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ORIGINAL ARTICLE

The role of endothelial microparticles in autoimmune disease patients with Raynaud's phenomenon

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Abstract *Background and aim:* Raynaud's phenomenon (RP) is a microvascular disorder characterized by episodic peripheral vasospasm and ischemia and is commonly found in patients with autoimmune diseases (AID). The vasomotor homoeostasis and endothelial cells damage are involved in RP. Endothelial microparticles (EMPs) may act as a biomarker for endothelial damage. The aim of this study is to investigate the correlation between the levels of microparticles (MPs) and microvasculopathy in AID with RP.
Methods: Thirty-seven patients with AID and RP (RP group) and 27 patients with AID but without RP (non-RP group) were enrolled. The microvasculopathy score of RP was graded by nailfold capillary microscopy. The plasma levels of MPs were measured by flow cytometry utilizing specific labels for endothelial MPs (CD105 and CD144) and annexin V staining for phosphatidylserine bearing-MPs (annexin V+MPs).
Results: The levels of circulating EMPs (CD105+ $p = 0.005$, CD144+ $p = 0.004$), and the annexin V+ MPs ($p < 0.001$) were significantly elevated in the RP group compared with the non-RP group. Moreover, the high microvasculopathy scores were closely related with annexinV+ MPs levels in the RP group ($p = 0.041$).

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Conclusions: Levels of circulating EMPs and annexin V+ MPs are elevated in AID patients with RP indicate the endothelial damage and endothelial dysfunctions. In addition, levels of annexin V+ MPs can predict the severity of microvasculopathy in AID with RP.

Introduction

Raynaud's phenomenon (RP) is a microcirculatory disorder with episodic vasospasm of small arteries and arterioles of the digits in response to cold or emotional stimuli. RP is classified as either primary or secondary based on whether there is an associated disorder. The pathophysiological abnormalities leading to RP are probably different between the primary and secondary forms.[1] Secondary RP may have more severe symptoms and cause digital ischemia, ulcerations, and even gangrene. Autoimmune diseases (AID) are associated with secondary RP, most notably systemic sclerosis (SSc) in $>$ 90% cases. RP also occurred in overlap or mixed connective tissue diseases (85%), systemic lupus erythematosus (SLE; 10—45%), primary Sjögren's syndrome (33%), dermatomyositis or polymyositis (20%), and rheumatoid arthritis (12.3%).[2,3] Nailfold capillary microscopy provides a morphologic approach to evaluate the severity of microvasculopathy in RP.[4]

In RP associated with AID, the vasospasm is more sustained, long-lasting, and highly recurrent, and is associated with structural alterations of the vessel walls. Structural alterations of small and medium-sized arteries can be seen in secondary RP with the features of endothelial proliferation and ineffective neoangiogenesis, which lead to vascular remodeling.[5] CD105, abundantly expressed in angiogenic endothelial cells, is associated with proliferation and induced by hypoxia.[6] VE-cadherin (CD144) is also an endothelial protein, and the transition of its function from the quiescent to the angiogenic state increases its content through the activation of its promoter.[7] Microparticles (MPs) are small vesicles that are released by budding plasma membranes after cell activation or apoptosis.[8] Endothelial MPs (EMPs) are directly indicative of endothelial cell stress/damage, and may also reflect endothelial inflammation, increased coagulation, and vascular tone.[9] MP-associated procoagulant activities rely on the exposure of phosphatidylserine (PS) and are considered to involve the vascular homeostasis.[10] Annexin V interacts strongly and specifically with PS,[11] and its presence on MP is a true reflection of procoagulant activity.[12] It is interesting to correlate the expression of annexin V+ MP and EMPs of CD105 and CD144 with the severity of nailfold capillaroscopy in AID with RP.

Materials and methods

Eligibility of study participants

Sixty-four patients with AID from our Rheumatology clinic were recruited in this study. Patients with AID who complained of cold intolerance and met the diagnosis of RP were enrolled as the RP group and those without the symptoms and signs of RP were enrolled as the non-RP group. Patients with rheumatoid arthritis, SSc, or SLE fulfilled the American College of Rheumatology classification criteria,[13—15] undifferentiated connective tissue disease fulfilled criteria according to LeRoy et al,[16] Sjögren's syndrome fulfilled American—European Consensus Group,[17] and dermatomyositis fulfilled Bohan criteria[18] were included. The patients taking vasodilator drugs, such as endothelin-1 receptor antagonists, prostacyclin analogs, type 5 phosphodiesterase inhibitors, or those having an active infection or thrombosis proven by clinical and laboratory evaluations were excluded. The study protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital and the protocol strictly adhered to the Declaration of Helsinki. Signed and informed consent was obtained from each participant before blood samples were taken.

