Original Article

Appearance of WBC-Platelet Complex in Acute Ischemic Stroke, Predominantly in Atherothrombotic Infarction

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Aim: Platelet aggregates with white blood cells (WBC-platelet complex) have recently been proposed as a marker of activated platelets, in addition to well-known molecular markers. We aimed to investigate the colocalization of activated platelets and WBC-platelet complex by means of flow cytometry, in patients with ischemic stroke.

Methods: Eighty-six patients with cerebral infarction (CI) in the acute phase (58 males, 28 females; 65 ± 14 years old) and 62 non-CI controls (23 males, 39 females; 53 ± 14 years old) were registered. The appearance of WBC-platelet complex was quantified using 3-color flow cytometry.

Results: The appearance rate of WBC-platelet complex was significantly higher in the CI group than in the controls. The appearance rate of WBC-platelet complex was significantly higher in atherothrombotic infarction (AT) than in lacunar infarction (LA) ($p < 0.05$). Furthermore, positive rates of both monocyte-platelet complex and granulocyte-platelet complex, but not lymphocyte-platelet complex, were significantly higher in the AT group than in the controls.

Conclusion: We concluded that WBC-platelet complex, especially involving monocytes and granulocytes, is a novel marker of platelet activation in the acute phase of ischemic stroke, mainly in AT.

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Key words; WBC-platelet complex, Cerebral infarction, Flow cytometry

Introduction

Atherothrombosis is the result of the progression of atherosclerosis, and its potentially life-threatening clinical consequences include coronary artery disease, cerebrovascular disease and peripheral artery disease. These events are mostly secondary to atherosclerotic plaque disruption and subsequent *in situ* thrombus formation¹. Similar pathophysiological changes in carotid arteries, as well as intracranial arteries, would contribute to the onset of ischemic stroke. Platelets are essential for primary hemostasis and repair of the endothelium, but also play a key role in the development of acute coronary syndromes and cerebrovascu-

lar events. Under various conditions, including shear stress, inflammation, hypertension, sleep apnea, etc., platelets are activated. They then form aggregates, release their contents, and subsequently participate in the formation and extension of atherosclerotic plaques. This activation is associated with the expression of P-selectin (CD62P), which translocates from the membrane of α -granules to the platelet surface. P-selectin mediates the adhesion of platelets to leukocytes². Thus, activated platelets, which adhere to the vessel wall at sites of endothelial cell damage, contribute to the development of chronic atherosclerotic lesions, ultimately leading to the acute onset of arterial thrombosis by plaque rupture.

Leukocytes have also been implicated in the pathophysiology of ischemic vascular disease. Adhesion molecule-mediated leukocyte recruitment is associated with increased tissue damage in stroke. Activated granulocytes release various inflammatory mediators, such as proteases and reactive oxygen species,

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Table 1. Characteristics of patients and controls

	AT (n=33)	LA (n=53)	Controls (n=62)
Males/Females	26/7	32/21	23/39
Age (years, mean $\pm$ SD)	65 $\pm$ 16 ***	65 $\pm$ 12 ***	53 $\pm$ 14
Risk factors			
Hypertension % (n)	51.5 (17) ***	41.5 (22) ***	9.7 (6)
Dyslipidemia % (n)	24.2 (8)	41.5 (22)	40.3 (25)
Diabetes Mellitus % (n)	15.2 (5) **	22.6 (12) **	1.6 (1)
Carotid intima-media thickness (>1.1) % (n)	69.7 (23) ***	49.1 (26) ***	6.5 (4)

LA: lacunar infarction, AT: atherothrombotic infarction

*** $p < 0.001$ ** $p < 0.01$ (vs control)

leading to endothelial cell damage^{3, 4}. Granulocyte elastase, a protease released from activated granulocytes, especially damages endothelial cells, because it is most active at neutral pH and has broad substrate specificity⁵. The processes of cellular adhesion, monocyte and macrophage attachment, and transmigration of immune cells across the endothelium are crucial steps in early atherogenesis and in the later stages of mature plaque rupture, particularly the transition of unstable plaque at the time of acute thrombosis.

