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Surface Expression of P-selectin on Platelets Is Related with Clinical Worsening in Acute Ischemic Stroke

Platelet activation has a critical role in arterial disorders. In this study, we showed that the upregulation of P-selectin expression on platelets was related with clinical worsening in acute ischemic stroke. We serially (within 24 hr, at 72 hr, and 7 days) measured the expression of P-selectin on platelets in patients with acute ischemic stroke (n=45) and investigated the correlation between their extents and clinical severity of ischemic stroke. A significant relationship between the P-selectin expressions and National Institute of Health Stroke Scale (NIHSS) was observed at 72 hr and 7 days after ischemic stroke onset. Patients with clinical deterioration showed significantly increased expression of P-selectin on platelets as compared to those without deterioration. These results suggest that the P-selectin expression on platelets may contribute to the aggravation of clinical course in acute ischemic stroke. Thus, adequate manipulation of activated platelets is an important therapeutic strategy in acute ischemic stroke.

Key Words : *Blood Platelets; Arteriosclerosis; Cell Adhesion Molecules; Cerebrovascular Accident*

Jae-Kwan Cha, Min-Ho Jeong*, Eun-Kyung Kim*, Yeong-Jin Lim*, Byung-Ryp Ha, Sang-Ho Kim, Jae Woo Kim

Department of Neurology and Institute of Medical Science*, College of Medicine, Dong-A University, Busan, Korea

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Address for correspondence
Jae-Kwan Cha, M.D.
Department of Neurology, College of Medicine, Dong-A University, 1, 3ga, Dongdaeshin-dong, Seo-gu, Busan 602-715, Korea
Tel : +82-51-240-5266, Fax : +82-51-244-8338
E-mail : nrcjk@unitel.co.kr

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INTRODUCTION

Platelets play a key role in arterial thrombosis (1) and acute vascular events (2, 3). Activated platelets translocate and secrete P-selectin from their alpha granule (4-6). Once exposed on activated platelets, P-selectin on platelets interacts with leukocytes and then induces inflammatory signals to potentiate vascular injury (7, 8). In several experimental studies (9, 10), early manipulation for P-selectin attenuates the reperfusion injury and infarction volume in ischemic rat brain, suggesting that P-selectin on platelets may implicate in progression of ischemic stroke. Actually, it has been reported that the surface expression of P-selectin on platelets is significantly increased in patients with acute ischemic stroke compared to normal control (11, 12). However, most previous studies focused on the differences of P-selectin expression on platelets according to the subtypes of ischemic stroke and did not serially evaluate the relationship between their changes and clinical severity of ischemic stroke. Therefore, in this study, we serially measured the extent of P-selectin on the surface of platelets in acute ischemic stroke to elucidate its relationship with severity of ischemic stroke.

PATIENTS AND METHODS

Patients

Forty-five ischemic stroke patients evaluated within 24 hr of stroke onset. Excluded from the study were patients (1) who have been previously taking anti-platelet agents; (2) who had autoimmune, hepatic, renal, or cancerous diseases; (3) who had a significant infection (leukocytes counts >11,000) or history of recent infection (<2 weeks). The diagnosis was established based on medical history, clinical examination, and result of brain MRI and MRA scan. In patients with acute ischemic stroke, the following diagnostic tests were performed; complete blood counts, blood chemistry, EKG, chest radiography, transthoracic cardiac echocardiography, transcranial cranial Doppler (TCD), and carotid duplex sonography. As a result, twenty-five patients had a large artery atherosclerotic infarction (LAA) related with major arteries (internal carotid, vertebrobasilar, or middle cerebral artery et al.) stenosis (>50%) which explains the localization of stroke, 13 patients lacunar infarction and 7 patient cardio-embolic infarction. The clinical severity of acute ischemic stroke was evaluated upon admission (within 24 hr), after 72 hr, and after 7 days of stroke onset

using the National Institutes of Health Stroke Scale (NIHSS) (13). Clinical deterioration was defined as an increase by ≥4 points on NIHSS during the 7 days of observation compared with the scores at initial work-up. All patients received intravenous heparin or subcutaneous heparinoid during this observation period.

Twenty-four normal subjects were recruited as a control group in which no evidence of large vessel atherosclerosis, clinical history of vascular events, and vascular risk factors were found.

Blood sampling

Peripheral venous blood was taken at 3 time points; within 24 hr, at 72 hr, and at 7 days after ischemic stroke onset. The samples were anticoagulated with 3.2% sodium citrate. All patients or relatives gave informed consent. Among the 45 ischemic stroke patients in this study, 1 patient died and 2 patients were discharged within 48 hr.

