Regular Article

Levels and value of platelet activation markers in different subtypes of acute non-cardio-embolic ischemic stroke

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Abstract

Introduction: Platelet activation and its interaction with leukocytes are important in the pathophysiology of ischemic stroke. This study aimed to evaluate the value of platelet activation and platelet-leukocyte interaction in different subtypes of acute, non-cardio-embolic ischemic stroke.

Methods: Fifty-four patients with acute, non-cardio-embolic ischemic stroke, including 32 small-vessel and 22 large-vessel diseases, were evaluated. Platelet activation markers (CD62P, CD63, and CD40L) and platelet-leukocyte interaction were measured by flow cytometry at different time points (<48 hours and Days 7, 30, and 90 post-ischemic stroke). Markers were also evaluated in 28 other stroke patients in the convalescent stage (3 to 9 months after acute stroke) and in 28 control subjects.

Results: Patients with ischemic stroke had significantly increased circulating CD62P, CD63, platelet-monocyte interaction, and platelet-lymphocyte interaction in the acute stage compared with the convalescent stage and control groups. Levels of CD62P and CD63 were significantly higher in the large-vessel disease group than in the small-vessel disease group, and differences in CD62P were significant even at one month. The CD40L level in the poor outcome group was significantly higher than that in the good outcome group. Stroke patients with diabetes mellitus and large-vessel disease were associated with poor outcome.

Conclusions: Patients with large-vessel cerebral infarction elicit higher platelet activation and platelet-leukocyte interaction compared to small-vessel infarction. Further large scale trials are warranted to evaluate the relationship between platelet activation markers and outcome in stroke patients under different anti-platelet therapies, and to clarify optimal treatment.

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Introduction

Stroke is the third most common cause of mortality worldwide and is the major cause of permanent disability [1]. Ischemic stroke is classified into various categories according to the presumed mechanism of the focal brain injury, and the type and localization of the vascular lesion. Characteristically, non-cardio-embolic ischemic stroke subtypes present with two distinct patterns: large-artery atherosclerotic infarction and small-vessel occlusion (lacune) [2]. The pathogenesis of lacunar infarction is enhanced small-vessel permeability with mural deposition of serum protein in areas of blood-brain barrier breakdown, so called lipohyalinosis [3], while atherothrombosis is a major cause of large-vessel cerebral infarction [4]. Thus, different subtypes of ischemic stroke have different risk factor profiles, with consequences on prognosis [5,6].

Current studies have demonstrated that increased platelet activation markers (e.g. CD62P [P-selectin], CD63, and CD40L) are found during acute ischemic stroke and even in the chronic phase [7,8]. Furthermore, elevated levels of platelet-leukocyte interactions, especially platelet-monocyte and platelet-neutrophil aggregation, have also been demonstrated in patients with acute ischemic stroke [9,10].

Currently, very little clinical research has focused on either the value or serial changes in platelet activation markers and platelet-leukocyte interactions for predicting the clinical outcome of different stroke
subtypes under anti-platelet therapy. Several important questions remain unanswered, including the value of platelet activation markers and platelet-leukocyte interactions on therapeutic outcome, the correlation between platelet activation markers and non-cardio-embolic ischemic stroke subtypes, and serial change of platelet activation markers and platelet-leukocyte interactions after non-cardio-embolic ischemic stroke.

This prospectively designed study aimed to report on the relationship between serial platelet activation and platelet-leukocyte interaction levels with therapeutic outcome in non-cardio-embolic ischemic stroke patients.

**Patients and methods**

**Study Patients**

Consecutive patients admitted to the Chang Gung Memorial Hospital-Kaohsiung from January 2007 to January 2008 with acute ischemic stroke were evaluated in this study. Acute ischemic stroke was defined as sudden-onset loss of focal cerebral function persisting for more than 24 hours. Stroke diagnosis was established based on clinical presentation, neurologic examination, and brain magnetic resonance imaging (MRI) with magnetic resonance angiography (MRA) within one week of the event.

On the basis of clinical evaluation and results of imaging studies, all non-cardio-embolic ischemic stroke patients were divided to two major etiologic subtypes (i.e., large-artery disease and small-artery disease) according to the TOAST criteria [2]. Excluded were patients with: 1) cardio-embolic stroke, stroke of other determined etiology, and stroke of undetermined etiology; 2) evidences of fever after stroke or with a history of infection one week before the stroke; 3) intracranial hemorrhage, a history of recent surgery or trauma within the preceding 3 months; 4) underlying neoplasm, hematological disorders that affect platelet count or function, end stage renal disease, liver cirrhosis, congestive heart failure as well as those with a history of atrial fibrillation or valvular heart disease; and 5) comatose or considered unlikely to survive for more than 3 months.

