters. Due to a technical failure, platelet reactivity, as judged from the platelet fibrinogen binding after agonist provocation, was not analysed in the healthy control group.

An enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) was used to analyse soluble P-selectin [35]. In order to avoid platelet activation during blood processing, a platelet inhibitory mixture was used as anticoagulant. The latter comprises equal volumes of the following solutions (pH 7.4 at 25°C).

(1) 2.7 mmol/l of theophylline dissolved in 0.15 mol/l of TRIS chloride buffer;
(2) 0.15 mol/l of Na_2 citrate and 0.13 mol/l of Na_3EDTA; and
(3) 0.001 g/l of prostaglandin E_1 and 1 ml of 95% ethanol in H_2O.

All the chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA.

The platelet and neutrophil counts were determined electronically. High-sensitive C-reactive protein (CRP) was measured in EDTA anticoagulated blood, using a turbometric technique.

Results

Demographic data of stroke and AF patients

Participants with acute stroke were older ($p < 0.001$) (Table I) and more frequently of female gender ($p < 0.05$). At admission, their blood pressure was higher, and the *p*-values were $p < 0.01$ and $p < 0.001$ for the diastolic and systolic blood

Table II. Platelet reactivity, activity, and inflammatory parameters in acute stroke and AF.

	Acute stroke (mean $\pm$ SD) ($n = 72$)	AF (mean $\pm$ SD) ($n = 58$)	*p*-Value
Platelet counts ($\times 10^{12}$/l)	265 $\pm$ 84	241 $\pm$ 64	NS
Platelet-bound P-selectin			
—TRAP-6 (74 μmol/l) (%)	42 $\pm$ 20	41 $\pm$ 21	NS
—TRAP-6 (57 μmol/l) (%)	17 $\pm$ 10	18 $\pm$ 9	NS
Platelet-bound fibrinogen			
—TRAP-6 (74 μmol/l) (%)	56 $\pm$ 25	58 $\pm$ 25	NS
—TRAP-6 (57 μmol/l) (%)	45 $\pm$ 28	42 $\pm$ 29	NS
—ADP (8.5 μmol/l) (%)	59 $\pm$ 20	57 $\pm$ 24	NS
—ADP (1.7 μmol/l) (%)	28 $\pm$ 17	29 $\pm$ 19	NS
Platelet-bound P-selectin			
—Without stimulation (%)	3.7 $\pm$ 1.6	5.4 $\pm$ 3.5	<0.001
Soluble P-selectin (μg/l)	74 $\pm$ 44	102 $\pm$ 53	<0.01
Neutrophil counts ($\times 10^{12}$/l)	5.0 $\pm$ 1.7	4.5 $\pm$ 1.5	NS
CRP (g/l)	12 $\pm$ 17	4 $\pm$ 8	<0.01

Notes: NS, not significant. %, percentage positive cells (P-selectin or fibrinogen).

pressures, respectively. Then, most of the stroke sufferers were given a loading dose of aspirin (300 mg) and the prescription (75 mg daily) was continued on a daily basis in the ward. AF subjects were treated almost exclusively with vitamin K antagonists. The groups did not appear to differ with respect to risk factors, such as diabetes, hypertension, or smoking habits. The numbers of previous myocardial infarctions were found to be similar. As expected, AF subjects were taking more β-blockers at hospital admission ($p < 0.01$). Stroke subjects then had more Ca^{2+} blockers ($p < 0.05$). The prescriptions of heart-protective and lipid-lowering drugs did not differ significantly between the study groups.

Platelet reactivity, activity, and inflammation in acute stroke and AF

Table II compares stroke cases vs. AF patients with respect to laboratory characteristics. Platelet reactivity as estimated from the membrane P-selectin expression following TRAP-6 stimulation (74 and 57 μmol/l) did not differ between the two study groups. Similar results were obtained when comparing platelet–fibrinogen binding after TRAP-6 (74 and 57 μmol/l) and ADP (8.5 and 1.7 μmol/l) provocation (Table II). In contrast, acute stroke was associated with lower platelet activity such that circulating platelets displayed less membrane attached P-selectin *in vivo* ($p < 0.001$). The finding of lower circulating P-selectin, confirmed the latter findings indicating that decreased platelet and/or endothelial activity is a feature of acute cerebral infarction ($p < 0.01$). The table further shows that acute stroke was associated with increased CRP values ($p < 0.01$).

Platelet reactivity, activity, and inflammation in acute stroke and healthy volunteers

The comparison of stroke sufferers vs. apparently healthy older individuals is given in Table III. Platelet reactivity was estimated from the surface-attached P-selectin after TRAP-6

Table III. Platelet reactivity, activity, and inflammation in acute stroke and healthy volunteers.

	Acute stroke (mean ± SD) ($n = 72$)	Healthy elders (mean ± SD) ($n = 24$)	p-Value
Platelet counts ($\times 10^{12}$/l)	265 ± 84	260 ± 78	NS
Platelet-bound P-selectin			
TRAP-6 (74 µmol/l) (%)	42 ± 20	74 ± 15	<0.001
TRAP-6 (57 µmol/l) (%)	17 ± 10	28 ± 18	<0.01
Platelet-bound P-selectin			
without stimulation (%)	3.7 ± 1.6	5.3 ± 2.1	<0.01
Neutrophil counts ($\times 10^{12}$/l)	5.0 ± 1.7	3.8 ± 1.5	<0.01

Notes: NS, not significant. %, percentage P-selectin positive cells.

