Thrombosis Research 135 (2015) 375–381

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Elevated Circulating VE-Cadherin + CD144 + Endothelial Microparticles in Ischemic Cerebrovascular Disease

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ARTICLE INFO

Article history:
Received 7 July 2014
Received in revised form 1 December 2014
Accepted 2 December 2014
Available online 5 December 2014

Keywords:
Ischemic Cerebrovascular Diseases
Stoke
Endothelial Microparticles

ABSTRACT

Objectives: Circulating endothelial microparticles act as biological markers of endothelial function that reflect vascular injury. Here, we examined the hypothesis that the quantity of endothelial microparticles in the circulation is increased in patients with ischemic cerebrovascular diseases, and investigated the potential utility of various phenotypes of endothelial microparticles as specific biomarkers of endothelial cell dysfunction. We additionally focused on identifying endothelial microparticles that may be effectively utilized as biomarkers of stroke severity in acute ischemic stroke patients.

Methods: In total, 129 subjects, including 68 consecutive patients with acute ischemic stroke and 61 age- and sex-matched healthy controls, were included in the study. Levels of circulating endothelial microparticles (CD144 +/CD41a-, CD31 + CD41a-, CD62E +, Annexin V + CD62E +) and platelet-derived microparticles (CD41a +/CD144-) in platelet-free plasma of patients and controls were measured using flow cytometry.

Results: Levels of circulating endothelial CD144 +/CD41a-, CD31 + CD41a-, CD62E +, and Annexin V + CD62E + microparticles, but not platelet microparticles, were significantly increased in acute ischemic stroke patients, compared with control subjects ($p < 0.05$). Notably, levels of CD144 +/CD41a- microparticles were significantly correlated with stroke severity. A mild degree of correlation was evident between Annexin V + CD62E + microparticles and stroke subtype. No association with stroke was observed for other microparticle phenotypes.

Conclusions: Circulating endothelial microparticle amounts are increased in acute ischemic stroke patients, compared with healthy subjects. Levels of CD144 +/CD41a- microparticle, but not the other phenotypes examined, may be effectively utilized as a biomarker of ischemic severity in the clinic.

Introduction

Cerebrovascular disease is the second leading cause of death worldwide and the primary cause of disability. According to World Health Organization (WHO) data, the global incidence of stroke varies widely [1,2]. A variety of etiologies may be responsible for the occurrence of cerebrovascular disease. For instance, atherothrombosis, inflammation, endothelial cell dysfunction and hereditary factors may all contribute to the process of ischemic stroke [3]. Soler et al. [4] proposed that stroke is not a simple disease, but a manifestation of pathophysiological mechanisms of several diseases. We hypothesized that endothelial cell dysfunction causing vascular permeability changes and accumulation of harmful metabolites plays an important role in the process of ischemic stroke. Processes, such as inflammation and damage, aggravate imbalance of endothelial function, eventually leading to vascular disease.

Circulating microparticles (MPs) are small membranous fragments originating from cells in the circulation (such as erythrocytes, leucocytes and platelets), which range from 0.1 to 1 μm in size [5–9]. Endothelial microparticles (EMPs) are small particles shed from mature endothelial cells upon cell activation or apoptosis [9–12]. MPs were initially referred to as "cell dust" by Wolf et al. (1967), reflecting cellular activation and/or apoptosis *in vivo* [13]. Elevated levels of circulating MPs have been reported in various cardiovascular diseases, such as hypertension and acute coronary syndrome [14–17]. Platelet microparticles (PMPs) express antigens originating from platelets, including glycoprotein IX (CD42a), glycoprotein Ib (CD42b) and CD41a. EMPs also express several antigens on the surface,

Abbreviations: WHO, World Health Organization; MPs, microparticles; EMPs, Endothelial microparticles; PMPs, Platelet microparticles; AIS, acute ischemic stroke; ICVD, ischemic cerebrovascular diseases; CT, computed tomography; MRI, magnetic resonance imaging; NIHSS, National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; L, arge-artery atherosclerosis; S, small-vessel occlusion; CE, cardioembolism; U, undetermined etiology; OCSP, Oxforshire community stroke project; TACI, total anterior circulation infarcts; PACI, partial anterior circulation infarcts; POCI, posterior circulation infarcts; LACI, lacunar infarct; WBC, white blood cell; Hb A1c, hemoglobin A1c; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; PFP, platelet-free plasma; PBS, phosphate buffer solution; APC, Allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate; AV, Annexin V; FSC, forward scatter; SSC, side scatter; EDTA, Ethylene Diamine Tetraacetic Acid

