Comparison of erythrocyte microparticle levels between acute coronary syndrome and stable angina pectoris patients

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Abstract: Objective: By analyzing the difference in the level of plasma erythrocyte microparticles (MPs) between patients with acute coronary syndrome (ACS) and patients with stable angina pectoris (SAP), we sought to evaluate an association to predict potential diagnostic value. Methods: A total of 127 patients with ACS and 21 patients with SAP were diagnosed through the analysis of a peripheral blood sample, and 45 patients with non-coronary artery disease (non-CAD) were enrolled as controls. After peripheral blood from the studied subjects was collected and centrifuged, the Ca2+ vector A23187 was added to obtain MPs. Erythrocyte MPs were labeled with an antibody specific to erythrocyte particles (glycophorin A CD235a) and then quantitatively analyzed by flow cytometry. Results: The levels of erythrocyte MPs in the peripheral blood of the ACS and SAP groups were 27.1±14.83% and 41.58±16.50%, respectively, and the level of erythrocyte MPs in the non-CAD group was 19.29±16.54%. The level of erythrocyte MPs in the ACS group was significantly higher than that in the non-CAD group (P<0.05). When ACS sub-groups were analyzed, the difference was not statistically significant (P>0.05). The level of erythrocyte MPs in the SAP group was higher than that in the non-CAD and ACS groups (P=0.011 and P=0.001, respectively) and was also higher than that in the sub-groups of ACS patients (P<0.001 and P=0.040, respectively). Conclusions: The level of erythrocyte MPs was higher in the ACS group than in healthy controls, suggesting that erythrocyte MPs may be involved in the development of ACS and may be associated with acute cardiovascular events in ACS.

Keywords: Erythrocyte microparticles, microparticles (MPs), acute coronary syndrome (ACS), pathophysiology

Introduction

Similar to the high mortality rate of ischemic heart disease, the incidence of acute coronary syndrome (ACS) has been rising over the last few years. ACS is an acute, severe, life-threatening ischemic event, and its pathophysiology is characterized by unstable atherosclerotic plaque in the coronary arteries and may include acute myocardial infarction (AMI) or unstable angina pectoris (UAP). ACS often leads to death due to acute myocardial ischemia, accounting for approximately half of the cases of cardiovascular death.

Growing evidence indicates that pro-coagulant microparticles (MPs) are locally present in atherosclerosis and are associated with plaque rupture in atherosclerotic coronary arteries [1, 2]. MPs are formed when phosphatidylserine from the inner layer of the cell membrane moves into the outer layer of the membrane, leading to budding of small bubble-like structures consisting of phospholipid vesicles with a diameter of 0.1-1 µm. The classification of MPs depends on their source cells, which can include erythrocytes, platelets, and endothelial cells [3], depending entirely on various stimuli, such as cell activation or apoptosis. In healthy people, MPs may be present at a physiological level, whereas in many diseases, such as hypertension, diabetes, and cancer, the level is pathologically increased [4]. Leroyer et al. extracted pellets from carotid plaques and plasma from patients undergoing carotid endarterectomy and found that MPs from erythrocytes in atherosclerotic plaques are associated with the rupture of small blood vessels within
the plaques [5]. We hypothesized that small vessels in localized plaque in the coronary arteries produce MPs derived from erythrocytes and are involved in the development of ACS. The aim of this study was thus to analyze differences in the level of erythrocyte MPs between an ACS group and a stable angina pectoris (SAP) group. The aims of this study were thus to analyze differences in the level of erythrocyte MPs between an ACS group and a stable angina pectoris (SAP) group. Differences between ACS sub-groups were also analyzed, and the correlation between erythrocyte MPs and ACS was further investigated.

Methods

Study population

To avoid coronary angiography and its associated interventional effects on peripheral blood erythrocyte MP levels, 2 mL peripheral blood was collected from all patients prior to coronary angiography and MP levels were subsequently detected. During the sampling process, the following preventive measures were taken: (1) the tourniquet was not maintained for a long time to avoid induction of cell activation or apoptosis, and (2) the anticoagulant and blood was in full contact and immediately centrifuged.

