Characterisation of circulating endothelial microparticles in Behçet's disease: new markers of chronic endothelial damage?

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Abstract

Objective

Endothelial cell-derived microparticles (EMPs) are directly indicative of endothelial cell activation or apoptosis, and may also reflect endothelial inflammation, increased coagulation, and vascular tone. The aim of this study is to investigate whether EMPs would be able to evaluate system involvement and be a new indicators of disease activity in Behçet's disease (BD).

Methods

Thirty-nine consecutive BD patients (who fulfilled the modified 1990 International Study Group on Behçet's disease or the 2006 International Criteria for Behçet's Disease) and 30 age- and sex-matched healthy controls were enrolled. The plasma levels of EMPs were measured by flow cytometry utilising specific labels for endothelial MPs ($CD31^+$ and $CD42b^-$).

Results

The levels of circulating EMPs ($CD31^+$ and $CD42b^-$) were significantly elevated in the case group compared with the healthy control group ($p = 0.000$). Moreover, BD patients plasma EMPs were positively correlated with BD current activity form ($r = 0.802$, $p = 0.000$). Vascular and gastrointestinal involvement in BD patients were significantly increased ($p = 0.004$ and $p = 0.011$, respectively) with respect to patients without vascular and gastrointestinal EMPs.

Conclusion

Levels of circulating EMPs are elevated in BD patients and correlate with the disease activity; the elevated EMPs may be a potential indicator to predict disease activity of BD. The plasma level of EMPs was increased, which indicated the increased risk of vascular and digestive tract involvement in BD.

Key words

Behçet's disease, cell-derived microparticles endothelial dysfunctions, hypertension, disease activity

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Introduction

Behçet's disease (BD) is an inflammatory disease characterised by recurrent ocular inflammation, oral, genital ulcers, skin damage and systemic involvement. Vascular involvement (VBD) is observed in up to 40% of patients and is the major cause of mortality and morbidity (1). Inflammation of vessel wall is seen in both arterial and venous systems. Venous wall inflammation manifests mainly as thrombosis, and deep venous thrombosis (DVT) of lower extremities is the most common form of VBD (up to 80%) (2). Currently, both the BD classification criteria and its activity assessment are based on clinical assessment, data assessing the veins is limited.

Endothelial microparticles (EMPs) are membrane-bound subcellular microparticles (MPs) produced by endothelial cells in response to a variety of triggers, and may act as a biomarker for endothelial damage. EMPs are increased in acute coronary syndromes, cerebrovascular disease and hypertension (3, 4). Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. EMPs are also elevated in some diseases which impaired endothelial function, such as Kawasaki disease, systemic lupus erythematosus (SLE), pre-eclampsia, thrombocytopenic purpura, multiple sclerosis, and nocturnal paroxysmal haemoglobinuria, all of which have in common the presence of endothelial cell activation or a hypercoagulable state, suggesting that EMP levels not only reflect the activation or damage of endothelial cells, but also play a role in regulating the inflammatory response, coagulation and vascular function. To date, there are no studies examining whether EMPs act as biomarkers of endothelial damage and cardiovascular risk in BD.

In the present study, we investigated whether active BD is associated with increased endothelial damage (EMPs), compared with age- and gender-matched controls.

Materials and methods

Patients and healthy subjects

From January 2019 to January 2022, 39 consecutive, unselected patients with BD and 30 age- and sex-matched healthy controls (HCs) from our centre were recruited. All patients fulfilled the modified 1990 International Study Group on Behcet's disease or the 2006 International Criteria for Behçet's Disease (5). We excluded subjects with a recent acute infection (≤1 month), recent cardiovascular event (≤3 months), any chronic infection, pregnant/lactating patients, and patients with chronic kidney disease (estimated glomerular filtration rate ≤20 ml/min). All subjects gave written informed consent, and ethics approval was obtained from Shanxi Bethune hospital.

Clinical and laboratory assessment

Patients with BD were assessed before the change in therapy and again approximately 4–6 months later. All subjects underwent a full history and physical examination at each visit, with CVD history and drug exposures documented. Patients underwent detailed assessment of their current and past BD features and therapeutic history. All subjects underwent a full history and physical examination at each visit, with CVD history and drug exposures documented. Patients underwent detailed assessment of their current and past BD features and therapeutic history. Two composite scores of disease activity were recorded at each visit: BD current activity form (BDCAF) (6) and Birmingham Vasculitis Activity Score (BVAS) (21).