Grading of microvasculopathy by nailfold capillary microscopy

Nailfold capillary microscopy (SMZ-10 stereoscopic microscope, Olympus, Tokyo, Japan) was performed on bilateral second to fourth fingers at room temperature. Grading of vascular enlargement, hemorrhagic spots, and avascularity was performed as in our previous study on RP.[19] Vascular enlargement, vasodilatation, grading (V score), avascularity (A score), and nailfold hemorrhagic spots (H score) were assessed. The H, A, and V scores were calculated as the summation of scores from the bilateral second to fourth nailfold in each category and the microvasculopathy score was obtained by summing these three scores.

Measurement of circulating MPs

Venous blood was collected by atraumatic antecubital venipuncture using a 21-gauge needle. A 2.5-mL sample of blood was then collected into a sodium citrate (0.109M) Vacutainer tube for platelet poor plasma preparation by one step centrifugation at 1500 *g* for 20 minutes at room temperature. The levels of circulating MPs were measured within 2 hours of blood collection. MPs were defined as events with a size $\leq$ 1 μm and positively stained by specific fluorochrome-labeled monoclonal antibodies. All MP analyses were performed using a Coulter EPICS XL flow cytometer with EXPO32 ADC analytical software (Beckman Coulter, Miami, Fla, USA). The determination of MP gate was adapted from the methods described by Robert.[20] Briefly, the light scattered and fluorescence channel were set as a logarithmic gain and calibrated fluorescent beads (Megamix, mixed 0.5-μm, 0.9-μm,

and 3-μm microbeads; Biocytex, Marseille, France) were used for the initial settings and identified in forward scatter versus side scatter intensity dot plot presentation. A 25-μL sample of platelet poor plasma was incubated for 30 minutes in the dark at room temperature with either specific monoclonal antibodies (mAbs; 10 μL/test) or the respective isotype-matched control. The mAbs used for MP analysis were as follows: phycoerythrin (PE)-conjugated mAb for CD105 (BD Biosciences, San Diego, CA, USA); PE-conjugated mAb for CD144 (eBioscence, San Diego, CA, USA). MPs bearing phophatidylserine were labeled by fluorescein isothiocyanate (FITC)-conjugated annexin V solution (10 μL/test) (BD Biosciences). Calibrator beads (Flowcount beads, 10 μm, Beckman Coulter) with a known concentration were added to each samples and run concurrently with the MP samples. Before the flow cytometry analysis, the sample was diluted in phosphate buffered saline to a final volume of 500 μL. Sample analysis was stopped when a fixed number (2500) of calibrated beads had been counted. The concentration of MPs was determined by comparison with that of the calibrator beads.

Statistical analysis

Statistical analysis was performed with the SPSS software for Windows version 16.0 (SPSS Software, Chicago, IL, USA). Quantitative variables with non-normal distribution were log-transformed. Differences between the groups were calculated using Student *t* test or Mann–Whitney *U* test. The correlations between variables were presented as Spearman's correlation coefficients. A p value < 0.05 was considered statistically significant.

Results

Higher microvasculopathy score in RP group AID patients

A total of 64 AID patients were enrolled in the study, with 37 in the RP group and 27 in the non-RP group. The characteristics of the participants are shown in Table 1. All patients were female. Two patients with SLE in RP group had vasculitis. Of the patients with SSc, 90% demonstrated secondary RP. The SLE activity was similar in RP and non-RP SLE patients. The cardiac risk factors of DM, HTN, and hyperlipidemia, are similar the two groups. In nailfold capillary microscopy examination, the RP patients showed higher rates of hemorrhage, and more avascularity and vasodilation than the non-RP patients (Table 2). Five of the 27 patients without RP did not have any microvascular lesion and most cases had minor microvascular abnormalities. In contrast, all of the patients in the RP group had marked microvascular morphological changes and higher microvasculopathy scores.

