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Significant Association Between Neutrophil Aggregation in Aspirated Thrombus and Myocardial Damage in Patients With ST-Segment Elevation Acute Myocardial Infarction

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Background This study was designed to clarify the relationship between myocardial damage and platelet-neutrophil aggregation in patients with acute myocardial infarction (AMI).

Methods and Results The study group comprised 107 patients with ST-segment elevation AMI, in whom aspiration catheter was used during emergency percutaneous coronary intervention. Patients were divided into 2 groups according to the cellular density of neutrophils in the aspirated sample: group L (n=53), ≤100 neutrophils/0.025 mm^2 thrombus; group H (n=54), >100 neutrophils/0.025 mm^2 thrombus. Myocardial blush grade (MBG) ≤1 and ST-segment resolution (STR) <50% were more frequently found in group H than in group L. Peak creatine kinase level tended to be higher and left ventricular ejection fraction (LVEF) at 6 months after onset was lower in group H than in group L. Multivariate analysis showed that high neutrophil density in aspirated thrombus was an independent predictor of MBG ≤1, STR <50%, and low LVEF at 6 months after onset.

Conclusions Platelet-neutrophil aggregates retrieved from ruptured plaque may be associated with impaired coronary microcirculation and resultant myocardial necrosis/dysfunction. These findings underscore the clinical importance of the interaction between thrombosis and inflammation in the pathogenesis of AMI. (*Circ J* 2009; **73:** 139 – 144)

Key Words: Inflammation; Lesion; Leukocytes; Myocardial infarction; Thrombus

Patients with acute coronary syndrome (ACS) are characterized by increased platelet activation within the coronary circulation[1] Thrombus formation at a ruptured or eroded plaque and distal embolization of platelet aggregates eventually lead to myocardial necrosis[2] Several lines of evidence in experimental ischemia–reperfusion models suggest the pathological significance of neutrophil–platelet interaction in ACS[3–6] However, the importance of this interaction in humans, especially its in vivo significance, remains unknown.

Thrombus aspiration devices can retrieve thrombi from the culprit coronary lesion and the aspirated samples may provide intraluminal pathological information. In a previous flow cytometric study, neutrophil–platelet aggregate formation indicated activated thrombus[7] so in the present in vivo study we sought to evaluate the specific role of platelet–neutrophil aggregation in myocardial reperfusion injury, infarct size, and left ventricular (LV) remodeling in patients with ST-segment elevation acute myocardial infarction (STEMI).

Methods

Study Patients

From January 1, 2003 to July 31, 2005, 267 consecutive patients with STEMI were treated with emergency percutaneous intervention (PCI) within 24 h of the onset of chest pain. Inclusion criteria of STEMI were (1) continuous chest pain that lasted >30 min, (2) ST-segment elevation ≥0.1 mV in 2 or more contiguous leads on the 12-lead electrocardiogram (ECG), (3) angiographically detected culprit lesion with diameter stenosis ≥75% and/or Thrombolysis In Myocardial Infarction flow grade 0 or 1, (4) subsequent increase in the serum creatine kinase (CK) level to more than 3-fold of the upper limit of normal.

We used an aspiration device in 147 of the 267 patients. The choice to use aspiration was made by the operator and was based on angiographic images. In total, 40 of the 147 patients without aspirated thrombus were excluded, so in the final analysis, we used the data from 107 patients with STEMI (77 men, 30 women; age 66±12 [mean ± standard