WBC-platelet complex has also been described as an important factor in the pathogenesis of vascular ischemic syndromes⁶⁻⁹. Primary attachment of platelets to leukocytes occurs by tethering of the platelet's P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, so the heterotypic adhesion of platelets and leukocytes is not just a marker of ongoing platelet activation, but also has important consequences for leukocyte function. Adhesion protein combines with leukocytes on the surface of activated platelets to form WBC-platelet complex^{10, 11}, which eventually causes further endothelial injury. Thus, we believe that WBC-platelet complex might have value as a novel marker of platelet activation in the acute phase of ischemic events.

Method

Patients

The study group included 86 patients with cerebral infarction (CI) and 62 non-CI controls (**Table 1**). We registered 86 CI patients who had not been treated with anti-platelet agents and from whom blood samples had been taken during the acute phase (within 14 days) of ischemic stroke. These CI patients were classified into subtypes of ischemic stroke on the basis of the NINDS III classification¹². Based on this classification, we included atherothrombotic (AT) and lacu-

nar (LA) stroke in the CI group, and excluded cardio-embolic stroke and others. We also selected 62 control patients, who had complained of headache or dizziness, from among 1476 patients at our clinic in Tokai University Hospital.

Written informed consent was obtained from all subjects. This study was approved by the Ethics Committee of Tokai University.

Sample Collection

In the CI group, blood samples were taken within 14 days after the onset of stroke. Citrate-anticoagulated whole blood was obtained from the ante-cubital vein with the aid of a light tourniquet. The first 2 mL blood was discarded, and then 4.5 mL blood was collected slowly into a plastic syringe fitted with a 21-gauge needle containing 0.5 mL of 3.14% sodium citrate. To obtain the absolute cell count of leukocytes, white blood cells were simultaneously counted in EDTA-anticoagulated whole blood.

Direct Immunofluorescence Staining

Blood (25 μ L) was gently added to the micro-centrifuge tubes with 10 μ L of a cocktail (Oncomark; Becton Dickinson Biosciences, San Jose, CA) containing monoclonal antibody to CD45 (MoAb-CD45) to identify white blood cells, anti-glycophorin antibody to identify red blood cells and MoAb-CD41a to identify platelets. The reaction mixture was gently stirred without vortexing, followed by incubation for 15 min at room temperature in the dark. Subsequently, cells were fixed in 500 μ L cold lysing solution.

Flow Cytometric Measurements and Analysis of WBC-Platelet Complex

A BD FACS Calibur (Becton Dickinson Biosciences) was used to analyze white blood cells, which were identified based on CD45 fluorescence, forward

Fig. 1. Whole-blood flow cytometric analysis of WBC-platelet complex.

White blood cells were identified based on CD45 fluorescence, forward scatter and 90-degree light scatter. These populations were further gated as granulocyte (C), monocyte (D) and lymphocyte (E) subpopulations in the white blood cell area. These groups were further divided into CD41a-positive (platelet-bound: “areas (2)” in Fig. 1C, D, E) and CD41a-negative (platelet-free: “areas (1)” in Fig. 1C, D, E) subpopulations in the granulocyte, monocyte and lymphocyte areas.

light scattering and 90-degree light scattering (Fig. 1A, B). This population was further gated into granulocyte, monocyte and lymphocyte subpopulations (Fig. 1C, D, E). These groups were further divided into CD41a-positive (platelet-bound: “areas (2)” in Fig. 1C, D, E) and CD41a-negative (platelet-free: “areas (1)” in Fig. 1C, D, E) subpopulations in granulocyte, monocyte and lymphocyte areas. The cutoff point of CD41a positivity with granulocytes, monocyte, and lymphocyte was defined based on the histogram, which indicated two peaks of positive and negative populations. The percentages of monocyte-platelet complex, granulocyte-platelet complex and lymphocyte-platelet complex were analyzed in the presence of a WBC marker (CD45). To avoid the influence of the individual WBC count, the quantitative amount of WBC-platelet complex (count/ μ L) was adjusted by using complete WBC counts as the index of the fixed

quantity evaluation, and presented as a percentage of the WBC-platelet complex count by adjusting WBC counts.