At baseline, demographic data, history of risk factors (i.e., hypertension, diabetes mellitus, dyslipidemia, cigarette smoking, cardiovascular disease, and asymptomatic carotid stenosis), and history of previous vascular events (i.e., myocardial infarction, angina, old stroke history) were obtained. Vascular risk factors included the following: hypertension, on anti-hypertensive treatment or blood pressure >140/90 mmHg at 2 readings; diabetes mellitus (DM), on anti-diabetic treatment or elevated hemoglobin A1c or elevated blood glucose at 2 readings; and dyslipidemia, on lipid-lowering medication or total cholesterol >200 mg/dL or triglycerides >180 mg/dL [11].

The therapeutic regimens of ischemic stroke were based on the American Heart Association (AHA)/American Stroke Association (ASA) guidelines for the prevention of stroke in patients with ischemic stroke and transient ischemic attack [12]. In order to avoid the confounding factor of various anti-platelet therapies, patients with anti-platelet therapy except aspirin (100 mg/day) were excluded. Patients received the first dose of aspirin orally within 24 hours after the acute stroke. The Chang Gung Memorial Hospital’s Institutional Review Committee on Human Research approved the study protocol and all of the subjects provided informed consent.

**Control subjects**

For comparison, 28 patients with non-cardio-embolic ischemic stroke in the convalescent phase were also evaluated. The convalescent phase was defined as 3-9 months after onset of acute ischemic stroke. All of the 28 patients were under aspirin treatment for secondary prevention of stroke or cardio-vascular disease. Another control group was composed of 28 age- and gender-matched subjects with no clinical evidence of acute cerebral infarction within one year.

**Clinical assessments**

All of the subjects underwent complete neurologic examinations upon enrollment and during the follow-up period. Brain MRI with MRA, Duplex ultrasound study of the carotids, and trans-cranial color code sonography (TCCS) were performed in the acute and convalescent phase of the ischemic stroke. The control subjects only underwent Duplex ultrasound of the carotids and TCCS upon enrollment.

Stroke severity was assessed using the National Institutes of Health Stroke Scale (NIHSS). The physical disability and handicap of stroke patients were evaluated using the Barthel index (BI) and a modified Rankin Scale (MRS) during the acute and convalescent phases. Therapeutic outcomes were evaluated three months after discharge. Good outcome was defined as a 3-month BI > 60 without any cardio-vascular event, while poor outcome was any of the following end points: BI = 60 or MRS > 3 at 3 months, recurrent stroke, and death [13].

**Blood sampling and assessment of platelet and leukocyte activity**

Blood samples were collected from acute stroke patients within 48 hours of the stroke, and on day 7, day 30, and 3 months after the stroke, and were also taken from the convalescent stroke patients and control groups. Under minimal tourniquet pressure, blood was extracted from the antecubital vein using a sterile 19-gauge needle syringe in a single attempt with the subjects sitting for at least 10 minutes. The first 4 ml of blood was for complete blood count, while the following 9 ml was used for flow cytometry study. Another 4.5 ml of blood was used for blood chemistry study, including HDL-cholesterols and hemoglobin A1c.

The flow cytometry used was a Coulter EPICS XL (Beckman Coulter) and procedural details were as previously described [14]. Briefly, sodium citrate containing blood was centrifuged for 15 min at 1500 rpm at room temperature. The supernatant platelet-rich plasma was used to assess platelet activity. Platelet activation marker was determined with CD62P, CD63, and CD40L. The samples were incubated with saturating concentrations of phycoerythrin (PE)-labeled antibodies (Becton Dickinson Biosciences) against CD62P (clone AK-4), CD63 (Clone H5C6), and CD40L (Clone TRAP1), with fluorescein isothiocyanate (FITC)-labeled antibodies against CD61 (clone VI-PL2) for 30 min at room temperature in the dark. As control experiments, platelets were incubated with PE-coupled unspecific mouse IgG1 (Becton Dickinson Biosciences) with the same ratio and concentration of fluorochrome-to-protein as the specific IgG. After immuno-labeling, the samples were analyzed by Coulter Epics XL flow cytometry (Beckman Coulter). Forward light scatter and expression of CD61 was used for platelet identification. Platelet-bound anti-CD62P, anti-CD63, and CD40L antibodies were determined by analyzing 10000 platelets for PE fluorescence. Results were expressed as a percentage of antibody-positive platelets.