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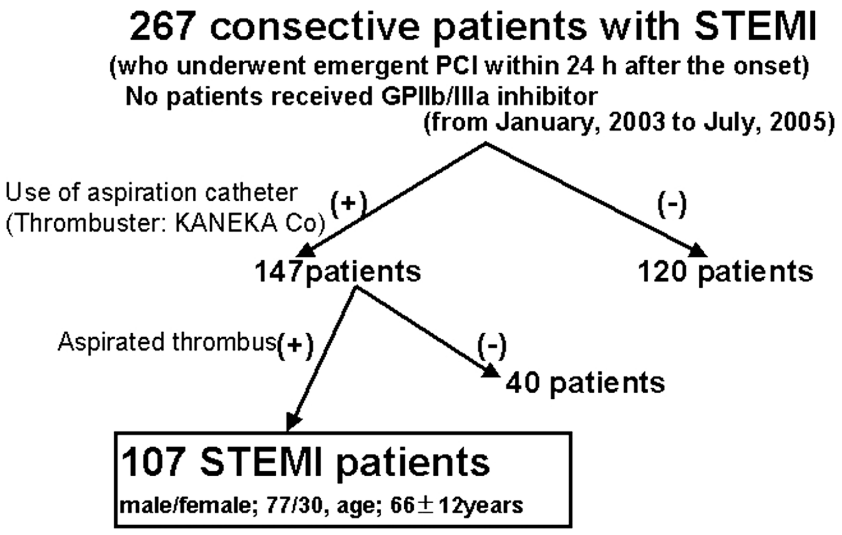

Fig 1. Flowchart of the study. STEMI, ST-segment elevation acute myocardial infarction; PCI, percutaneous intervention. GP, glycoprotein.

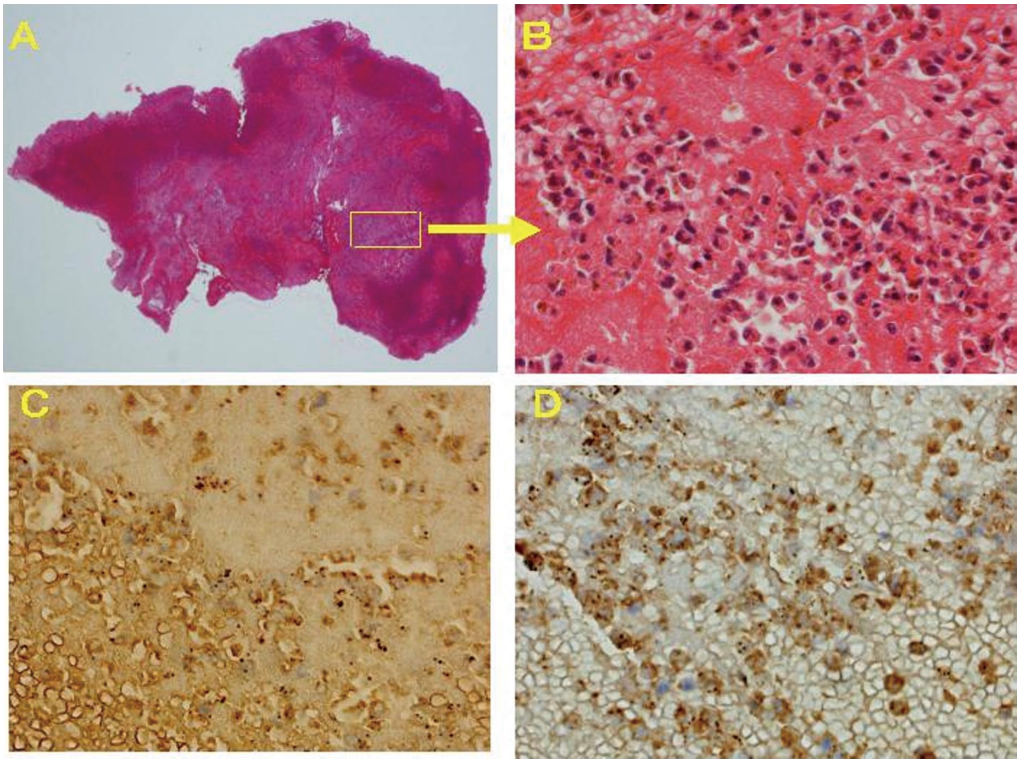

Fig 2. Histological analysis of thrombus burden aspirated from the culprit lesion. (**A**, **B**) Fragment of aspirated thrombus (H&E; A, ×20; B, ×20). (**C**, **D**) Serial sections examined by immunostaining for myeloperoxidase (MPO) and CD66 (C, ×400; D, ×400, respectively). In the quantitative analysis, the numbers of MPO- and CD66-positive cells/0.025 mm^2 were counted in at least 3 high-power fields (×400). The average of the counts from 3 fields with the highest numbers of neutrophils was taken as the neutrophil density.

