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ORIGINAL ARTICLE

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Circulating soluble CD40 ligand mediates the interaction between neutrophils and platelets in acute coronary syndrome

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Abstract Following plaque rupture, activated platelet will induce subsequent inflammatory process including neutrophil recruitment. In vitro study demonstrated an interaction between neutrophils and platelets via a mechanism involving CD40–CD40 ligand. However, whether this mechanism exists in the clinical setting remains unknown. Fifty-four patients with acute myocardial infarction (AMI) and 25 with unstable angina of pain onset of ≤24 h were enrolled consecutively. Acute myocardial infarction was diagnosed from three diagnostic criteria, i.e., anginal pain, electrocardiogram ST-T changes, and cardiac enzyme elevation. Unstable angina was diagnosed in patients without elevated cardiac enzymes. Peripheral venous blood was drawn at admission for routine blood count and soluble CD40 ligand (sCD40L) measurement. Neutrophil count was determined by an automated blood cell counter. Circulating sCD40L was measured using a standard enzyme-linked immunosorbent assay. Neutrophil count was significantly higher in AMI as compared with unstable angina ($P < 0.001$), whereas circulating sCD40L did not significantly differ. Despite marked elevation, no correlation was observed between neutrophil count and circulating sCD40L in AMI. Interestingly, we observed a strong and positive significant correlation between neutrophil count and circulating sCD40L level ($r = 0.607$, $P = 0.002$) in unstable angina. Circulating sCD40L is associated with neutrophil count and may mediate interaction between neutrophils and platelets in acute coronary syndrome, particularly in unstable angina.

Key words Soluble CD40 ligand · Neutrophil · Activated platelet · Acute coronary syndrome

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Introduction

Coronary atherosclerosis is a chronic inflammation process,[1] which occurs from initial stage into complicated stage of atherosclerotic plaque rupture.[2,3] Elevated leukocyte count has been shown in patients with acute myocardial infarction (AMI)[4,5] and is associated with higher risk for adverse events during the acute phase.[6] High leukocyte count during AMI is predictive of reduced epicardial blood flow and myocardial reperfusion, thromboresistance, and clinical adverse outcomes.[7] Leukocyte response in AMI is a major component of the systemic inflammation response to myocardial injury, and the greater the area of the myocardial necrosis, the greater the leukocyte response.[8] High leukocyte count associates with the level of high C-reactive protein (CRP), an acute-phase reactant and the most widely used marker of systemic inflammation, in acute coronary syndrome (ACS).[9]

CD40 ligand is a transmembrane protein of the tumor necrosis factor superfamily.[3] Activated platelet is a major source of CD40 ligand, which is subsequently cleaved by proteolytic process within a period of minutes to hours to form soluble CD40 ligand (sCD40L).[10] This circulating protein has the unique property of promoting both inflammatory and thrombotic processes.[11] Soluble CD40L is found to be elevated in coronary artery disease, particularly in patients with acute coronary syndrome (ACS).[12] The enhanced activity of sCD40L is more prominent in the setting of AMI, in which the upregulation of sCD40L release mechanism from platelets leads to elevated sCD40L level.[13]

Neutrophil is among the first leukocytes in acute inflammatory response to tissue injury, and is detected to be present in atherosclerotic plaques underlying both unstable angina and AMI.[14] Accumulation of neutrophils, with adherence of fibrin-platelet thrombus, occurs at the site of endothelial cell erosion after coronary artery bypass grafting.[15] Circulating neutrophil count is associated with the risk of AMI.[16] Hence, neutrophil activation may be one of the inflammatory components of acute myocardial infarction.[14]

In vitro study showed that CD40 expression in neutrophil is important in neutrophils and platelets interaction via the CD40–CD40L mechanism. This results in the activation of both neutrophils and platelets, enhancement of aggregation, and further stimulation of neutrophil and platelet conjugate formation.[17] However, the neutrophil and platelet interaction via the CD40–CD40L mechanism is unknown in the clinical setting. In this study, we aim to investigate further whether circulating sCD40L may mediate the interaction between neutrophils and platelets in ACS patients.

