Int J Clin Exp Med 2015;8(9):16704-16708
www.ijcem.com /ISSN:1940-5901/IJCEM0011120

Original Article

Alteration of circulatory platelet microparticles and endothelial microparticles in patients with chronic kidney disease

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Received June 6, 2015; Accepted July 28, 2015; Epub September 15, 2015; Published September 30, 2015

Abstract: Objective: To compare plasma platelet microparticles (PMPs), P-selectin, endothelial microparticles (EMPs), and von Willebrand factor (vWF) between a normal control group and patients with chronic kidney disease (CKD) and to explore the significance of PMPs and EMPs in CKD. Methods: Levels of plasma PMPs, P-selectin, EMPs and vWF in 122 CKD patients and 20 normal controls were detected by flow cytometry and enzyme-linked immunosorbent assay (ELISA). Relationships between PMPs, EMPs and blood pressure, creatinine clearance rate, 24-hour urine protein, hemoglobin, and cholesterol were analyzed. Results: (1) Plasma PMPs, P-selectin, EMPs and vWF levels in CKD patients were significantly higher than those of the control group. Plasma PMPs and P-selectin levels for nephrotic syndrome (NS) were significantly higher than for other CKD groups. No significant difference was found between other CKD groups. Plasma EMPs and vWF in NS, lupus nephritis (LN) and hypertensive nephropathy groups were significantly higher than that of diabetic nephropathy (DN) and chronic glomerulonephritis (CGN) groups. (2) Plasma PMPs, P-selectin, EMPs and vWF in stage I-II CKD patients were significantly higher than those of stage III-V CKD patients, no significant difference was found within stage I-II CKD patients or stage III-V CKD patients. (3) PMPs and EMPs were positively correlated with blood pressure and 24-hour urinary protein, but no significant correlation was found with the creatinine clearance rate, hemoglobin or cholesterol. P-selectin and vWF were positively correlated with PMPs and EMPs respectively. Conclusion: CKD patients have significant platelet activation and endothelial dysfunction, which was involved in CKD's occurrence and development; high blood pressure and proteinuria are important reasons for platelet activation and endothelial dysfunction in patients with CKD; PMPs and EMPs can be used as new markers for dysfunctional platelet activation and endothelium.

Keywords: Platelet microparticles, endothelial microparticles, microparticles, chronic kidney disease

Introduction

Microparticles refer to the different sizes (diameter: 0.1-1 μm) of particles that are shed from the cytoplasmic membrane after the cells are stimulated. The particles contain parental cell antigen, which mirrors the characteristics and status of the parental cells. Over the past decade, many scholars have found that plasma platelet microparticles (PMPs) or endothelial microparticles (EMPs) were significantly increased in patients with cardiovascular disease (such as acute coronary syndrome, acute myocardial infarction), inflammatory diseases (such as ulcerative colitis) or blood disorders (such as heparin-induced thrombocytopenia) [1-3]. Microparticles derived from various cells, especially PMPs and EMPs, had become indicators of diagnosis, efficacy evaluation and prognosis for some diseases. Specific cell microparticles analysis became a method and approach for exploring the pathological mechanism of various diseases. Cardiovascular disease is the main cause of death in chronic kidney disease (CKD) patients. Dysfunctional platelet activation and endothelium are closely related to the occurrence and development of cardiovascular disease [4, 5]. We examined plasma PMPs and

EMPs in CKD patients and analyzed their relationship to clinical indicators to explore the significance of PMPs and EMPs in CKD.

Data and methods

Study population

During the period February 2007 to April 2009, 122 hospitalized CKD patients in the nephrology department of The First Affiliated Hospital of Soochow University were included, with an average age of 47.5 ± 13.6 years. The staging of CKD was in accordance with K/DOQI guidelines. Twenty-five patients were chronic glomerulonephritis (CGN), 23 were diabetic nephropathy (DN), 25 were nephrotic syndrome (NS), 29 were hypertensive nephropathy, and 20 were lupus nephritis (LN). Twenty patients were in the healthy control group, with an average age of 43.4 ± 11.7 years. Hypertension, hyperlipidemia, diabetes, cardiovascular and cerebrovascular disease, kidney disease and a variety of acute infectious diseases had been ruled out.