The study population in the present analysis was selected from the inpatient Department of Cardiology at the People’s Hospital of Xinjiang Uygur Autonomous Region from September 2014 to September 2016. A total of 1215 patients whose clinical data were collected for the first time were selected according to the diagnostic inclusion and exclusion criteria. The study is a three-group cross-sectional observation study encompassing 68 AMI patients and 37 UAP patients compared to subjects with SAP (n=21) or non-coronary artery disease (non-CAD) (n=45) patients. Only non-CAD patients whose status was confirmed by coronary angiography were analyzed.

Biochemical parameters included total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), lipoprotein A (LPa), glycosylated hemoglobin (HbA1c), and red blood cell count.

Blood samples were collected in sodium citrate anticoagulant tubes (BD) within 24 hours of hospitalization. Additionally, clinical data were collected from clinical charts, including age, sex, smoking status, diabetes status, and hypertension status. The clinical baseline characteristics of the ACS, SAP, and non-CAD groups were similar (Tables 1 and 2).

The diagnostic criteria were based on previous studies, as follows: 1. UAP was newly occurring angina pectoris that had emerged within the past month, angina pectoris that had worsened within the past month (with grading of the angina pectoris increasing by at least one grade or reaching Grade III according to CCS grading), or angina pectoris that appeared when at rest. ECG showed new or dynamic changes in ST segments or T waves via at least two adjacent leads, and cardiac troponin T (cTnT) results were normal. 2. Patients with AMI experienced chest pain for over 20 minutes, and ECG of AMI patients showed elevation or depression of ST segments via at least two adjacent leads within 24 hours of onset. In addition, cTnT >0.1 µg/L (normal value <0.05 µg/L).

The exclusion criteria were as follows: 1. serious liver or kidney dysfunction; 2. cancer or other debilitating disease; 3. disease occurring in the hematopoietic system; 4. uncontrolled infection; and 5. infarction in another location in the body, such as cerebral infarction or pulmonary embolism.

Lesions in the coronary arteries of the enrolled patients were confirmed by coronary angiography, and patients shown to have angiographically normal coronary arteries were considered as non-CAD subjects.

Laboratory testing

Isolation of erythrocyte MPs: The blood was centrifuged at 2,000 g at room temperature for 10 minutes, followed by three centrifugations at 23,000 g at 4°C for 45 minutes. Afterwards, 200 µl phosphate-buffered saline (PBS) was added, and the samples were mixed for 5 minutes. Each sample was labeled with the date of collection and the patient admission number.
### Table 2. Clinical characteristics of the non-CAD, ACS, and SAP groups

<table>
<thead>
<tr>
<th>Baseline clinical characteristics</th>
<th>Non-CAD ①</th>
<th>AMI ②</th>
<th>UAP ③</th>
<th>ACS</th>
<th>SAP</th>
<th>SAP vs ACS</th>
<th>① vs SAP</th>
<th>① vs ACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55±10</td>
<td>59±12</td>
<td>59±9</td>
<td>58±12</td>
<td>62±13</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Male (n)</td>
<td>26 (45)</td>
<td>58 (68)</td>
<td>29 (37)</td>
<td>87 (105)</td>
<td>17 (21)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>29 (45)</td>
<td>30 (68)</td>
<td>15 (37)</td>
<td>45 (105)</td>
<td>21 (21)</td>
<td>0.008</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>7 (45)</td>
<td>14 (68)</td>
<td>10 (37)</td>
<td>24 (105)</td>
<td>2 (21)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>11 (45)</td>
<td>26 (68)</td>
<td>15 (37)</td>
<td>41 (105)</td>
<td>21 (21)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.27±1.30</td>
<td>4.90±4.71</td>
<td>4.04±1.05</td>
<td>4.57±3.77</td>
<td>4.22±0.69</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>0.92±0.21</td>
<td>0.84±0.21</td>
<td>0.88±0.17</td>
<td>0.86±0.20</td>
<td>1.03±0.32</td>
<td>0.011</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.68±2.02</td>
<td>2.69±0.95</td>
<td>2.42±0.98</td>
<td>2.58±0.97</td>
<td>2.43±0.61</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.69±0.88</td>
<td>1.79±1.22</td>
<td>1.87±1.39</td>
<td>1.82±1.28</td>
<td>1.60±0.78</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>LPA (mg/L)</td>
<td>134.97 [75.85, 263.34]</td>
<td>133.27 [76.08, 234.39]</td>
<td>146.81 [88.44, 313.66]</td>
<td>122.54 [75.25, 177.77]</td>
<td>110.81 [42.90, 410.38]</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.58±1.49</td>
<td>6.76±1.70</td>
<td>6.97±1.58</td>
<td>6.83±1.65</td>
<td>6.67±1.56</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>RBC (10^9/L)</td>
<td>4.71±0.50</td>
<td>4.66±0.50</td>
<td>4.65±0.38</td>
<td>4.65±0.46</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>
and then immediately stored at -80°C for subsequent analysis of erythrocyte MPs.