Materials and methods

Patients

We consecutively enrolled patients with acute myocardial infarction (AMI) admitted to the Intensive Coronary Care Unit (ICCU), Dr. Sardjito General Hospital, Yogyakarta, Indonesia, from June 2008 until November 2008. The inclusion criteria were patients with AMI, either ST-elevation AMI (STEMI) or non-ST-elevation AMI (NSTEMI), of ≤24 h duration and who gave informed consent. We excluded patients with chronic kidney disease in routine hemodialysis, history of severe chronic heart failure (New York Heart Association class >II), liver cirrhosis, known valvular heart disease, acute stroke, acute complication of diabetes mellitus (ketoacidosis or hyperglycemic hyperosmolar state), acute infection and sepsis, known chronic inflammatory disease, venous thromboembolism, malignancy, and pregnancy.

Acute myocardial infarction was diagnosed from three diagnostic criteria, i.e., anginal chest pain, ST-T changes on electrocardiography, and cardiac enzyme elevation. ST-elevation AMI was determined as anginal chest pain lasting more than 20 min, and electrocardiography examination revealing ST-segment elevation >1 mm in two or more consecutive limb leads and elevation >2 mm in two or more consecutive precordial leads. Non-ST-elevation AMI was determined as anginal chest pain and elevated cardiac enzyme (troponin I ≥0.6 ng/ml) but without ST-segment elevation.

We also enrolled patients admitted to ICCU with acute coronary syndrome but without any evidence of acute infarction (troponin I <0.6 ng/ml). These patients had a final diagnosis of unstable angina.

All patients gave their informed consent. The ethical clearance was approved by the ethics committee of the Faculty of Medicine, Gadjah Mada University.

Laboratory examination

Peripheral venous blood was drawn on admission for routine blood examination and before coronary revascularization for sCD40L measurement. Blood sampling was obtained after patients received the initial standard antiplatelet therapy of our hospital, i.e., oral loading dose 320 mg of acetosal and 300 mg of clopidogrel. Those initial drugs were administered less than 1 h before patients underwent coronary revascularization, i.e., medical thrombolysis or primary percutaneous coronary intervention (PCI) for STEMI and anticoagulant therapy, i.e., heparin for NSTEMI and unstable angina pectoris. Hematologic examination and clinical biochemistry parameters were determined by hospital standard laboratory methods. Total leukocyte, neutrophil, and lymphocyte counts were determined with an automated blood cell counter (Sysmex, Kobe, Japan). Troponin I was measured using the Abbott AxSYM system (Abbott Laboratories, Abbott Park, IL, USA) with normal range <0.6 ng/ml according to laboratory values. Serum for sCD40L measurement was taken after 30 min coagulation at room temperature followed by 5000 rpm centrifugation for 15 min, and stored at −80°C until final analysis. The analysis of circulating sCD40L was measured using a standard enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Quantikine Human soluble CD40 Ligand Immunoassay, R&D Systems, Minneapolis, MN, USA) and was read in a 450-nm standard plate reader.

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) version 13.0 for Windows (SPSS, Chicago, IL, USA). Continuous data were expressed as mean ± standard deviation and compared using the Student *t*-test for normally distributed data and the Mann–Whitney *U*-test for not normally distributed data. Tests for normality of data were conducted with the Kolmogorov–Smirnov test. Categorical data were expressed as proportion and compared using the chi-squared test. We further analyzed the correlation between continuous data using the Pearson correlation test and the Spearman rho test for a nonparametric alternative. Statistical significance was considered when the P value was less than 0.05.