deviation (SD)] years) (**Fig 1**). Elapsed time to reperfusion was 6.02±0.40 [mean ± SD] hours in these patients.

PCI

PCI was performed after intravenous administration of 10,000 IU heparin using a 6 or 7F sheath and catheters. Antiplatelet therapy before PCI consisted of aspirin only, because glycoprotein (GP) IIb/IIIa inhibitors and clopidogrel are not available in Japan. Facilitated thrombolysis with intravenous tissue plasminogen activator (tPA) was performed in 24 patients. After crossing the target lesion with the guidewire, coronary thrombus-aspiration was performed several times (depending on the operator's judgment). Aspiration was performed by 6 or 7F Thrombuster (Kaneka Co, Tokyo, Japan), which is a rapid exchange catheter with a central aspiration lumen and soft, flexible, non-traumatic tip with a large hole that communicates with the central lumen. A 20-ml screw syringe was connected to the proximal hub of the central lumen for thrombus-aspiration. In 23 patients, a distal protection catheter, PercuSurge GuardWire Plus (Medtronic Corp, Santa Rosa, CA, USA) was also used with the aspiration catheter at the operator's

Table 1 Baseline Clinical Characteristics and Coronary Angiographic Findings

	Group L (n=53) Neutrophil density≤100	Group H (n=54) Neutrophil density>100	P value
Age (years)	67.7±1.6	63.7±1.6	0.083
Gender (male, n (%))	42 (78%)	34 (64%)	0.119
Coronary risk factors, n (%)			
Hypertension	34 (63%)	36 (68%)	0.589
Diabetes mellitus	39 (74%)	32 (63%)	0.235
Hyperlipidemia	38 (70%)	31 (58%)	0.199
Smoking	33 (61%)	27 (54%)	0.092
Family history of CAD	11 (20%)	12 (23%)	0.755
Drugs n (%)			
Anticoagulant	12 (24%)	14 (26%)	0.821
Statins	16 (32%)	11 (21%)	0.194
ACEI/ARB	12 (24%)	6 (12%)	0.152
Ca-antagonists	11 (22%)	16 (30%)	0.344
β-blockers	3 (6%)	6 (11%)	0.334
Nitrates	7 (13%)	3 (6%)	0.210
Inflammatory markers (peripheral blood)			
WBC (/μl)	9,615±455	12,172±451	<0.001
Neutrophil (/μl)	6,609±426	9,218±418	0.001
CRP (mg/dl)	1.12±0.286	0.81±0.286	0.210
Previous MI, n (%)	6 (11%)	8 (14%)	0.424
Pre MI angina, n (%)	28 (52%)	21 (40%)	0.204
Time to reperfusion (h)	4.70±0.54	7.33±0.54	<0.01
Prior tPA, n (%)	16 (30%)	8 (15%)	0.061
Distal protection, n (%)	10 (19%)	13 (24%)	0.512
Killip score 1 vs 2/3/4, n (%)	46/7 (87/13%)	51/3 (94/6%)	0.169
RCA/LAD/LCX/LMT, n (%)	26/21/6/0 (50/39/11/0%)	26/22/5/1 (49/41/9/1%)	0.825
Multivessel disease, n (%)	34 (55%)	26 (58%)	0.431
Pre-reference diameter (mm)	3.42±0.08	3.48±0.09	0.595
Post-reference diameter (mm)	3.41±0.08	3.56±0.09	0.186
Pre-minimum diameter (mm)	0.19±0.05	0.16±0.05	0.748
Post-minimum diameter (mm)	3.20±0.07	3.33±0.08	0.275
Pre %stenosis (%)	96.04±1.34	96.64±1.25	0.748
Post %stenosis (%)	5.03±1.10	6.54±1.21	0.359
Bare metal stent (%)	46 (87%)	48 (89%)	0.970
Final TIMI 0/1/2 vs 3, n (%)	6/47 (11/89%)	7/47 (13/87%)	0.795