To evaluate platelet-leukocyte interaction, platelet–leukocyte aggregation assay of whole blood was diluted 1:10 with warmed buffer and 2 aliquots with an end volume of 50μL, which was incubated with CD61-FITC (an activation-independent sub-unit of platelet glycoprotein (GP) IIb-IIIa complex) to immunologically identify all platelets. Simultaneously, the sample was double-stained with CD45 to identify leukocytes. After incubation for 15 min, the process was stopped using cold buffer and immediately followed by flow cytometry. First, the leukocytes were identified by PE-Cy5-CD45 fluorescence positive, and then forward and sideward scatter properties of CD45-positive leukocytes were used to discriminate leukocyte subsets (neutrophils, monocytes, and lymphocytes). With simultaneous detection of CD61-FITC labeled platelets, platelet–leukocyte aggregates and platelet–leukocyte subset aggregates were recorded. For numeric evaluation, the percentage of leukocytes with aggregated platelets was quantified in relation to all detected leukocytes and leukocyte subgroups, respectively.
Results

Baseline characteristics of the study patients

Eighty (80) adults aged 18-85 years with acute non-cardio-embolic ischemic stroke were evaluated. Twenty-six of the 80 were subsequently excluded due to incomplete follow-up, use of other anti-platelet therapies (clopidogrel, aspirin/extended-release dipyridamole), fever or infection during the platelet therapies (clopidogrel, aspirin/extended-release dipyridamole), or incomplete follow-up, use of other anti-embolic therapies. The characteristics of these groups were similar by age and gender (Table 1). There was no significant difference in cardio-vascular risk factors among the three groups. In terms of laboratory data (Table 2), there were no significant differences among the groups in white blood cell (WBC), red blood cell (RBC) and platelet counts, serum triglyceride, prothrombin time (PT), activated partial thromboplastin time (APTT), and glycosylated hemoglobin (HbA1c). However, serum total cholesterol level was significantly higher in acute stroke group than in the convalescent and control groups (P<0.005).

Table 1
Baseline characteristics of stroke in the acute stage, convalescence stage and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Acute Stroke (n=54)</th>
<th>Convalescent Stroke (n=28)</th>
<th>Control (n=28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y) (mean±SD)</td>
<td>68.1±10.2</td>
<td>64.6±12.7</td>
<td>67.0±9.9</td>
<td>0.39</td>
</tr>
<tr>
<td>Male (% (n)</td>
<td>59 (32)</td>
<td>71 (20)</td>
<td>54 (15)</td>
<td>0.37</td>
</tr>
<tr>
<td>Hypertension % (n)</td>
<td>76 (41)</td>
<td>79 (22)</td>
<td>79 (22)</td>
<td>0.05</td>
</tr>
<tr>
<td>Diabetes mellitus % (n)</td>
<td>41 (22)</td>
<td>39 (11)</td>
<td>43 (12)</td>
<td>0.96</td>
</tr>
<tr>
<td>Hyperlipidemia % (n)</td>
<td>33 (18)</td>
<td>57 (16)</td>
<td>54 (15)</td>
<td>0.07</td>
</tr>
<tr>
<td>Cardiac disease % (n)</td>
<td>9 (5)</td>
<td>11 (3)</td>
<td>7 (2)</td>
<td>0.22</td>
</tr>
<tr>
<td>Current smoking % (n)</td>
<td>38 (12)</td>
<td>36 (10)</td>
<td>25 (7)</td>
<td>0.12</td>
</tr>
<tr>
<td>Previous stroke history % (n)</td>
<td>28 (15)</td>
<td>46 (13)</td>
<td>21 (6)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 2
Laboratory data of stroke in the acute stage, convalescence stage and control groups.