Preparation of whole blood for flow cytometry

The citrated whole blood samples were diluted at 6 folds in 30 μL of HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 20 mmol/L HEPES, 1 mg/mL bovine serum albumin, 3.3 mmol/L NaH_2PO_4, pH 7.4). The population of platelets was detected by using phycoerythrin (PE)-conjugated anti-CD42a (Pharmingen, SanDiego, U.S.A.). A 5-μL fluorescein isothiocyanate (FITC)-conjugated P-selectin (Pharmingen, SanDigo, U.S.A.) monoclonal antibody was used for surface staining to detect the activated platelets. After careful mixing and incubation for 15 min at room temperature, 2.5 mL of HEPES buffer containing 0.2% formaldehyde was added.

The stained platelets were analyzed by FACscan (EPICS XL, Coulter Electronics, Miami, U.S.A.). The assay that we used showed reproducible results and has been verified in other clinical studies (14, 15). The recognition of platelet population was found to be ≥99% for platelets-specific CD42a antigen. The extent of antibody binding was expressed as the mean fluorescence intensity (MFI) of total platelet population and was used as a quantitative measure for glycoprotein surface expression.

Statistical analysis

All data were presented as mean±standard deviation (SD). Statistical analysis was performed by using the SAS program. Statistical intergroup difference was examined with the chi-square and Fisher's exact test for categorical variables. Continuous variables were compared with t-test or ANOVA. To compare the platelet activation markers at 3 different time points, independent sample t-test was performed. To examine the correlation between the extent of P-selectin and NIHSS scores, Pearson's correlation coefficient was used. $p<0.05$ was considered statistically significant.

RESULTS

Platelet activation in acute ischemic stroke

Patient characteristics, including risk factors, and laboratory data taken on admission day are shown in Table 1. Forty-five

Table 1. Characteristics and the extent of P-selectin on platelets in patients and normal subjects

		Acute ischemic stroke	Normal subjects
Number of patients		45	24
Male (%)		22 (48.9)	12 (50)
Age (yr)		64.3±11.5	61.7±14.9
Hypertension (%)		25 (56)	-
Diabetes mellitus (%)		11 (24)	-
HbA_{1c}		8.2±1.8	-
Smoking (%)		19 (42)	-
Old myocardial ischemia		5 (11)	-
Leukocytes count (/μL)		7,270±2,440	6,520±1,700
Platelets count ($\times 10^3$/μL)		212±58	231±58
Total cholesterol (mg/dL)		203±47	180±40
LDL cholesterol (mg/dL)		123±35*	89±33
Triglyceride (mg/dL)		132±120	89±39
P-selectin (MFI)	initial 24 hr	108.2±38.3*	75.3±9.1
	at 72 hr	103.2±39.5*	
	at 7 days	101.7±41.3*	

*$p<0.01$ versus normal subjects.
MFI, mean fluorescence intensity.

Table 2. Comparison of basic characteristics and the extents of P-selectin on platelets in patients with and without clinical deterioration

	Clinical deterioration	Non-deterioration
Number of patients	9	31
Male (%)	6 (66.6)	14 (45.1)
Age (yr)	56.9±9.2	64.9±11.3
NIHSS	12.0±9.4	8.4±3.9
Hypertension (%)	2 (22.2)	22 (70.9)
Diabetes mellitus (%)	2 (22.2)	8 (25.8)
HbA_{1c}	8.4±0.5	8.8±1.2
Smoking (%)	6 (66.6)	13 (41.9)
Old myocardial ischemia	2 (22.2)	2 (6.4)
Leukocytes count (/μL)	9,300±3,200*	6,700±1,900
Platelets count ($\times 10^3$/μL)	226±72	207±54
Total cholesterol (mg/dL)	236±66	192±37
LDL cholesterol (mg/dL)	143±51	118±29
Triglyceride (mg/dL)	150±71	122±134
P-selectin (MFI)	110.7±39.5	103.7±33.9

*$p<0.05$ versus non-deterioration group.
LAA- large artery atherosclerotic infarction. NIHSS, National Institute of Health Stroke Scale. MFI, mean fluorescence intensity.