Data are mean±SD or median value (25–75th percentile range) or n (%).
CAD, coronary artery disease; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; WBC, white blood cell; CRP, C-reactive protein; MI, myocardial infarction; tPA, tissue plasminogen activator inhibitor; RCA, right coronary artery; LAD, left coronary artery; LCX, left circumflex; LMT, left main trunk; TIMI, Thrombolysis In Myocardial Infarction.

discretion. Bare metal stents were implanted in cases of coronary dissection or suboptimal results after balloon angioplasty. Quantitative coronary angiographic analysis was performed by QCA-CMS version 5.0 (MEDIS Medical Imaging Systems, Leiden, The Netherlands).

Assessment of Coronary Microcirculation by ST-Segment Resolution (STR) and Myocardial Blush Grade (MBG)

A 12-lead ECG was recorded immediately (≤30 min) before recanalization, and 1 h after recanalization. ST-segment elevation was measured at 80 ms after the J-point by 2 independent observers who were unaware of all clinical and angiographic findings. The sum of the ST-segment elevations in 3 contiguous leads with the highest ST-segment elevations was calculated. STR was defined as a reduction of at least 50% in ST-segment elevation on ECGs obtained 1 h after recanalization compared with the initial value[8] In this study group, there were no patients with left bundle-branch block or pacemaker rhythm.

MBG scale was determined by 2 observers, who were unaware of the clinical and angiographic findings, using a previously reported grading scale[9]

Assessment of LV Function and Infarct Size

LV function was evaluated on right anterior oblique views of left ventriculograms (LVG) obtained immediately and then 6 months after PCI. LV end-diastolic volume index (LVEDVI), and LV ejection fraction (LVEF) were determined by the centerline method (QLV-CMS version 5.0, MEDIS Medical Imaging Systems).

Blood samples were obtained on admission and at 3-h intervals until the CK level peaked and that value was used as the enzymatic marker of infarct size.

Measurement of Neutrophil Density in Aspirated Thrombi

The thrombi obtained by aspiration were immediately fixed in 10% buffered formalin solution for 6 h at 4°C and embedded in paraffin. Sections (4-μm thick) were stained with hematoxylin-eosin, and serial sections were immunostained for myeloperoxidase (MPO) and CD66 to identify neutrophils. After deparaffinization, tissues were pretreated with heat-induced epitope retrieval and the endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in methanol for 10 min. The sections were then incubated with a monoclonal antibody against human CD66 (DAKO Japan, Kyoto, Japan) and a polyclonal antibody against human MPO (DAKO Japan). Antibodies were used at a dilution of 1:50 and 1:600, respectively. Sections were washed with phosphate-buffered saline followed by incubation with Envision+ (DAKO Japan) for 30 min. After

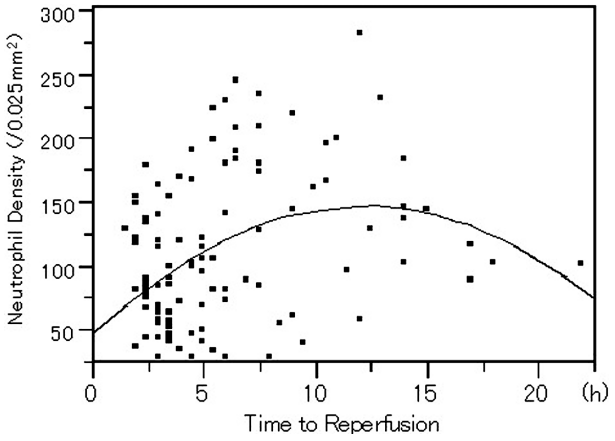

Fig 3. Association between neutrophil density and time to reperfusion. Regression analysis showing that the curve has a peak of neutrophil density around 13 h (P<0.01, r=0.285); the increased neutrophil density was not a simple function of time.