Results

Characteristic of patients with AMI and unstable angina

During our study, we enrolled 54 patients with AMI and 25 patients with unstable angina. The characteristics of the patients are shown in Table 1. There were no differences in gender, age, diabetes mellitus, and dyslipidemia between patients with AMI and unstable angina. Hypertension (P = 0.015) was significantly more frequent in unstable angina as compared with AMI, whereas current smoking habit (P = 0.013) was significantly more common in patients with AMI. The laboratory examination showed no significant differences in lymphocyte count, platelet count, circulating sCD40L level, creatinine level, and lipid profile examination. Both leukocyte and neutrophil counts were significantly higher in patients with AMI as compared with unstable angina (P < 0.001 for both differences).

Table 1. Characteristics of 54 patients with acute myocardial infarction (AMI) and 25 patients with unstable angina

	AMI (n = 54)	Unstable angina (n = 25)	P value
Demography and risk factors			
Gender			0.219
Male	47 (87.0)	19 (76.0)	
Female	7 (13.0)	6 (24.0)	
Age (years)	55.8 ± 8. 1	54.3 ± 7.2	0.411
Diabetes mellitus	11 (20.4)	4 (16.0)	0.764
Hypertension	23 (42.6)	18 (72.0)	0.015
Current smoker	28 (51.9)	5 (20.0)	0.013
Dyslipidemia	18 (33.3)	11 (44.0)	0.360
Laboratory examination			
Leukocyte count ($\times 10^3$/ml)*	11.3 ± 3.7	8.2 ± 2.3	<0.001
Neutrophil count ($\times 10^3$/ml)*	8.3 ± 3.6	5.4 ± 2.3	<0.001
Lymphocyte count ($\times 10^3$/ml)*	2.0 ± 1.0	1.9 ± 0.6	0.742
Platelet count ($\times 10^3$/ml)	238.0 ± 72.8	242.2 ± 70.1	0.858
sCD40L (pg/ml)	8119.0 ± 2305.3	7861.4 ± 3651.3	0.748
Creatinine (mg/dl)*	1.4 ± 0.5	1.1 ± 0.3	0.055
Cholesterol total (mg/dl)	200.2 ± 57.0	194.7 ± 33.0	0.549
LDL cholesterol (mg/dl)	147.9 ± 44.3	132.4 ± 26.3	0.056
HDL cholesterol (mg/dl)	39.2 ± 17.7	38.5 ± 8.6	0.854
Triglyceride (mg/dl)*	130.8 ± 79.2	151.6 ± 78.0	0.126
Troponin I (ng/ml)*	14.3 ± 19.6	0.06 ± 0.11	<0.001

Data are presented in mean ± standard deviation for continuous data and n (%) for categorical data

LDL, low-density lipoprotein; HDL, high-density lipoprotein

*Not normally distributed data; comparisons were analyzed using Mann–Whitney U-test

Table 2. Correlation between neutrophil and leukocyte counts with circulating sCD40L and troponin I in patients with AMI

	Neutrophil count		Leukocyte count	
	Pearson correlation	Spearman rho	Pearson correlation	Spearman rho
Circulating sCD40L				
Coefficient (r)	0.226	0.204	0.284	0.256
P value	0.110	0.151	0.038	0.062
Troponin I				
Coefficient (r)	–0.85	–0.025	0.013	–0.046
P value	0.573	0.869	0.931	0.756

Correlation between neutrophil count with circulating sCD40L level in patients with AMI

The correlations between neutrophil and leukocyte counts with circulating sCD40L and troponin I levels in AMI patients are shown in Table 2. We found a positive significant correlation between leukocyte count and circulating sCD40L in patients with AMI, although strength of correlation was weak (Pearson correlation, $r = 0.284$, $P = 0.038$) but in Spearman rho test the correlation was not significant ($P = 0.062$) (Fig. 1). However, no significant correlation was detected between neutrophil count with sCD40L level in both Pearson correlation and Spearman rho tests ($P = 0.110$ and 0.151, respectively) (Fig. 2). Moreover, no significant correlation was observed between neutrophil and leukocyte count with troponin I level.