Sample collection

Fasting cubital venous blood was collected in the morning. 2 ml of blood was put into an EDTA tube for anticoagulation and centrifuged for 15 min at 3000 r/min. Platelet poor plasma (PPP) was collected and frozen at -80°C for P-selectin content detection. 2 ml of blood was put into another EDTA tube for anticoagulation and 11 μl of prostaglandin E1 (PGE1, platelet activation inhibitor) and 5 μl Apyrase were immediately added, then centrifuged for 15 min at 1000 r/min at low temperature to obtain platelet-rich plasma (PRP). Then PPP was obtained after centrifuging for another 15 min at 3000 r/min, the upper plasma layer was collected and frozen at -80°C for plasma PMPs and EMPs detection. 2 ml blood was put into an anticoagulant tube with 3.8% sodium citrates, after centrifugation for 15 min at 3000 r/min, the plasma was collected and frozen at -80°C for vWF detection.

Reagents and detection methods

Determination of plasma PMPs and EMPs: PE-labeled anti-CD41 (platelet glycoprotein IIb) antibody was purchased from Genetics, PE-labeled anti-CD62E (E-selectin) antibody and 0.3 μm and 0.8 μm standard microspheres were purchased from Becton Dickinson, Annexin V was purchased from Biouniquer Technology, and Flow-Count™ fluorescent microspheres (966/μl) were purchased from Beckman Coulter. Frozen PPP was thawed in ice water, 40 μl PPP was taken from each tube and mixed fully with 10 μl PE-labeled anti-CD62E antibody and PE-labeled anti-CD41 antibodies respectively. After 20 minutes of dark incubation, 420 μl Bindbuffer and 5 μl Anti-Annexin V were added. After another 20 minutes, 5 μl Flow-Count™ fluorescent microspheres were added and PMPs and EMPs were detected by flow cytometry after full mixing. 5 μl mouse anti-human IgG-PE was used in the control tube, 462 μl Bindbuffer, 5 μl Annexin V, 1. 5 μl 0.3 μm and 1.5 μl 0.8 μm standard microspheres were added, then 5 μl Flow-Count™ fluorescent microspheres were added, detection by flow cytometry was performed after full mixing. Parameter data is displayed as a two-dimensional dot matrix graphic. The abscissa refers to fluorescein isothiocyanate (FITC), the ordinate refers to Phycoerythrin (PE). With the appropriate cross gate set, L1, L2, L3, L4 of the cross gate stand for PE single-positive cells, PE and FITC double-positive cells, negative cells, and FITC single-positive cells respectively. Determination of PMPs and EMPs: the diameter between 0.3 μm and 0.8 μm, the surface markers CD41 and Annexin V were double positive or CD62E and Annexin V were double positive. 10,000 microspheres were read and PMPs and EMPs concentrations were calculated in accordance with numbers of standard fluorescent microspheres.

Determination of GMP140 and vWF antigen content: Double-antibody sandwich ELISA (DAS-ELISA) was used and the specific procedure was carried out according to kit instructions (provided by the Thrombosis and Hemostasis Research Center of Soochow University).

Statistical analysis

SPSS 10.0 was used for statistical analysis. The data are measured as $\bar{x} \pm s$. Analysis of variance was used for comparison among groups, and t test was used for comparison between two groups. Correlation analysis was used for variables with a correlative tendency, $P < 0.05$ meaning significant difference.

Table 1. Plasma PMPs, P-selectin, EMPs and vWF in chronic kidney disease group and healthy control group ($\bar{x} \pm s$)

Group	Cases	PMPs (num/μl)	P-selectin (ng/ml)	EMPs (num/μl)	vWF (%)
Control	20	38.6 ± 28.5	13.5 ± 5.3	63.8 ± 40.8	72.5 ± 50.2
CKD	122	68.4 ± 24.6▲	38.4 ± 10.2▲	151.5 ± 66.7▲▲	124.5 ± 59.8▲▲

Note: Compared with control group, ▲P < 0.05, ▲▲P < 0.01.