Elevated circulating levels of EMPs and annexin V+ MPs in the AID patients with RP

The AID patients with RP revealed significantly elevated circulating levels of EMPs and annexin V+ MPs than the non-RP group. The median of CD105+ EMPs (Figure 1A), CD144+ EMP (Figure 1B) and Annexin V+ MP (Figure 1C) in RP group and non-RP was 1164 versus 402 events/μL ($p = 0.005$), 1250 versus 588 events/μL ($p = 0.004$) and 764 versus 132 events/μL ($p < 0.001$), respectively. Stratified analysis of patients with SLE showed similar results between two groups with RP and non-RP (Figure 2). The correlations of the MPs and microvasculopathy scores in the RP and non-RP groups are presented in Figure 3. Two EMPs, CD105+ EMPs and CD144+ EMPs, had a positive correlation in both the RP ($r = 0.6$, $p < 0.001$) and non-RP groups ($r = 0.8$, $p < 0.001$). The EMP levels were significantly correlated with annexin V+ MPs in the RP group (CD105+ EMP, $r = 0.45$, $p = 0.006$, CD144+ EMP, $r = 0.51$, $p = 0.001$), but not in the non-RP group. The annexin V+MP levels was positively associated with the avascular and microvasculopathy scores in the RP group ($r = 0.43$, $p = 0.008$; $r = 0.34$, $p = 0.041$, respectively).

Table 1 Baseline characteristics of autoimmune disease patients with and without Raynaud's phenomenon (RP).

	RP group	Non-RP group	*p*
n	37	27	
Age (y)	49.7 ± 2.2	47.2 ± 2.2	0.427
Diabetes	0	1	0.238
Hypertension	10	8	0.819
Smoke	4	0	0.078
RA	4	4	0.632
SSc	5	1	0.184
SLE	13	12	0.451
UCTD	11	6	0.502
Sjögren's syndrome	4	2	0.645
Dermatomyositis	0	2	0.093
Disease duration (y)	6.29 ± 0.98	6.5 ± 1.07	0.89
ACL-G	6.82 ± 1.89	4.64 ± 1.07	0.389
SLEDAI	3.6 2 ± 0.84	3.5 ± 0.42	0.905
White blood cells (10^9 /L)	5.98 ± 0.38	5.78 ± 0.37	0.731
Hemoglobin (g/dL)	12.6 ± 0.2	12.46 ± 0.23	0.68
Platelets (10^{10} /L)	20.8 ± 1.2	22.4 ± 2.1	0.486

Data are presented as mean ± standard error of the mean or *n*. ACL-G anticardiolipin G antibody; RA = rheumatoid arthritis; SSc = systemic sclerosis; SLE = systemic lupus erythematousus; SLEDAI systemic lupus erythematousus disease activity index; UCTD = undifferentiated connective tissue disease.

Table 2 The microvasculopathy score graded by the nailfold capillary microscopy examination.

	RP group	Non-RP group	*p*
H (hemorrhage)	4.4 ± 0.8	0.6 ± 0.2	< 0.001
A (avascular)	10.1 ± 0.7	0.9 ± 0.2	< 0.001
V (vasodilation)	1.8 ± 0.4	0.2 ± 0.1	< 0.001
Total microvasculopathy score	16.3 ± 1.4	1.9 ± 0.4	< 0.001

Data are presented as mean ± standard error of the mean. RP = Raynaud's phenomenon.