EMP assay

Patients were enrolled and 2 ml of venous blood was collected on the second day of admission under fasting conditions and placed in a vacuum raftersate anticoagulation tube (blue cap), 4 h for testing. In the healthy control group, blood was collected once and the method was the same as above. Blood samples were centrifuged at 160 g for 10 min to obtain platelet-rich plasma, followed by centrifugation at 1,000 g for 10 min to obtain platelet-poor plasma. The blood samples were stored at -80°C in the refrigerator for testing.

Briefly, blood samples were drawn into citrated Vacutainer tubes (5 ml) and were centrifuged for 10 min at 160 g

to prepare platelet-rich plasma (PRP). The PRP was then centrifuged for 6 min at 1000 g to prepare platelet-poor plasma (PPP). The PPP (50 ml) in a 12×75 mm polypropylene tube was incubated with 5ml of phycoerythrin (PE) anti-CD31 (BD, Franklin Lakes, NJ, USA) plus 5 ml of FITC anti-CD42 (BD) for 20 min with gentle (100 rpm) orbital shaking. Then 940 ml of 0.2 mm filtered PBS was added and the sample was ready for flow cytometry on a FACSCalibur flow cytometer using CellQuest Pro software (BD). EMPs were defined as CD31+ /CD42 particles with a diameter of <1.5 mm (calibrated with flow cytometry size calibrations beads; Invitrogen, Eugene, OR, USA). The number of CD31+/CD42 microparticles (per microlitre of PPP) was calculated by dividing the number of CD31+/CD42 events in the final volume of the sample (50 ml of PPP, 10 ml of antibody suspension, 940 ml of PBS) by the volume of PPP in the test sample (50 ml). To assess the reproducibility of CD31+ /CD42 EMP measurements, circulating EMPs were measured in two separate blood samples from the same participants (in 30 HCs). A very close correlation between the two measurements of CD31+/ CD42 EMPs was obtained (r=0.89, p<0.001).

Statistical analysis

All data analysis was performed using SPSS v. 17.0 (IBM, Armonk, NY, USA). The Mann-Whitney U-test was employed to compare BD and control groups. Spearman's correlation coefficient and binary logistic regression were used to identify a possible association between clinical/serological variables and either EMPs. The significance level was two-sided and set at p<0.05.

Results

39 patients with BD and 30 healthy controls were assessed. Baseline characteristics of cases and controls are described in Tables I and II. epidemiological features and traditional CV risk factors were equally distributed between patients and controls. In particular, factors potentially affecting EMP concentration, including age, menopausal status, hypertension, cholesterol levels and smoking, were matched between the two groups.

Among the 39 patients with BD, 19 were male and 20 were female, female:male ratio was 1.05:1. The age of onset ranged from 11 to 46 years, and the age of predilection ranged from 15 to 40 years, accounting for 33 cases (84.62%), and the duration of the disease ranged from 1 to 50 years. This group of cases involved several organ systems throughout the body (Table II), with the mouth (100%), genitalia (71.78%), and skin (48.72%) being the most frequently involved sites. Ophthalmia (11%), vascular (12%), nervous system (5%), and digestive system involvement (7%) were rare, and the results are shown in Table II. All BD patients were negative for autoimmune antibodies except for 3 patients with low titre positive ANA1:100. the BDCAF score ranged from 1 to 5 with a mean of 2.87±1.03. the BVAS score ranged from 2 to 20 with a mean of 9.05±5.36.

Patients with BD had significantly elevated CD31+/CD42b– EMPs (7.62±0.59 *vs*. 1.28±0.23; p=0.001). The results are shown in Figure 1, and Table III. Correlation analysis of CD31+/CD42b– EMPs EMPs levels with BDCAF, BVAS, ESR and CRP in BD patients showed that CD31+/CD42b– EMPs levels were positively correlated with BDCAF and BAVA in BD patients, p=0.000, while EMPs levels ESR and CRP in BD ptients were not significantly correlated, (Fig. 2, Table IV).

CD31+/CD42b– EMPs levels were significantly higher in patients with BD with vascular involvement compared to patients without vascular involvement (8.11±0.30 *vs*. 7.37±0.51, p=0.000) (Fig. 3A), CD31+/CD42b– EMPs were higher in patients with BD with gastrointestinal involvement compared to patients without gastrointestinal involvement, (8.08±0.54 *vs*. 7.56±0.59, p=0.011) (Fig. 3B). There were no significant differences in plasma EMPs levels between patients with BD in

Table I. Characteristics and traditional CV risk factors of primary BD patients and controls.