Correlation between neutrophil count with circulating sCD40L level in patients with unstable angina

Table 3 shows the correlation between neutrophil and leukocyte counts with circulating sCD40L in patients with unstable angina. We found a strong and positive significant correlation between leukocyte count and circulating sCD40L level in both Pearson correlation ($r = 0.607$, $P = 0.001$) and Spearman rho tests ($r = 0.748$, $P < 0.001$) (Fig. 3). Furthermore, a strong and positive significant correlation was also observed between neutrophil count and circulating sCD40L level in both Pearson correlation ($r = 0.607$, $P = 0.002$) and Spearman rho tests ($r = 0.730$, $P < 0.001$) (Fig. 4).

Table 3. Correlation between neutrophil and leukocyte counts with circulating sCD40L in patients with unstable angina

	Neutrophil count		Leukocyte count	
	Pearson correlation	Spearman rho	Pearson correlation	Spearman rho
Circulating sCD40L				
Coefficient (*r*)	0.607	0.730	0.607	0.748
P value	0.002	<0.001	0.001	<0.001

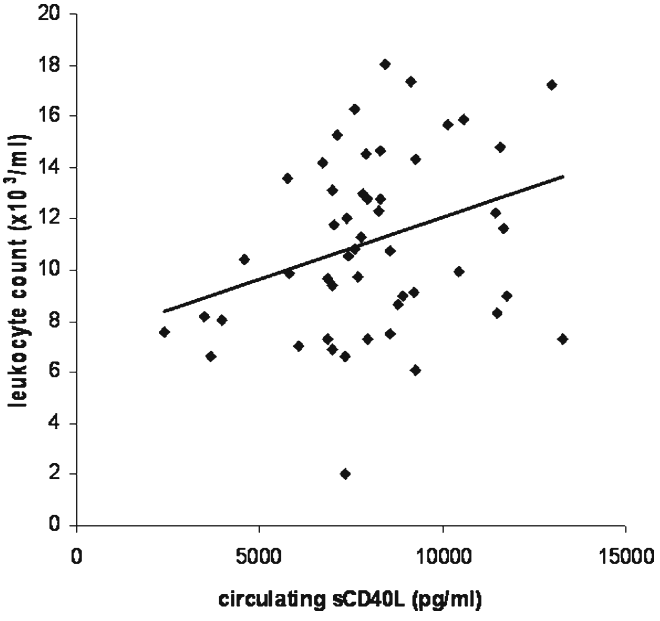

Fig. 1. Positive correlation between leukocyte count with circulating sCD40L in patients with AMI ($r = 0.284$, $P = 0.038$)

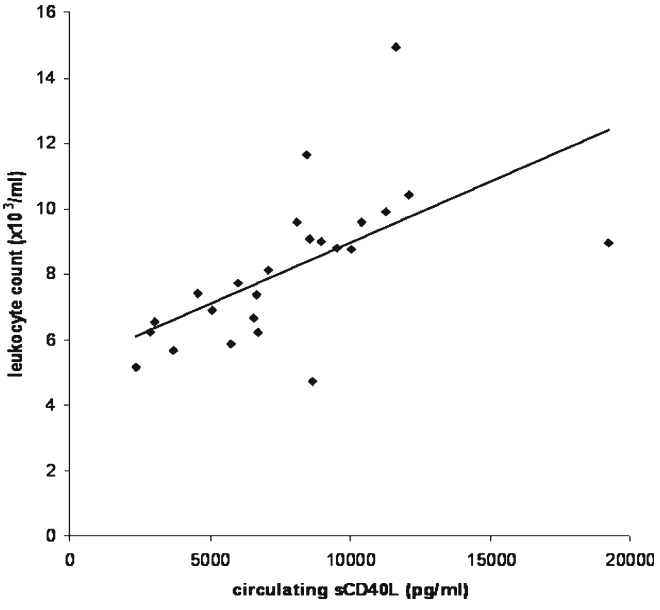

Fig. 3. Positive correlation between leukocyte count with circulating sCD40L in patients with unstable angina ($r = 0.607$, $P = 0.001$)