further washes, the sections were incubated with 0.005% 3,3'diaminobenzidine solution diluted with 5% hydrogen peroxide and counterstained with Meyer's hematoxylin. As the negative control for immunostaining, normal mouse immunoglobulin G was used instead of the primary antibody. The tissue from a lung abscess obtained at autopsy was used as the positive control.

The following quantitative analysis was performed by a pathologist who was unaware of the clinical information. As shown in **Fig 2**, the number of neutrophils in each aspirated thrombus was counted in a 0.025-mm^2 recutanglar region of interest (ROI). The neutrophil density in the thrombus was taken as the average of 3 ROI.

Statistical Analysis

Data are expressed as mean ± SD for continuous variables and as percentages for categorical variables. For testing of significance, continuous variables were compared using Student's t-test, and categorical data were compared using the chi-square test. To examine the relationship between neutrophil density and time to reperfusion, we constructed a quadratic regression curve and also assessed linear trend by analysis of variance. The variables included the clinical baseline (age, gender, medications, coronary risk factors, time to reperfusion, systolic blood pressure and heart rate on admission, peripheral inflammation data, culprit lesion, previous angina, the use of thrombolytic agents, and distal protection) and the neutrophil count in thrombi. Univariate and multivariate analyses were performed by logistic regression to determine independent predictors of impaired myocardial reperfusion and LV dysfunction (LVEF<45%) 6 months after onset. In this analysis, factors associated with the dependent variable at P<0.20 in the univariate analysis were entered into the multivariate model and eliminated using a backward procedure. A P-value<0.05 was regarded as statistically significant. All analyses were performed using the JUMP 5.1 software (SAS Institute, Cary, NY, USA).

Results

Neutrophils were detected in all of the aspirated thrombi. Patients were divided into 2 groups on the basis of the median density of all samples, which was 100.33 (27.33–287.33): group L, neutrophil density ≤100/0.025 mm^2 (n=53), and group H, neutrophil density >100/0.025 mm^2 (n=54).

Comparison of Patients' Characteristics and Angiographic Findings

Clinical characteristics and angiographic findings are summarized in **Table 1**. The majority of the parameters, with the exception of the white blood cell (WBC) neutrophil counts in peripheral blood, and the time to reperfusion, were similar between groups. The relationship between neutrophil density and time to reperfusion was not linear and the regression curve peaked at approximately 13 h (**Fig 3**). Additionally, there was not a linear relationship even when the patients were divided into 4 groups according to time to reperfusion: <6 h, 93±7/0.025 mm^2 (n=66); 6–12 h, 148±9/0.025 mm^2 (n=31); 12–18 h, 138±20/0.025 mm^2 (n=8); 18–24 h, 102±39/0.025 mm^2 (n=2).