	BD Patients n=39	Controls n=30	p value
Age(year), mean ($\bar{x} \pm s$)	38.95 ± 13.21	38.82 ± 2.66	0.965
Female	20 (52.3)	15 (50)	0.86
BP systolic (mmHg)	133 (102,146)	120 (111,128)	0.74
BP diasolic	78 (67,89)	79 (70,81)	0.81
BMI (kg/m^2)	25.3 (20.1,29.5)	25.8 (23.1,29.8)	0.44
Total cholesterol	4.2 (4.0,5.3)	4.6 (3.6,5.9)	0.56
HDL-cholesterol	1.31 (1.13,1.62)	1.34 (1.20,1.69)	0.34
LDL-cholesterol	2.69 (1.90,3.62)	3.01 (2.71,3.88)	0.36
Triglycerides	1.34 (0.86,1.88)	1.03 (0.82,1.30)	0.03
Lipid-lowering	3 (7)	0 (0)	0.16
Glucose (mmol/L)	4.4 (4.2,5.1)	4.8 (4.7,5.2)	0.07
Diabetes	0 (0)	0 (0)	1
Family history of CVD	9 (23.7)	5 (16.7)	0.09

BMI: body mass index; BP: blood pressure; CVD: cardiovascular disease; HDL: high-density lipoprotein; hsCRP: high-sensitivity C-reactive protein; LDL: low-density lipoprotein; BD: Behçet's disease.

Table II. Disease-specific clinical and immunological features of primary BD patients (n=39).

Feature (n=39)	n (%) or median (IQR)*
Disease duration, mean (SD) year	5 (6)
Symptom duration, mean (SD) year	8 (7)
BDCAF (1-5), $\bar{x} \pm s$	2.87 ± 1.03
BVAS (2-20), $\bar{x} \pm s$	9.05 ± 0.36
CRP, mean ($\bar{x} \pm s$), mg/ml	12.76 ± 2.06
Cutaneous involvement, n (%)	19 (48.72%)
Mouth ulcer, n (%)	39 (100%)
Genital ulcers, n (%)	19 (48.72%)
Neurologic involvement, n (%)	5 (12.82%)
Viremian, n (%)	7 (17.95%)
Vascular involvement, n (%)	12 (30.77%)

BDCAF: Behçet's disease current activity form; BVAS: Birmingham Vasculitis Activity Score.

Table IV. Correlation analysis of plasma EMPs levels in patients with BD with BDCAF, BVAS, ESR, CRP.

	EMPs	
	r	p*
CRP	0.287	0.081
ESR	0.193	0.247
BDCAF	0.802	0.000*
BVAS	0.607	0.000*

Table III. Endothelial function in BD cases compared with controls.

Group	EMPs ($\bar{x} \pm s$,%)	T	p*
BD patients (n=39)	7.62±0.59	49.35	0.000
Controls (n=30)	1.28±0.23		

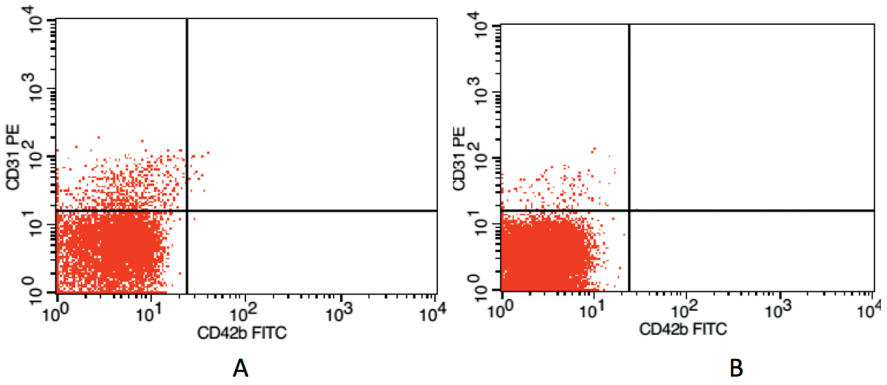

Fig. 1. Comparison of plasma EMPs levels between the BD group and healthy controls.
A: Plasma CD31+/VD42b-EMPs level of 7.92% in BD patients; **B**: Plasma CD31+/VD42b-EMPs level of 0.86% in a healthy control group.