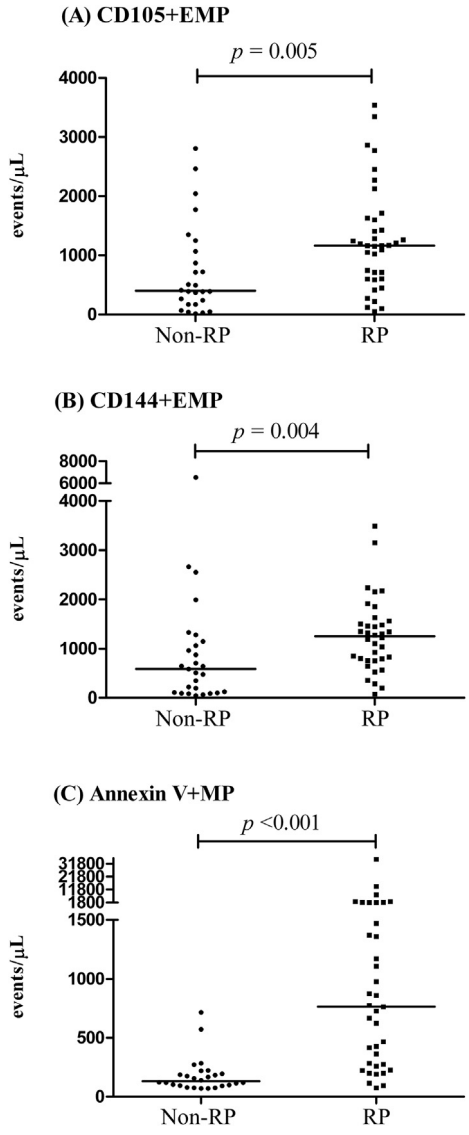

Figure 1. Circulating (A,B) endothelial microparticle (EMP) and (C) annexin V+ microparticle (MP) levels in autoimmune disease patients. RP = Raynaud's phenomenon.

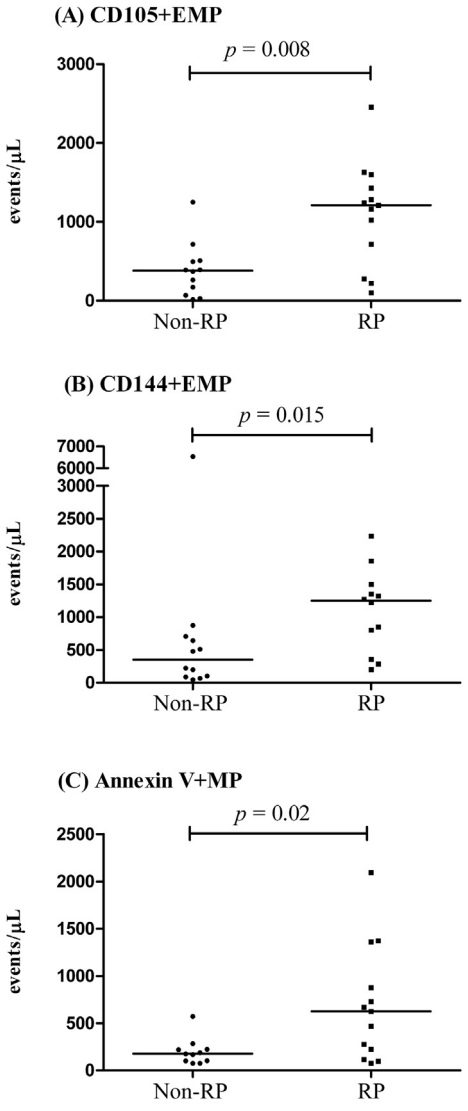

Figure 2. Circulating (A,B) endothelial microparticle (EMP) and (C) annexin V+ microparticle (MP) levels in systemic lupus erythematous patients. RP = Raynaud's phenomenon.

Discussion

Endothelial damage is thought to contribute to the microvascular injury in secondary RP as SSc patients have a higher level of von Willebrand factor compared with patients with primary RP and healthy controls.[21] Several vasoactive mediators, such as nitric oxide,[22] endothelin-1,[23] and angiotensin II[24] have also been reported to be increased in the blood levels of patients with secondary RP. These findings suggest the potential role of endothelial activation in the pathogenesis of secondary RP. The current study demonstrated increased EMP levels in the AID and SLE subgroup patients with RP. Circulating EMPs have been considered to serve as a potential marker of endothelial activation and damage in various autoimmune disorders, and higher EMP levels have been detected in patients with vasculitis and associated with disease activation.[25,26] Higher plasma EMPs have been reported in SLE patients with a state of low disease activity compared to healthy donors.[27] EMPs were elevated in active SLE and associated with increased endothelial damage and dysfunction, which decreased after suppression of clinical activity and inflammation.[28] The present study demonstrated that the level of EMPs in the SLE patients with RP is higher than those without RP. This suggests that more endothelial dysfunction in SLE patients with RP. Furthermore, elevated levels of circulating EMPs in other AID patients with RP are also found. EMPs may serve as a potential surrogate biomarker to reflect the endothelial activation and damage in secondary RP.