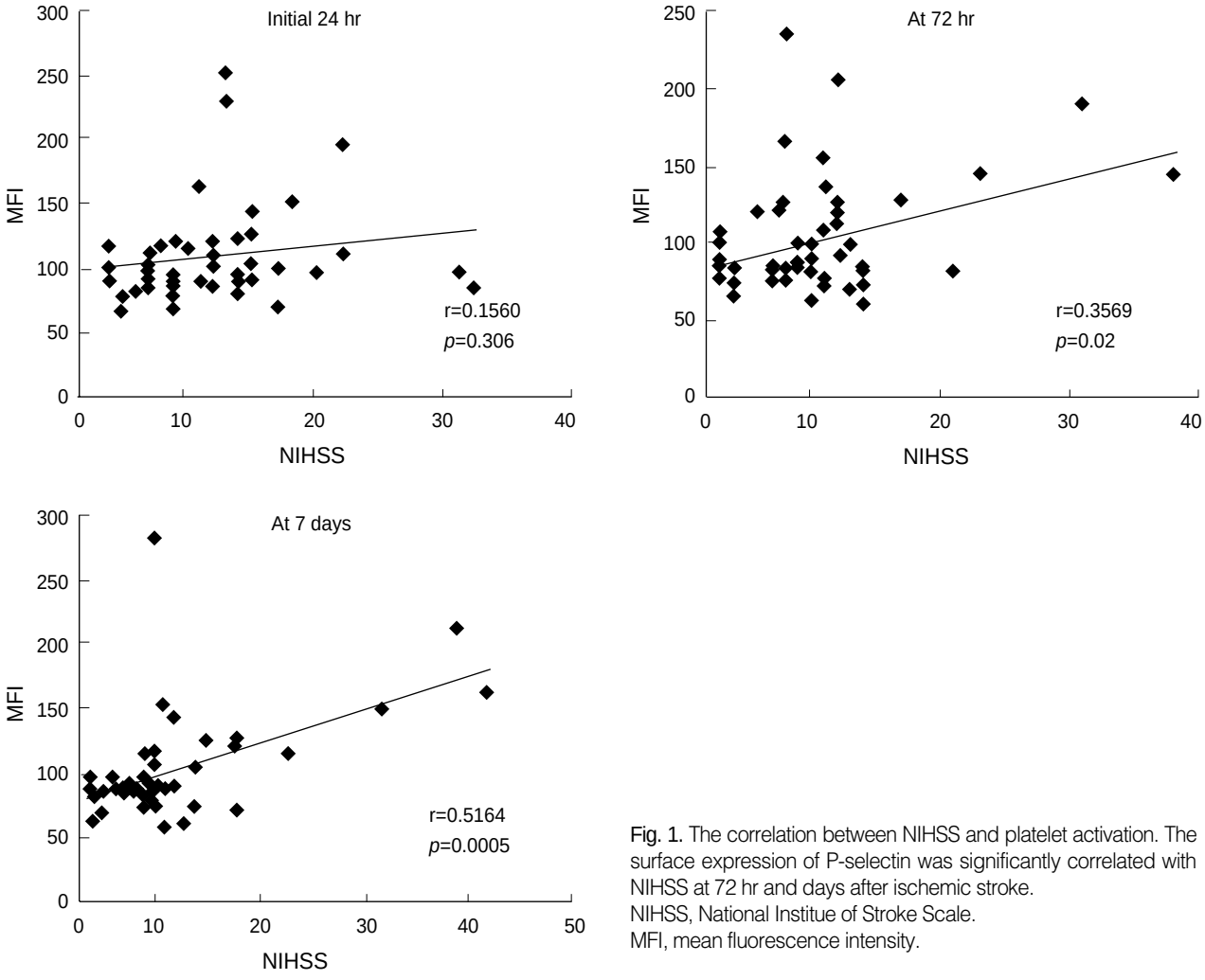

Fig. 1. The correlation between NIHSS and platelet activation. The surface expression of P-selectin was significantly correlated with NIHSS at 72 hr and days after ischemic stroke.
NIHSS, National Institue of Stroke Scale.
MFI, mean fluorescence intensity.

patients with acute ischemic stroke (22 men and 23 women) aged 31 to 83 yr (64.3±11.5) and normal subjects (n=24; 12 men and 12 women) aged 27 to 89 yr (61.7±14.9). During the initial 24 hr of onset, the surface expressions of P-selectin on platelets were significantly elevated in acute ischemic stroke patients compared to normal subjects. This elevation was sustained throughout the observation period of seven days in patients with acute ischemic stroke (Table 1).

P-selectin and clinical course of ischemic stroke

The mean NIHSS score was 10.2±6.2 at initial 24 hr, 9.0±7.0 after 72 hr, and 8.6±8.6 after 7 days of acute ischemic stroke. No significant correlation was observed between the extent of P-selectin on platelet and the NIHSS at the initial 24 hr. However, the surface expression of P-selectin correlated well with the NIHSS stroke scale after 72 hr. It became more prominent after 7 days (Fig. 1).