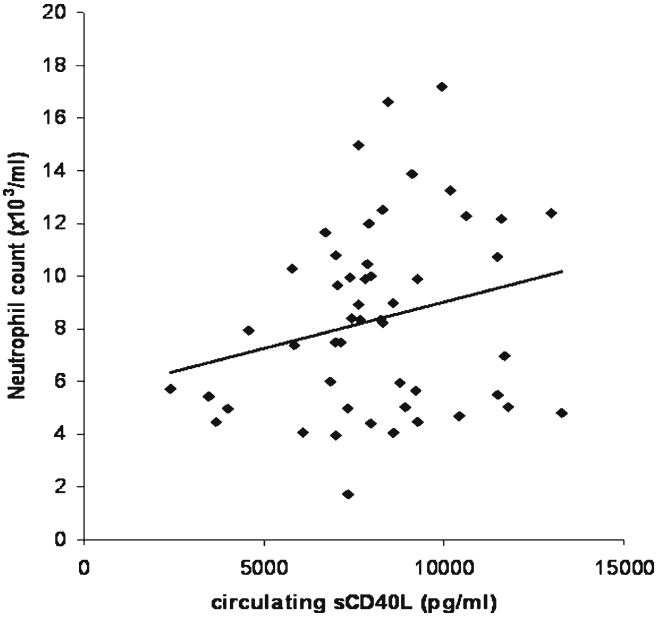

Fig. 2. No significant correlation between neutrophil count with circulating sCD40L in patients with AMI ($P = 0.110$)

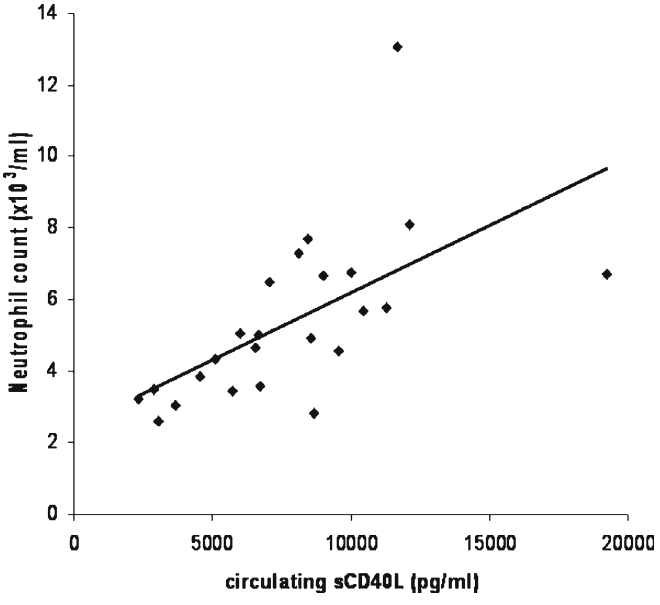

Fig. 4. Positive correlation between neutrophil count with circulating sCD40L in patients with unstable angina ($r = 0.607$, $P = 0.002$)

Discussion

Our study confirmed several previous studies that suggested an elevation of leukocyte and neutrophil counts in AMI. Marked elevation of leukocyte and neutrophil counts was found in patients with AMI compared with those with unstable angina. Myocardial necrosis induces an acute-phase inflammatory reaction,[18] and may account for the marked elevation of both leukocyte and neutrophil counts. After myocardial infarction, the release of chemoattractants stimulates the recruitment of neutrophils into the infarct zone for 6 h, and during the next 24 h they migrate into the myocardial tissue facilitated by cell-adhesion molecules.[19] Elevated neutrophil count is associated with peak creatine kinase concentration as a marker of myocardial damage in AMI patients, suggesting that the neutrophil count is increased in response to myocardial necrosis.[20] Plaque rupture itself might increase the peripheral neutrophil count.[21] The mechanism by which this process contributes to marked elevation in neutrophil count in AMI is still unelucidated.[20] This suggestion is in accordance with our study that elevated neutrophil count is markedly higher in AMI patients but not in those with unstable angina.