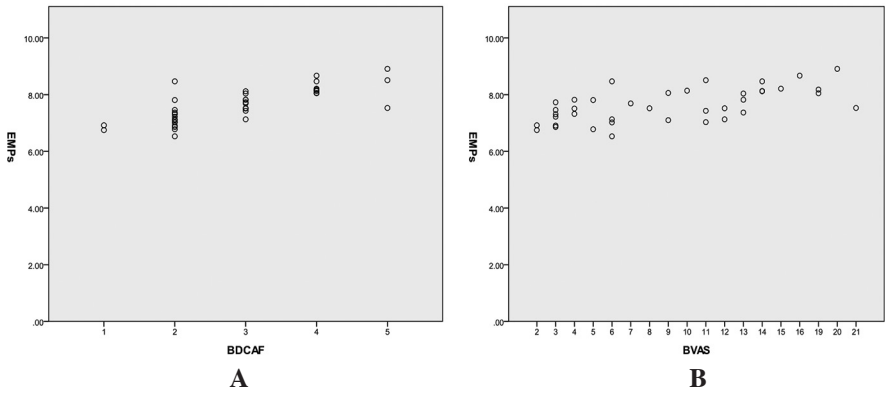

Fig. 2. Direct correlation between EMP number and BDCAF, BVAS.
A: Plasma EMPs levels in patients with BD are positively correlated with BDCAF.
B: Plasma EMPs levels in patients with BD are positively correlated with BAVS.

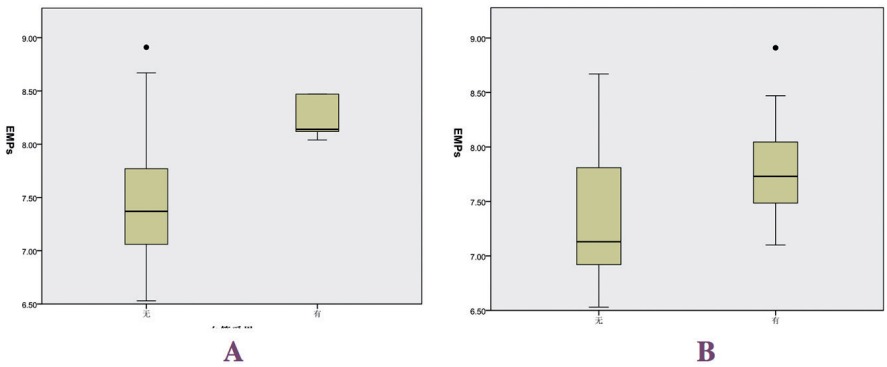

Fig. 3. A: Comparison of plasma EMPs levels in BD patients with and without vascular involvement.
B: Comparison of plasma EMPs levels in BD patients with and without vascular involvement.

terms of gender, rash, neurological involvement, ophthalmia, joint symptoms and whether the needle test was positive or not (Table V).

Discussion

To the best of our knowledge, our study is the first to confirm the levels of circulating endothelial particles in patients with BD and to assess the relationship between circulating endothelial particle levels and disease activity and vascular involvement.

This study showed that circulating endothelial particles were significantly elevated in BD patients compared to healthy controls. EMP has been found to be elevated in a variety of rheumatic and non-rheumatic diseases and has been shown to promote inflammation, activate endothelial cells and promote thrombosis. Our study confirms that EMP is elevated in BD and further confirms the presence of impaired vascular endothelial cell function in BD and correlates with disease activity. Currently, the conclusions of different studies on EMP and disease activity including SLE (7) and primary Sjögren's syndrome (8) are predominantly contradictory, and the choice of different surface markers to quantify EMP in different studies is the most important reason for this. Because EMP produces different surface markers in response to different pathological stimuli, we used a standard combination of markers to identify EMP (CD31+/CD42-). Indeed, CD31, a constitutive marker expressed at low levels on endothelial cells and on platelets, has been shown to be released during apoptosis of surface endothelial cells. CD42 is a platelet-specific molecule and CD31+/CD42- expression is considered to be the most reliable marker (9).