(74 and 57 µmol/l), provocation was lower in stroke. The levels of significance were ($p < 0.001$) and ($p < 0.01$), respectively. Platelets of stroke subjects demonstrated less surface bound P-selectin *in vivo* ($p < 0.001$). Consequently, they circulate less activated. Finally, as expected, the inflammatory response, as determined from increased neutrophil counts, was higher in acute stroke ($p < 0.05$).

Discussion

It is evident that the current platelet features reflecting reactivity and activity have little impact on the pathophysiology of acute cerebral infarctions. Indeed, the condition proved to be unrelated to platelet reactivity (Table II). Furthermore, stroke sufferers displayed reduced platelet activation *in vivo*, as determined from both surface-bound and circulating P-selectin. Comparison was carried out with two control groups, namely subjects with AF (Table II) and healthy older subjects (Table III). Ample evidence testifies the effectiveness of aspirin for reducing stroke recurrence [30]. With a few exceptions [6, 23], previous work has revealed enhanced reactivity and increased platelet activity in acute stroke [19]. It, therefore, appears that the findings of this study are not consistent with earlier science or with up-to-date clinical practice.

The acute cerebral infarction group was heterogeneous in that large-vessel disease, lacunar infarctions, and cardiogenic stroke were combined. A caveat is necessary as platelet features of the subgroups can differ. In everyday clinical practice it is difficult to categorize stroke into subgroups. Nevertheless, our stroke subjects were evaluated according to subgroups as well (Table I). Platelet characteristics reflecting reactivity and activity proved to be unrelated to stroke origin (data not shown). Consequently, despite possible disparities, all stroke sufferers were grouped together for assessment.

According to the literature, platelet activity increases coincidentally with acute cerebral infarction [19], whereas we found it to be lower. Consequently, we failed to replicate previous findings. Current laboratory techniques do not assess all pathways of platelet reactivity and activation. Apparently, stroke is characterized by an increased number of platelet–leukocyte aggregates [23]. These are believed to signify platelet activity. Platelets trapped in aggregates may be undetected by the flow cytometer and the current experimental protocol may under-estimate the number of activated platelets. It is conceivable that the platelet activity is associated with reactivity. In our study, acute stroke proved to be unassociated with platelet reactivity (Table II). This makes it less likely that platelet subpopulations are 'lost' during laboratory procedures. Furthermore, we used two different modalities when estimating the platelet activity such that determination of both platelet-bound and soluble P-selectin was carried out. Both measures proved to be significantly lower in acute stroke. Therefore, with a reasonable degree of certainty, decreased platelet activity can be said to be a feature of acute cerebral infarctions.

Some further caveats exist in that subjects with acute stroke frequently were treated with low-dose aspirin, whereas many of the AF participants had vitamin K antagonists. Furthermore, many stroke sufferers received a loading dose of aspirin in the ward. The drug does not affect platelet reactivity markers, such as surface-bound fibrinogen after agonist provocation [29]. Aspirin has acknowledged anti-inflammatory effects [31]. This may explain the favorable effects of the drug in preventing stroke recurrence [30] despite current data showing unaltered platelet reactivity and reduced activity concomitant with acute stroke.

From an ethical standpoint, it is not feasible to conduct blood sampling in cases of very severe disease. Consequently, comatose individuals and those considered unlikely to survive were excluded from the trial. Therefore, with a high degree of certainty, current science involves less gravely affected individuals than normally found on a stroke ward. This constitutes a possible source of error. It also offers an explanation as to why our study includes less large-artery disease than comparable investigations. Previous studies have been criticized on grounds of methology, limited patient numbers and for not assessing patients in the acute phase after an ischemic stroke. Frequently, apparently healthy age- and sex-matched individuals served as controls [19], whereas we used individuals with AF and healthy older subjects as controls.

This study has ascertained that the P-selectin expression of non-stimulated circulating platelets is reduced in the acute phase of cerebral infarction. This is in keeping with a previous study showing fewer activated platelets in stroke convalescence [23]. The latter also demonstrated less platelet reactivity *in vitro* following agonist provocation. We failed to reproduce that finding, as platelet reactivity in acute stroke in our investigation was similar to platelet behavior in AF controls (Table II). This study further demonstrates that platelet activity, as estimated from both surface-bound and circulating P-selectin, was lower in conjunction with stroke. It is apparent that the current results are not consistent with the daily practice for stroke treatment. In stroke bleeding, complications are possible following rigorous antiplatelet regimens. New effective platelet inhibitors are currently being introduced. Due to increased risk of severe bleedings, this study suggests that large-scale trials are necessary to evaluate relationships between platelets and stroke pathophysiology before introducing these new powerful drugs for stroke treatment.

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Declaration of interest: We declare no conflict of interest.

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