<table>
<thead>
<tr>
<th></th>
<th>Acute Stroke (n=54)</th>
<th>Convalescent Stroke (n=28)</th>
<th>Control (n=28)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (X10^3/ml)</td>
<td>7.5±2.5</td>
<td>7.2±2.1</td>
<td>6.4±2.6</td>
<td>0.17</td>
</tr>
<tr>
<td>RBC (X10^6/ml)</td>
<td>4.6±0.5</td>
<td>4.74±0.7</td>
<td>4.8±0.6</td>
<td>0.47</td>
</tr>
<tr>
<td>Platelet counts (X10^9/ml)</td>
<td>201±5.4</td>
<td>231±7.4</td>
<td>210±6.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>198.7±46.7</td>
<td>166.4±30.9</td>
<td>189.1±32.7</td>
<td>0.005</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>142.8±97.7</td>
<td>122.5±47.9</td>
<td>137.2±122.6</td>
<td>0.16</td>
</tr>
<tr>
<td>APTT (seconds)</td>
<td>27.8±2.3</td>
<td>29.0±2.6</td>
<td>28.5±4.9</td>
<td>0.30</td>
</tr>
<tr>
<td>PT (seconds)</td>
<td>10.0±0.5</td>
<td>9.6±0.4</td>
<td>9.8±2.1</td>
<td>0.40</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.5±2.1</td>
<td>6.8±1.0</td>
<td>6.5±1.0</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD; WBC, white blood cell; RBC, red blood cell; APTT, activated partial thromboplastin time; PT, pro-thrombin time.

Statistical analysis

Laboratory data and age were presented as mean±SD and comparison among acute stroke, convalescent stroke, and control groups was made by one-way ANOVA. Data of platelet activation markers and platelet-leukocyte interaction were presented as median and inter-quartile range (IQR) because the parameters were not normally distributed. The Kruskal-Wallis test was used for comparison of median values and the Chi-square test or Fisher’s exact test for comparison of proportions among groups, where appropriate. Repeated measures of ANOVA were used to compare platelet activity and platelet-leukocyte interaction at four different time points (<48 hours and on days 7, 30, and 90). Scheffe’s multiple comparison was used to analyze the intra-individual course of parameters over time and to compare parameters of two different groups. Laboratory data, platelet activation markers, and platelet-leukocyte interaction were compared between good and poor outcome groups using the independent t-test or Mann-Whitney U-test. Multiple logistic regression analysis was used to examine the independent influence of different predictive variables on each of a number of outcome measures of interest. A p value of <0.05 was considered statistically significant. All statistical calculations were performed using the SAS software package, version 9.1 (2002, SAS Statistical Institute, Cary, North Carolina).
The time courses of platelet activation markers in patients with acute ischemic stroke were listed in Table 3. Levels of CD62P were higher in the large-vessel disease group (median, 2.1%) at the acute stage of stroke than in the small-vessel disease group (median, 1.8%) (P = 0.075). Similarly, expressions of platelet-monocyte and platelet-lymphocyte interaction were significantly higher in the large-vessel disease group but the difference was not statistically significant at 3 months (P = 0.05). The levels of CD40L in the acute stage of stroke among three groups (P = 0.90).

The level of platelet-leukocyte interaction was significantly higher in the large-vessel disease group (median, 9.9%) than in the small-vessel and control groups (medians, 4.4% and 4.2%, respectively). Among those with good outcome, the median values (IQR) were 4.2% (2.3-10.8%), 19.1% (1.0-3.3%), 0.2% (0.1-0.4%), 26% (18.5-39.1%), 40.6% (38.9-51.8%), and 15.5% (10.0-20.3%), respectively. Among those with good outcome, the median values (IQR) of CD62P, CD63, CD40L, platelet-neutrophil interaction, platelet-monocyte interaction, and platelet-lymphocyte interaction in the poor outcome group were 5.1% (2.3-10.7%), 2.6% (1.7-3.8%), 0.4% (0.2-0.8%), 32% (24.2-43.0%), 54% (33.8-65.7%), and 16.5% (12.1-20.7%), respectively. Among those with good outcome, the median values (IQR) were 4.2% (2.3-10.8%), 19.1% (1.0-3.3%), 0.2% (0.1-0.4%), 26% (18.5-39.1%), 40.6% (38.9-51.8%), and 15.5% (10.0-20.3%), respectively. The statistical results revealed that CD40L in the poor outcome group was significantly higher than that in the good outcome group (P < 0.05; Mann-Whitney Test).

Statistical analysis of the clinical manifestations and laboratory data between the good and poor outcome groups revealed that DM...
The variables used in logistic regression included DM, large-vessel stroke, and CD40L on admission. After analysis, DM (p = 0.04, OR = 4.73, 95% CI = 1.01-22.18) and large-vessel disease (p = 0.03, OR = 5.49, 95% CI = 1.24-24.30) was independently associated with outcome. Furthermore, the area under the receiver operating characteristic (ROC) curve for CD40L at the time of admission was 0.70 (p = 0.04, 95% CI = 0.52-0.87). The cut-off value of CD40L on admission for poor outcome was 0.34% (sensitivity 70% and specificity 71%). Thus, this model discriminated well the patients with poor outcome.