Quantitation of erythrocyte MPs by flow cytometry: The extracted platelet-free plasma (PFP) was rapidly dissolved at room temperature. Next, 2.5 μL of sample, 100 μL of the Ca²⁺ vector A23187, 100 μL binding buffer, and 5 μL annexin V were mixed together well at room temperature for 20 minutes. Subsequently, 2.5 μL of an erythrocyte-specific monoclonal antibody (glycophorin A CD235a) was added, followed by thorough mixing at room temperature for 20 minutes. Finally, 175 μL PBS was added. FACSAria™ analysis was performed, and a total of 10,000 microspheres were collected. The final peripheral blood erythrocyte-derived particle size was expressed as a percentage (Figure 1).

Statistical analysis

Categorical variables are expressed as absolute counts and percentages. The data were processed with the SPSS 21.0 software package, and continuous variables are summarized as the mean ± standard deviation for normally distributed data. Cross-sectional data were analyzed by one-way analysis of variance. Unpaired two-tailed Student’s t-tests were used if data fulfilled the criteria of normal distribution and equal variance. The correlation of erythrocyte MPs with risk factors of cardiovascular disease was tested by multiple linear regression analysis. Fisher’s exact test was used for categorical data.

Results

The quantitative data of low-density LPa in the three groups did not meet the normality distribution, so the rank-sum test was used to yield medians and quartiles. The remaining data from the three groups were normally distributed and were thus expressed as the mean ± standard deviation, with a t test used to compare the three groups. There was no significant difference in age, sex, diabetes mellitus, the presence of hypertension, or smoking status (P>0.05), indicating that the clinical information was comparable among the three groups. There was also no significant difference in TC, TG, LDL-cholesterol, HDL-cholesterol, LPa, HbA1c, or erythrocyte count among the three groups (P>0.05), indicating that the clinical biochemical indicators were comparable among the three groups (Tables 1 and 2).

The level of erythrocyte MPs was recorded for the ACS, SAP, and non-CAD groups, with the ACS patients including AMI and UAP patients (Figures 2 and 3). The level of erythrocyte MPs was significantly higher in the ACS group than in the non-CAD group (P=0.005). The difference in the analysis of the sub-groups of ACS was not statistically significant (P>0.05). The level of erythrocyte MPs was higher in the SAP group than in the non-CAD and ACS groups (P<0.001 and P=0.001, respectively) or the sub-groups of ACS patients (P=0.011 and P=0.04, respectively) (Table 3).

All subjects were grouped according to the presence or absence of diabetes, hypertension, and a smoking history (P>0.05). Compared with non-hypertensive, non-smoking, and non-diabetic patients, there was no difference in the level of erythrocyte MPs in the peripheral blood of patients with hypertension, diabetes or a smoking history among the three groups (P>0.05). The levels of serum TC, TG, LDL-cholesterol, HDL-cholesterol, LPa, HbA1c, erythrocyte counts and erythrocyte levels were measured in all subjects, and there was a nega-
Erythrocyte microparticles between ACS and SAP

Figure 2. Levels of erythrocyte MPs in the ACS and non-CAD groups.

Figure 3. Levels of erythrocyte MPs in the ACS, SAP, and non-CAD groups.

Discussion

CAD is related to the pathological process of the artery. A large number of previous studies confirmed that many components of the blood, such as anticoagulant substances, fibrous factors, and inflammatory cells, play very important roles in the occurrence and development of CAD [6, 7]. In recent years, different cell-derived MPs have received much attention as biomarkers for assessing cardiovascular disease.