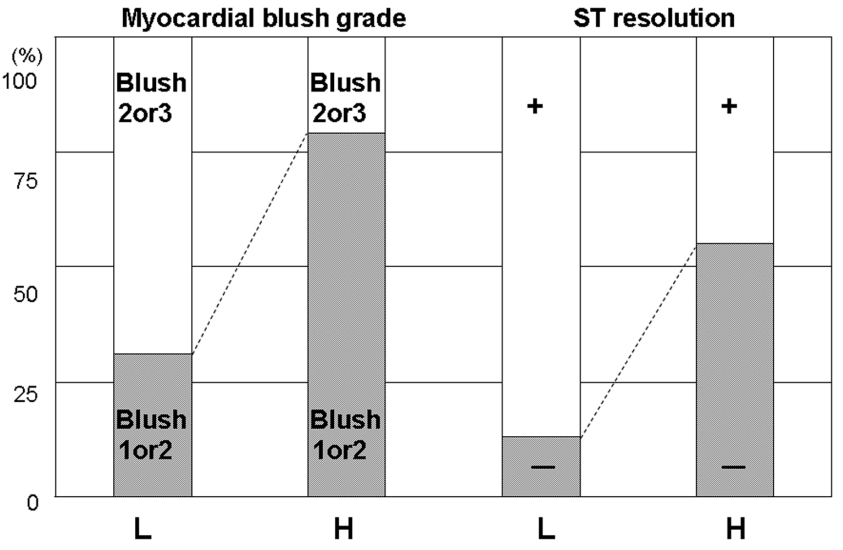

Fig 4. Comparison of myocardial reperfusion injury between group H (>100 neutrophils/0.025 mm^2) and group L (≤100 neutrophils/0.025 mm^2). (**Left**) Myocardial blush grade. Open bar indicates blush grade 2 or 3 and solid bar indicates blush grade 0 or 1. (**Right**) ST-segment resolution (STR). Open bar indicates STR and solid bar indicates no STR.

Table 2 Comparison of Groups L and H for Left Ventricular Function

	Group L (n=53)	Group H (n=54)	P value
LVEDVI (ml/m^2)			
Immediately after PCI	67.2±2.5	71.7±2.6	0.230
6 months after PCI	72.9±3.1	78.2±2.9	0.215
LVEF (%)			
Immediately after PCI	43.6±1.2	41.4±1.2	0.186
6 months after PCI	47.1±1.5	42.4±1.4	0.021

Data are mean±SD.
LVEDVI, left ventricular end-diastolic volume index; PCI, percutaneous coronary intervention; LVEF, left ventricular ejection fraction.

Table 3 Multivariate Analysis for the Incidence of Myocardial Reperfusion Injury and Left Ventricular Dysfunction

	OR (95%CI)	P value
Blush Grade 0 or 1		
LAD lesion	0.324 (0.118–0.826)	0.0216
Neutrophil count in thrombi	9.430 (3.777–25.912)	<0.0001
Absence of preceding tPA	2.450 (0.827–7.712)	0.1120
No ST-segment resolution		
RCA lesion	0.438 (0.163–1.117)	0.0898
Neutrophil count in thrombi	3.272 (1.249–9.070)	0.0180
Time to reperfusion	76.960 (6.595–1,385.694)	0.0013
LVEF <45% at 6 months		
Neutrophil count in thrombi	5.683 (2.044–17.474)	0.0014
LAD lesion	2.296 (0.830–6.728)	0.1164
Systolic BP on admission	0.024 (0.001–0.425)	0.0157

OR, odds ratio; CI, confidence interval; BP, blood pressure. Other abbreviations see in Tables 1,2.

Impaired Coronary Microcirculation, Myocardial Damage, and Myocardial Function

Impaired coronary microcirculation, defined as MBG of 0 or 1 (79% and 31%, respectively) and no STR (55% and 13%, respectively) occurred more frequently in group H than in group L (P<0.001) (**Fig 4**). Also, peak CK levels tended to be higher in group H than in group L (4,387±400 U/L vs 3,284±403 U/L, P=0.05).

Volumetric data immediately after and at 6 months after PCI are summarized in **Table 2**. LVG was performed at both time points in 38 patients in group L and in 42 patients in group H. LVEDVI and LVEF were similar for the 2 groups immediately after PCI, but at 6 months after the onset of AMI, LVEF was significantly lower in group H than in group L while LVEDVI remained similar in both groups.

Multivariate Analysis

To investigate which clinical variables and risk factors were associated with impaired coronary microcirculation and myocardial damage, we performed univariate and multivariate analyses (**Table 3**). Neutrophil density in thrombus was a common independent predictor for MBG 0 or 1, no STR, and LVEF <45% at 6 months. A significant association was found between LAD lesions and MBG 0 or 1, between time to reperfusion and no STR, and between low systolic blood pressure and LVEF <45% at 6 months. However, after incorporation of neutrophil count in thrombi into the multivariate regression model, the peripheral WBC and neutrophil count were not associated factors.