Discussion

The major findings of the present study are as follows: (1) Platelet activation markers (CD62P and CD63) and platelet-leukocyte interaction (platelet-monocyte and platelet-lymphocyte) are significantly enhanced in acute stroke patients than in convalescent stroke patients and control subjects; (2) Levels of CD62P and CD63 are significantly higher in large-vessel disease than in small-vessel disease during the acute stage of stroke. Furthermore, CD62P expression remained significantly higher in the large-vessel group until one month compared with the small-vessel group; (3) Platelet-neutrophil interaction increased in the first weeks after stroke and gradually decreased thereafter; and (4) Higher CD40L level (cut-off value of >0.34%) at presentation was associated with poor outcome in non-cardio-embolic stroke patients.

Our study demonstrated that the expression of CD62P and CD63, but not CD40L, was significantly higher in acute stroke patients than in convalescent stroke and control subjects. A previous study has demonstrated that platelets expressed CD40L on the cell surface within a very short period of time following stimulation by thrombin [15]. The number of CD40L-positive platelets on activated thrombi is increased in the first 24 hours is significantly higher in the majority of damage following ischemic stroke does not occur immediately, but rather develops gradually over the course of many hours and even days [25]. Our results corroborated previous studies that inflammatory and thrombotic events are progressing within first week after acute ischemic stroke. However, all three platelet-leukocyte markers in the current study do not show statistical significance between large- and small-vessels diseases during the entire observation period.

DM is a well-known risk factor for stroke and is frequently associated with poor neurologic outcome [26,27]. This study demonstrates that DM is a predictor of poor three-month outcome in acute non-cardio-embolic stroke patients. Yip and colleagues found that high CD62P expression (cut-off value >3.16%) is strongly associated with adverse clinical outcomes after acute ischemic stroke [28]. Our study demonstrated that higher CD40L level (cut-off value >0.34%) at presentation is associated with poor outcome and there is a trend of higher CD62P, CD63, and platelet-leukocyte expressions on presentation in patients with poor outcome, even though the difference is not significant. The discrepancy between these two studies may be attributed to different methodologies (e.g. stroke subtypes of patients on enrollment or heterogeneous anti-thrombotic agents), follow-up periods (one month vs. three months), and statistical methods.

Although it has been shown in many previous studies that platelet activity and platelet-leukocyte interaction are enhanced after acute stroke, there are some methodologic disadvantages, including: (1) assessing the platelet activation markers in acute stroke patients using heterogeneous anti-thrombotic agents with different dosages (e.g. aspirin, 50 to 325 mg qd; the combination of aspirin and extended-release dipyridamole; or clopidogrel) [29]; (2) the expression of the platelet activation markers are obtained once in acute stroke patients [30]; (3) assessing the expression of platelet activation markers in heterogeneous groups of acute stroke patients [8,31]; and 4) assessing serial changes of platelet activation markers, which do not correlate with the clinical outcome using scientific score or neuro-imaging studies [7,10,32].

There are several limitations in this study. First, patients who are comatose, those considered unlikely to survive for more than three months, and those with recurrent stroke have been excluded. Thus, there is uncertainty in assessing the expression of the platelet activation markers and platelet-leukocyte interaction in critically ill and high-risk patients. Second, physiologic assays, such as those for
determining platelet aggregation or adherence, have not been performed. Therefore, increased expression of platelet activation markers and platelet-leukocyte interaction may not necessarily be reflected in changes in platelet physiologic function. Finally, the expression of the platelet activation markers and platelet-leukocyte interaction may be influenced by other drugs (e.g. statins and calcium channel blockers) that are commonly used in stroke patients, which may cause potential bias in statistical analysis [33,34].

In conclusion, the present study demonstrates increased platelet activation and platelet-leukocyte interaction in the acute stage of non-cardio-embolic ischemic stroke. Large-vessel cerebral infarction elicits higher platelet activation and platelet-leukocyte interaction compared to small-vessel infarction. Large-scale trials to evaluate the relationship between platelet activation markers and outcomes in stroke patients under different anti-platelet therapy and to clarify optimal treatment are warranted.

Acknowledgments

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