MPs result from the process of phosphatidylserine moving from the inner layer of the cell membrane to the outer layer. Loss of phospholipids causes the originally neutral cell membrane to become negative, and phospholipid release depends on apoptosis and activation of the cell. MPs carry a variety of biologically active molecules from the source cell, such as cytokines, RNA and DNA, and regulate a series of biological effects by transferring these biologically active molecules to target cells [8-10].

Medical studies have shown that MPs are associated with a variety of biological processes, such as thrombosis and hemostasis, inflammatory responses, vascular and immune function, and apoptosis. Through the transmission of particulate surface proteins between cells, MPs can even promote an inflammatory response and activation of the coagulation system, leading to atherosclerosis, which is one of the important reasons for ischemia [11].

Erythrocytes are subjected to deleterious stimuli such as cell membrane oxidation, and damage forms MPs via exocytosis. The present study found that the level of erythrocyte MPs significantly increased during the course of cardiovascular disease. The level may indirectly reflect the state of apoptosis or activation, which in turn may reflect vascular injury, explaining the role of MPs in acute cardiovascular events from the perspective of pathophysiology [12, 13]. Giannopoulos G et al. performed a study comparing the numbers of erythrocyte and platelet MPs between 51 patients with acute ST-segment elevation myocardial infarction (STEMI) after percutaneous coronary intervention and 50 healthy subjects. The results suggested that after patients with AMI underwent initial angioplasty, the number of erythrocyte MPs increased significantly compared with the number in the healthy population and was closely related to the clinical events after myocardial infarction. In contrast, the levels of platelet MPs were not significantly different between the two groups and were not associated with clinical end points [12]. The level of erythrocyte-derived granulocytes in 60 patients with STEMI after coronary angioplasty was also analyzed. The level of erythrocyte-derived MPs was associated with myocardial injury, and erythrocyte-derived particles played an important role in the pathogenesis of ischemic injury [14]. Accumulating medical evidence shows that MPs can be used as biomarkers for sys-
Erythrocyte microparticles between ACS and SAP

In the current study, we examined the level of erythrocyte MPs in the peripheral blood of patients with ACS in Xinjiang Uygur Autonomous Region, with the goal of investigating whether the level of erythrocyte MPs in the peripheral blood was higher in patients with ACS than in the non-CAD group in order to reveal any correlation between erythrocyte MPs and acute cardiovascular events in coronary heart disease. The Xinjiang Uygur Autonomous Region is a multi-ethnic area where Uighurs was the first ethnic minority. The present study also analyzed ACS patients in the Han and Uygur groups ACS patients, and the results suggests that there were no obvious differences in erythrocyte MP levels, which may be related to the assimilation of living habits among different ethnic groups in the same region. This finding might also be related to the analysis of relatively small samples from each ethnic group, so there is a need for large-scale multi-ethnic clinical research studies to further characterize any differences.

In this study, multiple linear regression analysis of peripheral blood erythrocyte MP levels showed a negative correlation between serum LDL and the erythrocyte MP level. TG and cholesterol levels were significantly affected by recent diet in the study. LDL was significantly affected by drugs, whereas the acute phase of the disease had little effect on LDL. In this study, the clinical data collection process did not identify the timing and type of oral lipid-lowering drugs and did not determine the effects of any lipid-lowering drugs on lipid levels. Long-term oral lipid-lowering drugs ingested before admission may have resulted in low LDL-cholesterol levels. The level of erythrocyte-derived MPs began to rise during the pathogenesis of ACS, and the peak time was followed by further studies via blood sampling at different stages. The sample size of this study was relatively small, and the impact of drugs cannot be judged at present, so there is a need to further improve the quality of the sample data and to expand the sample size.

The level of erythrocyte MPs was significantly higher in the ACS group than in the non-CAD group. The difference in the analysis of the subgroups of ACS was not statistically significant. The main reason was the differences in the pathogenesis of the two subgroups: (1) AMI included STEMI and non-STEMI, with STEMI occurring due to thrombosis, which blocked the blood vessels via coronary artery lesions, resulting in severe and persistent corresponding myocardial ischemia and finally causing AMI. The occurrence of UAP was associated with instability of atheromatous plaque as part of secondary pathological changes, such as platelet internal bleeding, breakage of the fibrous surface cap of the plaque and platelet aggregation, which stimulated coronary artery spasm and caused severe myocardial ischemia. (2) Significant differences in erythrocyte MP levels may not have been demonstrated due to the small subgroup sizes for every subgroup. Therefore, the study needs to be expanded to more subjects.