EMP varies with fluctuations in disease activity in autoimmune diseases, and Parker *et al*. (7) found that EMP levels in SLE were positively correlated with disease activity and could be reduced by suppressing inflammation *in vivo*. In contrast, Bartoloni *et al*. (8) found that EMP and endothelial progenitor cells were elevated in patients with dry syndrome, but not correlated with disease activity. In this study, ESR, CRP, BDCAF, Iranian BD dynamic activity measure (IBDDAM) and the Behçet's Syndrome Activity Score (BSAS) were used to score disease activity. Our study found a positive correlation between plasma EMPs levels and BDCAF and BAVA in BD patients, suggesting that EMPs levels may be a new indicator of disease activity in BD.

Table V. Comparison of plasma EMPs levels of each system of involvement in patients with BD.

BD		EMPs (%)			
		n	$\bar{x} \pm s$	t	*p*
Gender	male	19	7.77 ± 0.63	1.601	0.118
	female	20	7.48 ± 0.52		
Genital ulcers	Yes	28	7.60 ± 0.59	0.121	0.905
	No	11	7.58 ± 0.52		
Vascular involvement	Yes	12	8.11 ± 0.30	4.707	0.000*
	No	27	7.37 ± 0.51		
Skin involvement	Yes	18	7.68 ± 0.58	0.869	0.391
	No	21	7.38 ± 0.43		
Gastrointestinal involvement	Yes	7	8.08 ± 0.54	2.673	0.011*
	No	32	7.56 ± 0.59		
Uveitis	Yes	10	7.81 ± 0.63		
	No	29	7.52 ± 0.54	1.399	0.17
Neurologic involvement	Yes	5	7.73 ± 0.76	0.563	0.577
	No	34	7.58 ± 0.54		

The mechanism of EMP release in BD is unclear. *In vitro*, various stimuli have been shown to induce EMP release from cultured endothelial cells under physiological and pathological conditions, with high-level systemic inflammation being one of the main stimuli capable of inducing increased circulating EMP release *in vitro* and *in vivo* (10, 11). BD is a vascular inflammatory disease with multisystem involvement of unknown origin and is thought to be a prototype for systemic inflammatory disease induced thrombosis. The systemic inflammatory response is considered to be the main trigger for thrombosis compared to common thrombophilic factors and may be associated with T lymphocyte, monocyte, neutrophil-mediated production of pro-inflammatory cytokines, and vascular endothelial dysfunction (12).

In this study, thrombosis reached 37% of patients, including arterial and venous thrombosis of all sizes, with deep and superficial lower limb vein thrombosis common. Our study found significantly higher levels of EMP in patients with vascular involvement, suggesting a higher level of endothelial impairment in patients with vascular involvement in BD, and this study is consistent with studies of diseases such as SLE and antiphospholipid syndrome. In 1999 Combes *et al*. (13) first found large amounts of EMPs in patients with LA and correlated with a history of thrombosis and little change in EMPs with anticoagulation. Sibikova *et al*. (14) found by *in vitro* experiments that MPs in SLE and RA patients increased the expression of adhesion molecules, chemokine production and structural alterations in macrovascular and microvascular endothelial cells exacerbating endothelial dysfunction in patients.

EMPs have paracrine and autocrine effects on vascular cells and there is growing evidence that EMPs act as mediators of intracellular signalling as they are able to deliver many bioactive molecules to receptor cells. These bioactive molecules include growth factors, proteases, adhesion molecules, DNA and mictro-RNA (15). Functional proteins such as VEGF and endothelial-type nitric oxide synthase have also been identified in EMPs (16, 17). Cell culture-derived EMPs have also been shown to inhibit angiogenesis in a mouse model of atherosclerosis (18), and in acute vascular stress, EMPs may have a vasoprotective endothelial role (19, 20). Thus, EMPs may act as inert injury markers, but rather as downstream pro-inflammatory delivery systems that are vasoprotective under acute inflammatory conditions but may persist in vascular dysfunction in chronic disease. This suggests that plasma EMPs have the potential to be markers of risk for vascular events in patients with BD and that high levels of EMPs further exacerbate endothelial cell dysfunction in patients, but the pathogenesis still needs further study and our laboratory is conducting experiments to further investigate their role in vascular dysfunction in BD patients.

Circulating endothelial particulate levels are elevated in BD patients compared to hypertensive patients and healthy individuals; circulating endothelial particulate levels in BD patients may be a new indicator for assessing their disease activity; elevated circulating endothelial particulate levels in BD patients may indicate an increased risk of vascular and digestive tract involvement; this study was a cross-sectional study and failed to detect dynamic changes in plasma EMPs levels in BD patients and the sample size was small. Further studies with larger sample sizes are needed to confirm these findings.

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