Table 2. Plasma PMPs, P-selectin, EMPs and vWF between different types of CKD patients ($\bar{x} \pm s$)

Group	Case	PMPs (num/μl)	P-selectin (ng/ml)	EMPs (num/μl)	vWF (%)
Chronic nephritis	25	59.7 ± 23.8★,▲	31.7 ± 8.2★★,▲	119.4 ± 85.6★★	90.4 ± 21.8★
Nephritic syndrome	25	73.8 ± 24.8★★	38.4 ± 6.8★★	183.8 ± 90.5★★,#	142.4 ± 34.6★★,#
Lupus nephropathy	20	62.3 ± 24.7★,▲	33.4 ± 10.2★★,▲	160.9 ± 61.3★★,#	130.8 ± 32.3★★,#
Diabetic nephropathy	23	61.7 ± 23.4★,▲	26.6 ± 6.3★★,▲	132.4 ± 70.9★	117.9 ± 34.6★★
Hypertensive nephropathy	29	53.3 ± 21.6★,▲	27.8 ± 5.8★★,▲	140.7 ± 42.7★,#	133.4 ± 31.4★,#
Control	20	38.6 ± 28.5	13.6 ± 5.4	63.8 ± 40.8	72.5 ± 50.2

Note: Compared with the control group, ★P <0.05, ★★P <0.01; Compared with the nephrotic syndrome group, ▲P <0.05, Compared with the chronic nephritis group and diabetic nephropathy group, #P <0.05.

Table 3. Comparison of plasma PMPs, P-selectin, EMPs and vWF in CKD patients of different stages ($\bar{x} \pm s$)

CKD stage	Cases	PMP (num/μl)	P-selectin (ug/L)	EMPs (num/μl)	vWF (%)
Stage I	29	88.4 ± 27.7★	45.7 ± 9.4★	177.6 ± 85.1★	133.9 ± 43.4★
Stage II	33	93.6 ± 24.8★	51.5 ± 8.1★	169.7 ± 75.3★	152.4 ± 51.6★
Stage III	28	56.8 ± 24.6	32.4 ± 8.7	128.1 ± 74.6	112.4 ± 43.2
Stage IV	17	54.7 ± 28.6	28.6 ± 8.7	113.6 ± 37.7	110.5 ± 34.4
Stage V	15	57.1 ± 25.3	25.2 ± 9.8	125.5 ± 71.5	116.3 ± 40.7

Note: compared with stage III-V, ★P <0.05.

Results

*Comparison of plasma PMPs, P-selectin, EMPs and vWF in chronic kidney disease group and healthy control group (**Table 1**)*

Compared with the control group, CKD patients had significantly higher levels of plasma PMPs, P-selectin, EMPs and vWF (P < 0.05).

*Comparison of plasma PMPs, P-selectin, EMPs and vWF in CKD patients of different types (**Table 2**)*

Plasma PMPs, P-selectin, EMPs and vWF levels in CKD patients were significantly higher than those of the control group, (P < 0.05, P < 0.01), plasma PMPs and P-selectin levels in nephrotic syndrome (NS) were significantly higher than those of other CKD groups (P < 0.05), No significant difference was found among other CKD groups (P > 0.05); Plasma EMPs and vWF in the NS, lupus nephritis (LN) and hypertensive nephropathy groups were significantly higher than those of the diabetic nephropathy (DN) and chronic glomerulonephritis (CGN) groups (P < 0.05).

*Comparison of plasma PMPs, P-selectin, EMPs and vWF in CKD patients of different stages (**Table 3**)*

Plasma PMPs, P-selectin, EMPs and vWF in stage I-II CKD patients were significantly higher than those of stage III-V CKD patients. No significant difference was found within stage I-II CKD patients and stage III-V CKD patients (P > 0.05).

Correlation analysis

The correlation analysis showed plasma PMPs of CKD patients were positively correlated with blood pressure (r = 0.38, P < 0.05) and 24-hour

urinary protein ($r = 0.31$, $P < 0.05$); No significant correlation was found between plasma PMPs and creatinine clearance rate ($r = -0.14$, $P > 0.05$), hemoglobin ($r = -0.11$, $P > 0.05$), or serum cholesterol ($r = 0.12$, $P > 0.05$). GMP140 and PMPs were positively correlated ($r = 0.36$, $P < 0.05$). Plasma EMPs of CKD patients were positively correlated with blood pressure ($r = 0.42$, $P < 0.01$) and 24-hour urinary protein ($r = 0.33$, $P < 0.05$); No significant correlation was found between plasma EMPs and creatinine clearance rate ($r =- 0.11$, $P > 0.05$), hemoglobin ($r =- 0.14$, $P > 0.05$), or serum cholesterol ($r = 0.07$, $P > 0.05$). vWF and EMPs were positively correlated ($r = 0.45$, $P < 0.05$).