The results of this study suggest that in terms of the levels of erythrocyte MPs in the peripheral blood in the non-CAD, ACS and SAP groups, the ACS group has a higher level than the non-CAD group. Additionally, differences between the two sub-groups were not statistically significant for the following reasons: (1) Different sub-types of pathogeneses of AMI were based on

### Table 3. Levels of erythrocyte MPs in the ACS, SAP, and non-CAD groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Erythrocyte MPs level</th>
<th>T value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-CAD</td>
<td>19.29±16.54</td>
<td>5.102*</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>SAP</td>
<td>41.58±16.50</td>
<td>-2.855**</td>
<td>0.005**</td>
</tr>
<tr>
<td>ACS</td>
<td>27.1±14.83</td>
<td>3.73***</td>
<td>0.001***</td>
</tr>
<tr>
<td>AMI</td>
<td>26.53±15.68</td>
<td>-0.533</td>
<td>0.595</td>
</tr>
<tr>
<td>UAP</td>
<td>28.15±13.27</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Non-CAD vs SAP, **Non-CAD vs ACS, ***SAP vs ACS.

### Table 4. Multiple linear indexes related to erythrocyte MPs and LDL in a regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>R</th>
<th>Standard error</th>
<th>Standardized R</th>
<th>T value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>34.34</td>
<td>4.83</td>
<td>-</td>
<td>7.12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL (X)</td>
<td>-3.85</td>
<td>1.78</td>
<td>-0.26</td>
<td>-2.16</td>
<td>0.035</td>
</tr>
</tbody>
</table>

In this study, the clinical data collection process did not identify the timing and type of oral lipid-lowering drugs and did not determine the effects of any lipid-lowering drugs on lipid levels. Long-term oral lipid-lowering drugs ingested before admission may have resulted in low LDL-cholesterol levels. The level of erythrocyte-derived MPs began to rise during the pathogenesis of ACS, and the peak time was followed by further studies via blood sampling at different stages. The sample size of this study was relatively small, and the impact of drugs cannot be judged at present, so there is a need to further improve the quality of the sample data and to expand the sample size.

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Erythrocyte microparticles between ACS and SAP

coronary artery disease, thrombosis leading to lumen occlusion, serious and persistent corresponding myocardial acute ischemia leading to myocardial necrosis. UAP was associated with unstable coronary atherosclerotic plaque-related secondary pathological changes, such as plaque bleeding, plaque fibrous cap cracks, and surface platelet aggregation, which stimulated coronary artery spasm, resulting in an increased risk of ischemia. (2) The study of the sub-types had small sample sizes, so subtype differences need to be further examined with larger sample sizes.

This study suggests that the level of erythrocyte MPs is higher in the SAP group than in the non-CAD group or the ACS group. Patients with SAP with mild clinical symptoms, without treatment or with irregular oral single or double antiplatelet drugs, may explain the presence of patients with SAP without coronary heart disease secondary to preventative drug use. In our study, the clinical case data collection process did not clarify the oral antiplatelet drug time or type, so there is a need to further improve the quality of the sample data.

In the healthy population, we could also detect MPs, which are common in the peripheral blood of the population. Therefore, if MP formation is due to cell apoptosis or activation, after the occurrence of a disease state, the level of the particles would increase dramatically. Our results suggest that the number of MPs is higher in patients with ACS than in the healthy population, consistent with our previous findings [18-20]. In the past, we have investigated the levels of erythrocyte granulocytes in the peripheral blood of patients with cardiovascular disease and found that the level of erythrocyte MPs was higher in patients with ACS than in healthy individuals. MPs may thus be involved in the pathogenesis of ACS. The specific mechanism by which erythrocyte MPs participate in acute thrombotic events in ACS is unclear. The specific mechanism of peripheral blood erythrocyte-derived particle involvement in ACS-related acute thrombosis is also unclear.

Our findings suggest that erythrocyte MPs are involved in the pathogenesis of ACS, but more randomized controlled trials, molecular biology studies and animal models are needed to determine the specific mechanism as well as the mechanism of acute hemangioma events in acute coronary syndromes.

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Disclosure of conflict of interest

None.

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