Statistical Analysis

Statistical analysis was performed with SPSS Software (version 18.0J for Windows). Differences in the amount of WBC-platelet complex and the percentage of monocyte-platelet complex, granulocyte-platelet complex and lymphocyte-platelet complex between CI and control groups were examined for statistical significance using non-parametric tests. We used the chi-square test for comparison of age, risk factors (hypertension, dyslipidemia, diabetes mellitus), and carotid intima-media thickness among subgroups, between CI and the control group. Inter-group differences in clinical profiles between CI and the control groups were evaluated by the Mann-Whitney *U* test.

Table 2. Counts of WBC and appearances of WBC-plt complex in CI and Control groups

	CI		Controls (<i>n</i> =62)
	AT (<i>n</i> =33)	LA (<i>n</i> =53)	
WBC (counts/ μ L)	7700 ^{***, \$\$} (6100-8400)	6300 ^{***} (5625-7600)	5400 (4682-6180)
Platelet ($\times 10^4$ counts/ μ L)	23.1 (19.3-26.1)	21.3 (19.2-25.0)	22.6 (19.0-28.5)
WBC-plt complex (counts/ μ L)	765 ^{***, \$\$} (548-1013)	598 ^{***} (397-797)	390 (196-598)

median (25-75th percentiles)

^{***} $p < 0.001$ vs control, ^{\$\$} $p < 0.05$ vs LA

WBC: white blood cells, plt: platelet, LA: lacunar infarction, AT: atherothrombotic infarction, CI: cerebral infarction

Inter-group differences in clinical profiles among the control group, LA and AT groups were also evaluated by the Kruskal-Wallis *H* test. The relation between WBC count and WBC-platelet complex was also analyzed by Spearman's rank correlation. $P < 0.05$ was considered to denote statistical significance.

Results

Characteristics of Patients with Cerebral Infarction and Control Group

Table 1 shows the profiles of the CI patients and controls, including sex, age, hypertension, dyslipidemia, diabetes mellitus, and carotid intima-media thickness. In the CI group, 33 and 53 patients were assigned to the AT and LA groups, respectively. There were significant differences in age (Student's *t* test), hypertension, diabetes mellitus, and carotid intima-media thickness (IMT), but not dyslipidemia, between the CI and control groups (chi-square test) (**Table 1**).

Baseline Blood Cell Counts and Markers of Platelet Activation in Patients with CI

As shown in **Table 2**, WBC count in the CI group was significantly higher than that in the control group (5400 counts/ μ L) in each subtype, i.e., AT (7700 counts/ μ L, $p < 0.001$) and LA (6300 counts/ μ L, $p < 0.001$). Furthermore, WBC count was significantly higher in the AT group than in the LA group ($p < 0.05$). There was no significant difference in platelet counts. The appearance rates of WBC-platelet complex in AT (765 counts/ μ L) and LA (598 counts/ μ L) were also significantly higher than in the control group (390 counts/ μ L, $p < 0.001$ in each case). In addition, the appearance rate of WBC-platelet complex was significantly higher in AT than in LA ($p < 0.05$).

Relationship between WBC Count and the Appearance of WBC-Platelet Complex

Significant correlations were observed between

the WBC count and the appearance of WBC-platelet complex in both the control (**Fig. 2A**) and LA groups (**Fig. 2C**) ($r = 0.28$, $p < 0.05$ and $r = 0.42$, $p < 0.01$, respectively), but not in the AT group (Spearman's correlation) (**Fig. 2B**).

Furthermore, in order to exclude the possibility that the WBC-platelet complex just reflected WBC counts, we analyzed all data by adjusting the WBC counts. Percentage of WBC-platelet complex counts / WBC counts in both AT and LA were significantly higher than in the control group ($p < 0.01$), although there was not significant difference between AT and LA (**Fig. 3**).

Appearance of Granulocyte-Platelet Complex, Monocyte-Platelet Complex and Lymphocyte-Platelet Complex in the Stroke Subtypes

The appearance rate (%) of granulocyte-platelet complex in AT was also significantly higher than in the control group (**Fig. 4A**; AT vs control group; 13.6 vs 9.1%, $p < 0.05$). The appearance rate (%) of monocyte-platelet complex in AT was significantly higher than in the control group (**Fig. 4B**; AT vs control group; 23.1 vs 14.3, $p < 0.001$). The appearance rate (%) of monocyte-platelet complex was also significantly higher in AT than in LA (**Fig. 4B**; AT vs LA group; 23.1 vs 17.5%, $p < 0.05$); however, the appearance rate (%) of lymphocyte-platelet complex was not significantly different among the 3 groups (**Fig. 4C**).