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http://dx.doi.org/10.1016/j.thromres.2014.12.006
0049-3848/

such as CD144 (vascular cadherin), CD62E and CD105. Both EMPs and a proportion of PMPs express surface CD31 antigen. The presence of distinct antigen molecules provides a means to distinguish different phenotypes of microparticles [18,19].

In the present study, we quantified various circulating EMP and PMP phenotypes in acute ischemic stroke (AIS) patients in comparison to control subjects. The main objective was to establish whether the circulating EMP quantity is increased in patients with ischemic cerebrovascular diseases (ICVD) and the potential utility of specific EMPs as a biomarker of endothelial cell dysfunction. We additionally focused on identifying the EMP phenotypes that may be effectively applied as biomarkers of stroke severity in AIS patients in the clinic.

Materials and Methods

Participants

Sixty-eight consecutive patients with AIS and 61 healthy control subjects were eligible for enrollment. Our patient population comprised inpatients at the Neurology Department in the First Affiliated Hospital of Guangxi Medical University (Guangxi, Nanning, China) between April 2013 and April 2014. Patients experiencing focal symptoms within 7 days of clinical symptom onset were recruited. Physical and neurological assessments were conducted for each patient. All patients underwent perfusion weighted imaging examination via either computed tomography (CT) or magnetic resonance imaging (MRI). Patients with inflammatory disease, renal, hepatic or hematological disorders, and autoimmune or malignant disease were excluded. Disease severity was measured using National Institutes of Health Stroke Scale (NIHSS) [20], whereby scores < 5 were defined as "mild stroke" and ≥ 5 as "moderate-severe stroke". Ischemic stroke was classified into the following subtypes according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) system [21]: large-artery atherosclerosis (L), small-vessel occlusion (S), cardioembolism (CE), and stroke of undetermined etiology (U). Simultaneously, ischemic stroke was classified into five subtypes, according to the Oxfordshire community stroke project (OCSP) [22]: total anterior circulation infarct (TACI), partial anterior circulation infarct (PACI), posterior circulation infarct (POCI), lacunar infarct (LACI) and uncertain. Baseline demographic (age, sex, presence of hypertension, diabetes, smoking, hyperlipidemia and previous stroke) and clinical (glucose, white blood cell [WBC], hemoglobin A1c [Hb A1c], erythrocyte sedimentation rate [ESR], C-reactive protein [CRP], fibrinogen and lipid profiles) data of the study population were collected for analysis. Subjects of the control group were selected from healthy individuals admitted to the Medical Center in the First Affiliated Hospital of Guangxi Medical University (Guangxi, Nanning, China) for regular check-ups between April 2013 and April 2014. All controls had no history of stroke or chronic hepatic or renal disease. Age and gender of control subjects were strictly matched to those of patients. Consent forms complying with the Declaration of Helsinki were signed by patients and their relatives [23–25]. The study protocol was approved by the Institutional Review Board of the First Affiliated Hospital of Guangxi Medical University.

Acquisition of Circulating Microparticles

Circulating MPs were isolated from fresh plasma samples. Briefly, 4 ml of peripheral venous blood from each of the 129 participants was collected into sodium citrate tubes with gentle agitation. Blood samples were sent to the laboratory within 1 h at room temperature, and centrifuged at $1{,}500 \times g$ for 10 min at 4 °C to remove blood cells, followed by centrifugation at $2{,}700 \times g$ for 30 min at 4 °C to obtain platelet-free plasma (PFP). After removal of MP-free plasma, PFP was packed into several small tubes at aliquots of 20 μl per tube, with care to avoid contamination. Aliquots of PFP were stored at −80 °C until assay. Frozen samples were thawed in a 37 °C water bath for 5 min prior to analysis. In preliminary experiments, MP counts from fresh plasma remained stable, compared to those from PFP subjected to a freeze-thaw cycle.