Discussion

To our knowledge, this is the first report of an analysis of the immunohistology of aspirated thrombi obtained from the culprit lesions in patients with STEMI. The major finding is that high neutrophil density in aspirated thrombus is associated with impaired coronary microcirculation, consequent myocardial damage (elevated CK level), and LV dysfunction.

A growing body of evidence suggests that inflammation plays an important role in ACS, followed by progression of atherosclerosis, plaque rupture and coronary occlusion because of thrombus formation. Aspiration devices have facilitated the recent study of aspirated thrombi, thus providing novel information regarding the histology of the culprit lesions.

Neutrophils stained with anti-MPO and anti-CD66 antibodies were detected in all of the aspirated thrombi in the present study (**Fig 2**). Local accumulation of neutrophils may be associated with increased platelet activity, as reported in a flow cytometric study[3] Neutrophil aggregation may be also involved in the primary etiology of increased platelet activity in the process of ACS. Activated neutrophils are reported to enhance platelet aggregation and thromboxane release in a P-selectin–P-selectin glycoprotein ligand-1 dependent manner, followed by an adhesion-strengthening interaction mediated by the β 2-integrin and Mac-1[4,10,11] Neutrophil-platelet aggregates are vehicles for the local delivery of surface-bound tissue factor, coagulation factor Xa and fibrinogen, each of which is a key component of the local coagulation response![12]

A recent randomized trial of patients with STEMI demonstrated that manual thrombus aspiration improved myocardial reperfusion, as compared with conventional PCI![13] The present study focused on the characteristics of aspirated thrombus and demonstrated that peak CK level tended to be higher in patients in which thrombus contained >100 neutrophils/0.025 mm^2 compared with those in which thrombus contained ≤100/0.025 mm^2. Moreover, although we did not obtained follow-up volumetric data in all patients

and medical treatments, especially the usage of statins, angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers, were not totally matched between the 2 groups, the paired data demonstrated that LVEF at 6 months after the onset of AMI was significantly different between the 2 groups (**Table 2**). Elevated peripheral inflammatory markers, which are associated with progression of myocardial damage after recanalization in patients with STEMI,[14–18] were not independent predictors for impaired coronary microcirculation and myocardial damage after inclusion of neutrophil count in thrombi into a multivariate model (**Table 3**). These findings indicate an important association between intraluminal platelet–neutrophil aggregates and clinical outcome following reperfusion therapy. Platelet–neutrophil aggregates could cause distal embolization by plugging the microvascular structures and activating the inflammatory cascade, leading to myocardial injury, probably because of increased production of reactive oxidants and diffusible radical species.[19]

Several experimental studies have shown that inhibition of neutrophil–platelet aggregation or neutrophil adhesion to endothelium with monoclonal antibodies can reduce infarct size; however, the studies in humans have been disappointing.[20–24] One possible explanation for the failure of anti-neutrophil therapy in humans is the pathway of administration. Systemic drug administration may not result in sufficient concentration to modulate local inflammation in the culprit lesion. In addition to local administration, a successful strategy against reperfusion injury may require targeting several pathways at once, rather than attempting to block a single final pathway. In fact, in a mouse AMI model, combined antibody therapy targeting both P-selectin and intercellular adhesion molecule-1 achieved a much greater reduction in infarct size, improved the LVEF, and regional myocardial blood flow compared with administration of either one of the antibodies alone.[25]

In conclusion, platelet–neutrophil aggregates retrieved from ruptured plaques may be associated with impaired coronary microcirculation and resultant myocardial necrosis/dysfunction. Although further prospective validation is needed, these findings indicate the clinical importance of the interaction between the pathways involved in thrombosis and inflammation in the pathogenesis of AMI.

Disclosures

None.

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