Among the 42 ischemic stroke patients who were evaluated for the complete observation period, 31 patients experienced improvement or remained stable, and 11 experienced clinical deterioration (LAA 7). Two patients with clinical deterioration had hemorrhagic transformation. To investigate the possible relevance of platelet activation to the clinical deterioration, data obtained from the clinical deterioration group (n=9) was compared with those from the non-deterioration group (n=31). Table 2 showed main baseline characteristics between clinical- and non-deterioration groups. The leukocyte count was significantly higher in deterioration group compared to non-deterioration group (Table 2). Surface expressions of P-selectin on platelets were statistically increased in patients with clinical deterioration than in the non-deterioration group after 72 hr and after 7 day compared to their counterpart (Fig. 2A). Because most of the acute ischemic stroke showing clinical deterioration belonged to the LAA group, the extents of P-selectin on platelets within LAA were re-analyzed. Table 3 summarizes the clinical and laboratory data between non- and deterioration group. All of clinical and laboratory findings showed no differences between two groups at initial 24 hr, except leukocytes count (Table 3). A significant increase of P-selectin expressions was observed after 72 hr and 7 days in the group that experienced clinical deterioration (Fig. 2B).

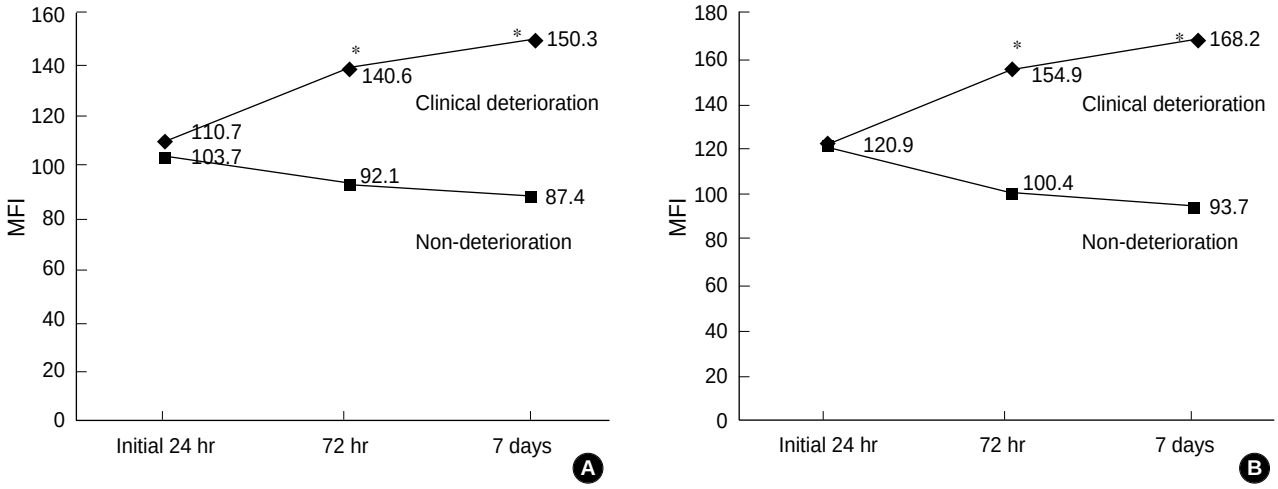

Fig. 2. Comparison of sequential changes of P-selectin on platelets in patients with and without deterioration. (A) Whole population of ischemic stroke (n=40). (B) Patients with large artery atherosclerotic infarction (n=22). MFI, mean fluorescence intensity. *p<0.05 versus non-deterioration group.

Table 3. Comparison of basic characteristics and the extent of P-selectin on platelets in large artery atherosclerotic infarition patients with and without clinical deterioration

	Clinical deterioration	Non-deterioration
Number of patients	7	15
Male (%)	5 (71.4)	9 (60)
Age (yr)	57.1±10.6	62.9±8.3
NIHSS	13.3±10.0	9.1±2.9
Hypertension (%)	1 (14.3)	9 (60)
Diabetes mellitus (%)	2 (28.6)	3 (20)
HbA_{1c}	8.4±0.5	8.2±0.5
Smoking (%)	4 (57.1)	8 (53.3)
Old myocardial ischemia (%)	2 (28.6)	2 (13.3)
Leukocytes count (/μL)	9,900±3,200*	7,400±1,800
Platelets count ($\times 10^3$/μL)	215±67	222±62
Total cholesterol (mg/dL)	251±67	192±32
LDL cholesterol (mg/dL)	150±56	117±27
Triglyceride (mg/dL)	154±70	98±34
P-selectin (MFI)	120.0±39.8	120.97±41.1

*p<0.05 versus non-deterioration group. LAA- large artery atherosclerotic infarction. NIHSS, National Institute of Health Stroke Scale. MFI, mean fluorescence intensity.