Antibodies and Flow Cytometry Analysis

For flow cytometry, MPs were suspended in 10 μl PFP diluted with phosphate buffer solution (PBS) (200 μl, 154 mmol/l NaCl, 1.4 mmol/l phosphate, 10.9 mmol/l trisodium citrate, pH 7.4). Suspensions of MPs were incubated with the following fluorescent monoclonal antibodies in the dark at 4 °C for 10–15 min: allophycocyanin (APC)-conjugated anti-human CD144 (5 μl; VE-Cadherin; eBioscience, San Diego, USA), phycoerythrin (PE)-conjugated anti-human CD41a (5 μl; eBioscience, San Diego, USA), PE-conjugated anti-human CD62E (E-selectin) (5 μl; eBioscience, San Diego, USA), fluorescein isothiocyanate (FITC)-conjugated anti-human CD31 (PECAM-1) (5 μl; eBioscience, San Diego, USA), and FITC-conjugated anti-human Annexin V (AV) (VAA-33) (10 μl; eBioscience, San Diego, USA). Isotype-matched (IgG) non-specific antibodies were added to another sample tube as a negative control. Samples were shaken gently and analyzed on a fluorescence-activated cell sorting (FACS) II flow cytometer (BD Biosciences) with a medium flow setting. MPs were measured using both forward scatter (FSC) and side scatter (SSC). The cellular origin of MPs was identified via simultaneous detection of antigens on the surface (platelet MPs: CD41a + CD144-, endothelial MPs: CD144 + CD41a-, CD31 + CD41a-, CD62E+, AV + CD62E+). Absolute cell counting beads 2 μm in diameter (Spherotech, Inc, USA) were used for size calibration. MPs were defined as particles smaller than 2 μm. Particles smaller than 0.5 μm in size cannot be detected accurately using a flow cytometer [26]. Amount of MPs were quantified and recorded as counts per μl PFP (counts/μl). All blood samples were analyzed by a skilled technician blinded to clinical data.

Statistical Analysis

Data were expressed as mean values ± standard deviation or percentage. Kolmogorov-Smirnov test for continuous variables was conducted. EMP and PMP values after log transformation (on a base 10 scale) were analyzed to meet with normal distribution. Categorical variables of baseline data between control subjects and patients were analyzed by x^2 test. Continuous variables for measurement

Table 1
Characteristics of the Study Population.

Demographic	Control Group (n = 61)	Acute Stroke Group (n = 68)	p value
Mean age ± SD,(yr)	60.32 ± 11.66	63.59 ± 13.33	0.139
Male sex,n (%)	40(65.57)	48(70.59)	0.541
Hypertension, n (%)	31(50.82)	45(66.18)	0.077
Diabetes,n (%)	11(18.03)	17(25.00)	0.338
Smoking, n (%)	21(34.43)	22(32.35)	0.803
Hyperlipidemia, n (%)	51(83.61)	58(85.29)	0.963
Previous Stroke, n (%)	12(19.67)	15(22.06)	0.739
Laboratory			
WBC ($/L \times 10^9$)	7.36 ± 2.32	8.34 ± 2.42	0.030*
Glucose (mmol/L)	4.86 ± 0.96	5.15 ± 0.86	.092
HbA1c (%)	5.94 ± 0.69	6.15 ± 0.89	0.402
CRP (mg/L)	0.51 ± 1.12	1.32 ± 1.43	0.000*
Fibrinogen (g/L)	3.62 ± 0.54	4.05 ± 0.83	0.075
Lipid profiles (mmol/L)			
Total cholesterol	5.20 ± 1.16	5.02 ± 2.18	0.589
Triglycerides	2.02 ± 2.10	1.63 ± 0.93	0.187
HDL	1.39 ± 0.41	1.25 ± 0.50	0.138
LDL	2.82 ± 0.89	2.69 ± 1.08	0.472

Note: WBC = white blood cell; Hb A1c = hemoglobin A1c; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; HDL = high-density lipoprotein; LDL = low-density lipoprotein. *$p < 0.05$ compared to control group.