Discussion

This study show that the appearance rate (%) of the WBC-platelet complex was significantly higher in the CI group than in the control group, and was significantly higher in the AT group than in the LA group.

Hypertension¹³⁻¹⁶, high total cholesterol¹⁶, high LDL-cholesterol and low HDL-cholesterol¹⁷ and diabetes^{18, 19} are known risk factors for ischemic stroke

Fig. 2. Relationship between WBC count and the appearance of WBC-platelet complex.

Significant correlations were observed between WBC count and the appearance of WBC-platelet complex in the control (A) and lacunar infarction (LA) groups (C), but not in the atherothrombotic infarction (AT) group (B)

and coronary heart disease. Increased IMT reflects inflammation in atherosclerosis²⁰, and is highly associated with the onset of ischemic stroke or myocardial infarction in middle-aged and elderly subjects^{21, 22}. These risk factors, which were present in ischemic stroke patients in this study (Table 1), may lead to the breakdown of homeostasis of vascular endothelial function, and may result in clot formation on damaged endothelial cells and plaque rupture. Our previous study demonstrated that the appearance rates of the platelet activation markers PAC-1 and CD62P were increased not only in patients with ischemic cerebrovascular disease presenting with carotid artery abnormality²³, but also in non-stroke patients with carotid artery abnormality. Thus, the appearance of both WBC-platelet complex and platelet activation may reflect active clot formation in the acute stage of atherothrombosis, especially in the acute phase of ischemic stroke.

We have developed a novel flow cytometric method using CD45 antibody to detect WBC-platelet complex, including granulocyte-, monocyte- and lymphocyte-platelet aggregates. This method is simple, convenient and inexpensive, and in these respects is superior to various existing methods for evaluating platelet activation, such as measurements of β -thromboglobulin, platelet factor 4²⁴ in plasma, platelet aggregation ability by flow cytometry²⁵, etc. Michelson *et al.*²⁶ used a method similar to ours to detect WBC-platelet complex with P-selectin (CD62P), and found that circulating WBC-platelet aggregates (especially monocyte-platelet aggregates) are a more sensi-

Fig. 3. Percentage of WBC-platelet complex counts/WBC counts in the CI and control groups.

Percentage of WBC-platelet complex counts/WBC counts in cerebral infarction (CI) groups were significantly higher than in the control group ($p<0.01$); however, the percentage of WBC-platelet complex counts/WBC counts was not significantly different between atherothrombotic infarction (AT) and lacunar infarction (LA).

tive marker of *in vivo* platelet activation than circulating P-selectin-positive platelets in patients with acute myocardial infarction. Furthermore, monocyte-platelet aggregates were significantly associated with the presence of carotid plaques in type 2 diabetic patients²⁷. On the other hand, the presence of WBC-platelet aggregates in other kinds of atherothrombosis,

Fig. 4. Appearance of monocyte-platelet complex, granulocyte-platelet complex and lymphocyte-platelet complex in the CI and control groups.

Granulocyte-platelet complex and monocyte-platelet complex in atherothrombotic infarction (AT) were significantly higher than in the control group. Appearance rate of lymphocyte-platelet complex was not significantly different among the AT, lacunar infarction (LA) and control groups. Monocyte-plt complex (%) = FACS monocyte-plt comp/FACS monocyte (count) $\times$ 100, Granulocyte-plt complex (%) = FACS granulocyte-plt complex/FACS granulocyte (count) $\times$ 100, lymphocyte-plt complex (%) = FACS lymphocyte-plt comp/FACS lymphocyte (count) $\times$ 100. Plt: platelet.