VE-cadherin (CD144) is an integral membrane protein of interendothelial adherens junctions that maintains vascular integrity.[29] Reduced VE-cadherin expression at the inter-endothelial junctions has been demonstrated to occur in response to the ischemia—reperfusion injury and to be associated with increasing microvascular permeability.[30]

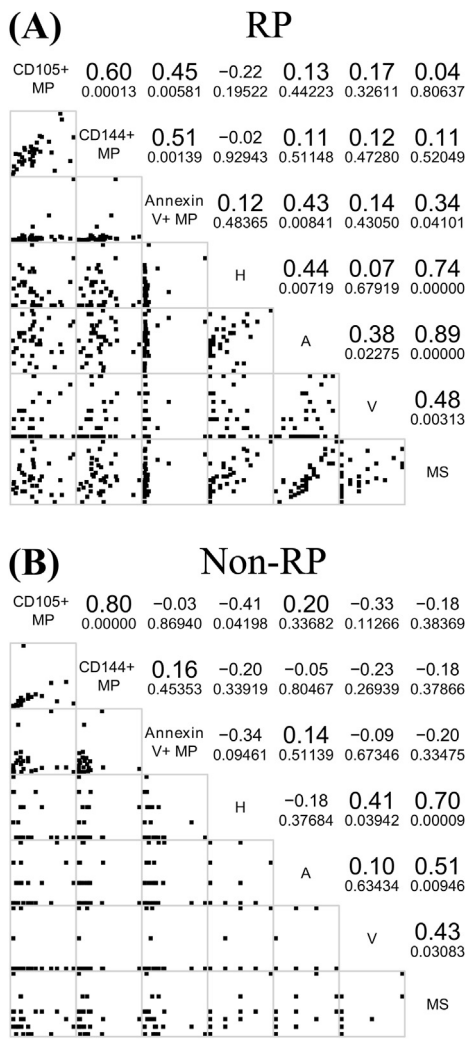

Figure 3. Correlograms of the microvasculopathy score and microparticle (MP) levels in autoimmune disease patients (A) with and (B) without Raynaud's phenomenon (RP). A = avascular area score; H = hemorrhage score; MS = total microvasculopathy score; V = vasodilatation score. A large font size number denotes a correlation with $p < 0.05$.

We demonstrated higher VE-cadherin+ MP levels in the AID patients with RP, which indicated changes in the integrity of the adherent junctions of endothelial cells. These findings suggest that ischemia–reperfusion injury plays an important role in the pathophysiology of RP, and that alterations of endothelial integrity are probably required to cause microvascular hemorrhage.

EMPs have an endothelium-dependent vasodilatation effect, as the EMPs isolated from patients with end-stage renal failure have been shown to impair endothelium-dependent vasodilatation *in vitro*.[31] In clinical studies, EMP level was reported to have an inverse correlation with artery relaxation.[32,33] These findings suggest that the endothelial-dependent vasoregulation may play a critical role in the pathogenesis of RP, although the exact effects of EMPs require future investigations.

Nailfold capillaroscopy is a noninvasive method to access microvasculopathy in patients with AID and RP. Widefield nailfold capillaroscopy using a stereomicroscope as in the present study has been shown to be significantly correlated with videocapillaroscopy in patients with RP.[34] Morphologic alterations of the microcirculation have been associated with systemic vascular damage and disease activity,[35] and microvascular abnormalities are relevant to tissue hypoxia. Loss of capillaries (the avascular area) represents critical tissue hypoxia and is associated with a poor prognosis. We demonstrated that the annexin V+ MP is significantly correlated with the severity of microangiopathy and avascular area, but EMP lacks similar correlation. In this study, we measured total Annexin V+ MPs shed from various cells, including platelets, white blood cells, and endothelium. The aforementioned results suggest the potential role of phosphotidylserine-bearing MPs in the pathogenesis of microvasculopathy in secondary RP, but not only shed from endothelium. In particular, platelet activation also contributes to the pathogenesis of RP,[5] and the activated platelets release MPs with procoagulant activities that rely on the exposure of PS and involve vascular injury.[10] However, the precise mechanisms require further investigation.

In conclusion, circulating EMPs and phosphatidylserine-bearing MPs are elevated in AID patients with RP, and annexin V+ MPs are significantly correlated with the severity of microangiopathy. EMPs may serve as a potential surrogate biomarker to reflect the endothelial activation and damage in secondary RP.

Conflicts of interest

The authors declare no conflict of interest with regard to the work.

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