Although the basic mechanism of AMI and unstable angina is similar, i.e., a rupture of unstable coronary plaque followed by intracoronary thrombus formation, the subsequent event is different. In AMI, the distal myocardium becomes infarcted and releases inflammatory mediators that may enhance inflammation. Abbate et al.[22] showed that the inflammatory response in myocardial infarction occurred not only in coronary artery but was also widespread within the myocardium. In AMI, increased leukocyte count is a common finding that reflects the infiltration of leukocytes into the necrotic myocardial tissue, and neutrophil is the first leukocyte to be found in the damaged myocardial area and are removed from myocardial tissue after phagocytosing the debris.[23] Bai et al.[24] reported that in rat myocardium with AMI, the necrotic myofibers at the periphery of the infarct area were replaced by the inflammatory cells and macrophages. In addition, in myocardial infarction the delay of neutrophil apoptosis may contribute to elevated number of circulating neutrophils.[25] Here we propose that increasing neutrophil count in AMI is not only a consequence of atherosclerotic plaque rupture, but also myocardial necrosis and apoptotic delay. We did not find a significant correlation between leukocyte and neutrophil counts with troponin I level in our study. This observation did not exclude the existence of significant correlation in AMI, since the measurement of troponin I level was only conducted once at admission (≤24 h since pain onset).

In our study, no significant differences in circulating sCD40L level between both groups were observed. More than 95% of circulating sCD40L is released by activated platelet within a period of minutes to hours.[26] After coronary angioplasty and stent placement, circulating sCD40L is raised within a 10-min period.[27] Plaque disruption leads to platelet activation and subsequent release of circulating sCD40L.[3,11] Therefore, both AMI and unstable angina might have enhanced levels of circulating sCD40L.[12,13,28,29]

To further clarify the interaction between neutrophils and platelets, we analyzed the circulating sCD40L level in the AMI group, and we did not find a significant correlation between circulating sCD40L with the neutrophil count. In this group, we observed marked elevation of both leukocyte and neutrophil counts, exceeding the upper normal value, whereas the circulating sCD40L level was not elevated compared with that in unstable angina. Among subjects with unstable angina, we found a strong and positive significant correlation between both leukocyte and neutrophil counts with circulating sCD40L level. In this group, no marked elevation exceeding upper normal limit for both leukocyte and neutrophil counts was observed. Interaction between neutrophils and platelets after plaque rupture is mediated by ligand–receptor interaction, such as P-selectin with PSGL-1 and β-integrin with GPIIB/IIIA.[30] The role of sCD40L in neutrophil and platelet interaction is mediated in this mechanism, although recent study found the presence of a CD40–CD40L mechanism.[17] Interestingly, in our study the correlation between neutrophil count and circulating sCD40L found in unstable angina did not occur in AMI, suggesting that this might underlie the difference in the pathogenesis of unstable angina as compared to AMI.

Based on this observation, we proposed that an observed association between circulating sCD40L level with neutrophil count in unstable angina is evidence of two-way CD40–CD40L interaction, whereas the lack of association between sCD40L and neutrophil count in AMI reflected another mechanism that led to marked elevation of neutrophil count exceeding sCD40L level. As we mention above, the possible role of myocardial necrosis in triggering early inflammatory response is responsible for this increase in neutrophil count, independent from the CD40–CD40L mechanism.

Limitations

In this study we enrolled patients with an episode of acute coronary syndrome. Healthy individuals and patients with stable coronary artery disease were not observed during our study. Therefore we could not exclude the possibility that the observed interaction between neutrophils and platelets might also occur in these populations.

Conclusion

Circulating sCD40L was strongly associated with neutrophil count and might mediate the interaction between neutrophils and platelets in acute coronary syndrome, particularly in unstable angina. Furthermore, in acute myocardial infarction we found no significant association between circulating sCD40L and neutrophil count. Our study suggests that a more complex mechanism should be further elucidated, involving the CD40/CD40L-mediated neutrophil and platelet interaction in patients with AMI, in clinical study as well as using experimental methods.

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