Controls: $n=62$, AT: $n=33$, LA: $n=53$

*** $p < 0.001$ vs control, * $p < 0.05$ vs control

such as cerebrovascular disease and peripheral arterial disease, has not yet been clarified. In the present work, we evaluated WBC subtype-platelet complexes, i.e., platelet aggregates with granulocytes, monocytes and lymphocytes, in patients with acute ischemic stroke for the first time. The strong relationship between WBC count and WBC-platelet complex in patients with ischemic stroke may reflect vascular inflammation in cerebral arteries.

Our results show that the appearance rate (%) of WBC-platelet complex and WBC count in each subtype, i.e., AT and LA, was significantly higher than in the control group, and was significantly higher in the AT group than in the LA group. Furthermore, significant correlations were observed between the WBC count and the appearance of WBC-platelet complex in both the control and LA groups, but not in the AT group. The appearance rate of WBC-platelet complex tended to be higher in patients with AT, even if the WBC count remained within the normal range. Then, we reanalyzed all data by adjusting the WBC counts. The data showed that the percentage of WBC-platelet complex was significantly higher in the CI group than in the control group. The percentage of WBC-platelet complex/WBC counts was significantly higher in both

AT and LA groups than in the control group, but was not significantly different between the AT and LA groups. Although the definite physiological role of WBC-platelet complex in both groups remains unclear, it may partly reflect the elevation of the WBC count, and others.

Recently, it has been shown that the three WBC subtypes show different kinetics of aggregate formation with platelets after ischemia. In the present study, we found that both monocyte-platelet and granulocyte-platelet complexes were very frequently observed in the acute phase of CI. Monocyte-platelet interaction occurs through P-selectin and P-selectin glycoprotein ligand-1²⁸⁾. The higher proportion of monocyte-platelet complex found in CD14^{high}CD16⁻ monocytes may result from higher expression of P-selectin glycoprotein ligand-1 in human CD14^{high}CD16⁻ monocytes and in murine Ly6C^{high} monocytes²⁹⁾. Clinically, platelet activation and monocyte-platelet interaction have been described in stroke patients^{11, 30)} and appear to be associated with a worse outcome^{31, 32)}. Furthermore, granulocyte-platelet adhesion was detected in unstable angina in association with increased granulocyte anti-CD11b and decreased anti-L-selectin immunofluorescence³³⁾. In general, the contribution of lym-

phocyte-platelet adhesion to stroke has not well been described in the literature. Recently, Marquardt *et al.*³⁴⁾ reported that the increase of monocyte-platelet aggregates is short-lived and may reflect an acute reaction to cerebral ischemia, whereas granulocyte-platelet aggregate formation persists into the subacute phase, and may be a particularly sensitive parameter reflecting both prothrombotic and inflammatory processes after stroke.

We found that both monocyte- and granulocyte-platelet complex counts were significantly higher in AT group, but not in LA group, as compared with the control group. In addition, the monocyte-platelet complex count in AT was significantly higher than that in LA. Our results are not in agreement with a previous report (McCabe *et al.*³⁵⁾), which suggested that the monocyte-platelet complex count, but not the granulocyte-platelet complex count, was higher in acute stroke patients than in controls. They also found that LA patients with the highest counts of granulocyte- and monocyte-platelet complexes in the acute phase had a history of mild hypertension, hyperlipidemia and excess alcohol intake. This discrepancy may be due to the different criteria used to define the acute phase (within 4 weeks in their study) and/or different techniques for detecting the complexes. Since endothelial damage and atherosclerotic plaque may be more prominent in AT than in LA, our results may reflect the severity of vascular inflammation in the whole body. Taken together, the differences in the WBC-platelet complex between the AT and LA groups may reflect not only elevation of the WBC count, but also elevation of the monocyte-platelet complex in patients with AT.

In conclusion, our results indicate that WBC (monocyte and granulocyte)-platelet complex in patients with ischemic stroke (especially AT) can be easily evaluated by means of flow cytometry, at low cost. Thus, WBC-platelet complex, with monocyte and granulocyte involvement, is suggested to be a novel marker of platelet activation in the acute phase of ischemic stroke, at least in AT. Finally, although the WBC-platelet complex may reflect both prothrombotic and inflammatory processes after stroke, it may be also influenced by other systematic conditions, such as sleep apnea syndrome, systemic infection, and/or malignancy.

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Disclosures

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