Discussion

P-selectin is a member of the selectin family belonging to cell adhesion molecules and is mainly found in platelet α-granule membrane and lysosome. It is one of the specific molecular markers for platelet activation and is involved in thrombosis [6]. vWF is a glycoprotein mainly synthesized and secreted by endothelial cells and stored in the Weibel-Palade body of endothelial cells. vWF is released into the blood when endothelial cells are injured, vWF may reflect the function and degree of injury of endothelial cells. vWF mainly mediates adhesion of platelet and subendothelial collagen and participates in the process of thrombosis and hemostasis. This study showed that P-selectin and vWF levels were positively correlated with PMPs and EMPs respectively. PMPs and EMPs can be used as new markers for dysfunctional platelet activation and endothelium.

The surface of the microparticle membrane contains a high level of phosphatidylserine, which carries the surface marker of the parental cell and mirrors partial parental cell function. In our experiment, PMPs and EMPs were marked by Annexin V and the specific antibody CD41, CD62E respectively. PPP was collected after high-speed centrifuging to eliminate the effects of residual cells, and the platelet activation inhibitor PGE1 and Apyrase were added to inhibit platelet activation in vitro when the specimens were collected, so the level of circulatory microparticles could be accurately determined. Experimental results showed that PMPs, P-selectin, EMPs and vWF in the CKD group were significantly higher than those of the normal control group, suggesting dysfunctional platelet activation and endothelium in CKD patients.

The main function of PMPs is the coagulant activity. PMPs are rich in membrane receptors of coagulation factor Va and can provide a catalytic surface for prothrombinase reaction, thus accelerating thrombin generation [6], which may be related to the susceptibility of CKD patients to cardiovascular and cerebrovascular diseases. PMPs can also promote mitosis of smooth muscle cells in coronary arteries and activate platelets and endothelial cells through arachidonic acid dependent transcellular activation [7]. Endothelial dysfunction in patients with CKD may be related to advanced glycation end products, (AGEs)- mediated inhibition of endothelial nitric oxide (NO) synthase, decreased fetuin-A level and elevated circulating Lignans [8, 9]. Recent studies have shown that [10] lipopolysaccharide (LPS), AGEs, proinflammatory cytokines (TNF-α, IL-1) or oxidized low-density lipoprotein in the plasma of patients with CKD and high blood pressure can cause increased EMPs release. Faure et al. [11] proved that uremic toxins such as p-cresol, indole-phenol sulfate can increase the release of EMPs by inhibiting endothelial cell proliferation and migration in cultured human umbilical vein endothelial cells in vitro [12]. Further analysis showed that in different clinical groups, plasma PMPs in the NS group were significantly higher those of other groups, indicating more pronounced platelet activation in patients with NS, which may be one of the mechanisms of thrombosis in the NS group. Plasma EMPs in the NS, LN and hypertensive nephropathy patients were very significantly higher, suggesting more pronounced endothelial injury in these patients. At different stages of CKD, EMPs and PMPs in stage I-II CKD patients were significantly higher than those of stage III-V CKD patients. No significant difference was found within stage I-II CKD patients and stage III-V CKD patients, suggesting that PMPs and EMPs may be related to primary disease rather than renal function. The correlation analysis found that the level of PMPs and EMPs was positively correlated with blood pressure and 24-hour urinary protein, indicating that high blood pressure and proteinuria play an important role in the development of NS. Actively controlling blood pressure has important significance in

the inhibition of abnormal platelet activation and the protection of endothelial function as well as in preventing CKD development and its complications.

NS patients have varying degrees of renal dysfunction and the mechanism and pathophysiological process are complex, but abnormal platelet activation and endothelial dysfunction is an important mechanism in the occurrence and development of CKD. PMPs and EMPs can be used as new markers for dysfunctional platelet activation and endothelium and a new indicator for an assessment of platelet and endothelial cell function, coagulation function and drug efficacy.

Disclosure of conflict of interest

None.

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