DISCUSSION

In this study, we measured the P-selectin expression on platelets by using whole blood flow cytometry. The advantages of this method include not being influenced by artificial platelet activation of centrifugation and directly reflecting the platelet activation in vivo (16). Therefore it has been known as an effective tool to screen the platelet function in clinical setting.

In this study, we used heparin or heparinoid in patients with acute ischemic stroke for initial 7 days. Previous reports (17, 18) showed that heparin or heparinoid could downregulate circulating level of P-selectin. In spite of this effect, our results revealed that surface expressions of P-selectin on platelets increased more significantly in patients with acute ischemia than in normal subjects, which is consistent with the results from the previous studies (11, 12). In this study the expression of P-selectin on platelets sustained during the 7 days after onset of ischemic stroke. Also, the surface expressions of P-selectin on platelets were correlated well with the clinical severity and significantly increased in patients with clinical deterioration at 72 hr and 7 days after ischemic insult. Thus, it is suspected that the P-selectin on platelets has important role in progression of ischemic stroke during 7 days.

It has been known that several different mechanisms are involved in the clinical deterioration of ischemic stroke. Among them, the inflammatory processes are important in progression of ischemic stroke. During the early stage of ischemic stroke, massive accumulation of leukocytes occurs at the ischemic areas and potentiates the ischemic neuronal injury by microvascular obstruction and by release of oxygen radicals and a cytolytic enzyme (19). In addition, various pro-inflammatory cytokines, chemokines, and adhesion molecules influence these processes (20-22). Interleukin (IL)-8 is a member of C-X-C chemokines (23), recruiting leukocytes, and was detected in brain tissues and blood during the early ischemia-reperfusion injury and finally contributed to the formation of brain edema in an animal study (24, 25). In line with the above mentioned facts, the leukocytes count could predict the clinical outcome after ischemic stroke (26, 27) and was significantly higher in the clinical deterioration group than in the non-deterioration group in our study. These inflammatory processes of ischemic events may be reciprocally affected by activated platelets (1, 7, and 8). Particularly, P-selectin on activated platelets interacts with P-selectin glycoprotein ligand (PSGL)-1 on leukocytes

and then secretes IL-8 and monocyte chemotactic protein (MCP)-1 to accumulate leukocytes and to aggravate the ischemic neuronal injury (28). In animal studies, P-selectin accumulates neutrophils in the ischemic brain and eventually leads to brain edema (9, 10). Therefore, it is hypothesized that overexpression of P-selectin on platelets may aggravate the progress of ischemic stroke. This hypothesis is partly supported by the observation that the increased expressions of P-selectin in platelets might predict the poor clinical outcome in acute coronary syndrome (29, 30). In the present study, we investigated the correlation between P-selectin and clinical deterioration in ischemic stroke. Therefore, we cannot clearly define whether the upregulation of P-selectin on platelets contribute to the development of clinical deterioration of ischemic stroke or simply represents a marker of the progression of brain ischemia. In an experimental study showing a causal relationship (31), animals that failed to express the P-selectin exhibited smaller infarcts and improved survival compared with control animals, suggesting that the P-selectin may be a critical factor for the provocation of neurological deterioration after ischemic stroke. However, it is still controversial (32) whether the gene deletion of P-selectin is beneficial or not in focal brain ischemia. Further studies are underway to clarify the causal relationship between P-selectin and clinical course of ischemic stroke.

In this study, no significant relationship was observed between the extent of P-selectin and clinical severity of ischemic stroke within 24 hr of ischemic onset. Although, we cannot exactly explain the cause, their extents within 24 hr of stroke onset may be revealed the differences according to the subtypes of ischemic stroke. Previous studies showed that the extent of P-selectin expression on platelets was significantly increased in atherosclerotic ischemic stroke than in other subtypes within 24 hr (11, 12).

Taken together, our data suggest that upregulation of P-selectin on platelets is related with the clinical course of acute ischemic stroke. Therefore, a therapeutic strategy to modulate the signal transduction through the membrane-bound P-selectin in activated platelets may help to prevent the progression and complication of acute ischemic stroke.

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