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Evaluation of renal vascular lesions using circulating endothelial cells in patients with lupus nephritis

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Objective. Currently the detection of renal vascular lesions (VLS) mainly depends on biopsy examination, and lacks surrogate biomarkers for clinical dynamic evaluation. The aim of this study is to find the correlation between numbers of circulating endothelial cells (CECs) and renal VLS in lupus nephritis (LN).

Methods. Thirty LN patients with VLS and 30 LN patients without VLS were recruited. Thirty age- and sex-matched healthy volunteers served as controls. CECs were isolated from peripheral blood with anti-CD-146-coated immunomagnetic Dynabeads and were counted under microscopy. Parameters of renal involvement, including blood urea nitrogen, serum creatinine, 24 h urine protein excretion and quantitative urine sedimentation were also measured.

Results. The number of CECs showed no difference between LN patients without VLS and controls. In patients with VLS, the number of CECs was significantly higher than those without VLS ($P < 0.01$). A strong positive correlation was found between CECs and serum creatinine ($r = 0.503$, $P < 0.01$) and mean blood pressure ($r = 0.423$, $P < 0.05$). In all LN patients with VLS, CEC number of the patients with thrombotic microangiopathy (TMA) significantly increased compared with those without TMA ($P < 0.01$).

Conclusion. Numeration of CECs may serve as a potential and useful marker for vasculopathy in LN. Dynamic observations of CEC number can be used not only to provide evidence for monitoring disease severity and disease activity, but also to determine therapy efficacy in LN patients.

Key words: Lupus nephritis, Renal vascular lesion, Circulating endothelial cells.

Introduction

Renal vascular complications are frequently encountered in SLE. Renal vascular lesions (VLS) have profound effects on the clinical course, prognosis and choice of therapy in patients with Lupus Nephritits (LN) [1, 2]. However, the presence and significance of renal VLS are often overlooked. Current observation of renal VLS mainly depends on renal biopsy examination, and surrogate biomarkers for clinical dynamic estimation of patients are greatly needed.

More than 30 yrs ago, Bouvier and colleagues [3] first reported the presence of non-haematopoietic cells of endothelial origin in the blood of rabbits after endotoxin injection. This was also confirmed by subsequent studies by Hladovec *et al.* [4, 5]. Circulating endothelial cells (CECs) have been associated with several pathological conditions that have common vascular injury [6–8], and endothelial cells or endothelial progenitors in circulation can home in to sites of ischaemia [9, 10] as well as play a role in the formation of non-thrombotic neointima and angiogenesis on vascular prosthetic surfaces *in vivo* [11, 12]. Identification of the origins of CEC and blood endothelial outgrowth may facilitate the use of these cells in clinical diagnosis. Also, measurement of CECs is useful in ANCA-associated small vessel vasculitis and renal transplantation [13–15].

Although circulating CECs have been documented in autoimmune conditions, including lupus and vasculitis, the relationship between CECs and renal vascular dysfunction in lupus nephritis has not been investigated [13, 16]. There is no report on the relationship between the number of CECs and histopathology features in LN patients with VLS also. Accordingly, in the present study, we want to determine the difference in the number of CEC in the circulation of LN patients with/without renal VLS and the association of CEC number and disease activity or renal function (serum creatinine levels).

Materials and methods

Materials

M-450 Dynabeads were purchased from Dynal (Oslo, Norway). Anti-CD 146 antibodies were obtained from Biocytex (Marseille, France). All other reagents were of the highest grade and commercially available.

Patients and control subjects

Thirty LN patients with renal VLS (LN with VLS) and 30 LN patients without renal VLS (LN without VLS), who were hospitalized and underwent renal biopsies at the clinical unit of the nephrology centre from February 2002 to December 2006, were recruited. All the patients were diagnosed as SLE according to the criteria set out by ACR in 1982 [17] and had a renal biopsy prior to the study to reveal cases of LN according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification [18]. The patients were divided into four pathological groups: Class II (mesangial proliferative LN, $n = 12$), Class III (focal LN, $n = 2$), Class IV (diffuse LN, $n = 31$), Class V (membranous LN, $n = 15$) (Table 1). Disease activity was assessed using the SLEDAI as described [19]. They had not received any strong immunosuppressive treatment of cyclophosphamide or mycophenolate mofetil (MMF) at least 2 months before renal biopsy. The diagnosis of LN with renal VLS was made according to the criteria of Weening and Appel [2, 18]. The criteria are as follows: (i) cellular crescent formation in glomeruli ($\geq$15%) (Fig. 1A), (ii) glomerular necrosis and intracapillary thrombi (Fig. 1B and C) and (iii) interstitial VLS. Interstitial VLS includes: (i) Vascular immune complex deposits: by light microscopy, the vessels usually appear normal. Less commonly, eosinophilic, periodic acid-Schiff-positive and fuchsinophilic deposits are seen beneath the endothelium or between the cells of the media. No thrombosis, necrosis or inflammatory infiltration of the vessel wall is present, and the lumen is usually not compromised (Fig. 1D). By immunofluorescence microscopy, the

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deposits may contain immunoglobulin G (IgG) (Fig. 1E). (ii) Non-inflammatory necrotizing vasculopathy: by light microscopy, smudgy eosinophilic, fuchsinophilic material that stains focally positive for fibrin occupies the lumen and intima, with frequent extension into the media. The endothelium is usually swollen or denuded, with occasional pyknotic nuclear fragments and smudgy degeneration of the medial monocytes. The elastic membrane of the interlobular arteries is usually disrupted. Rarely, a few lymphocytes may be seen in the lumen or intima (Fig. 1F). By immunofluorescence microscopy, variable staining for IgG, IgM, IgA, complement components and fibrin-related antigens is present in the wall and extends to the lumen (Fig. 1G). (iii) Thrombotic microangiopathy (TMA): there is marked luminal narrowing or total occlusion by intraluminal, subendothelial or medial accumulation of eosinophilic, fuchsinophilic material with staining properties of fibrin, invariably associated with endothelial swelling, denudation and sometimes fragmented and/or haemolysed erythrocytes (Fig. 1H and I). Since the true renal vasculitis is the least frequent renal VLS encountered in SLE, none of these cases have been studied. Thirty age- and sex-matched healthy volunteers served as controls. The local research ethics committee gave approval for the study. Written informed consent was obtained from each patient and healthy volunteer before blood sampling. All research work with human subjects was in compliance with the Helsinki Declaration.

TABLE 1. Pathological groups of patients according to the ISN/RPS 2003 classification

	LN with VLS ($n=30$)	LN without VLS ($n=30$)
Class II	0	12
Class III	2	0
Class IV	26	5
Class V	2	13

Renal histological examination

Percutaneous renal biopsy was performed in each lupus patient under ultrasonographic guidance. Formalin-fixed tissue was embedded in paraffin using routine procedures. Sections of 2 μm in thickness were stained with haematoxylin/eosin (HE), periodic acid-Schiff (PAS), silver methenamine and Masson's trichrome for microscopic pathological diagnosis. For immunofluorescence, renal tissues in optimum cutting temperature (OCT) compound were snap-frozen and kept in liquid nitrogen. Immunofluorescence staining was performed on 3 μm cryostat sections by using FITC-labelled rabbit anti-human IgG, IgA, IgM, Complement (C)3, C4, C1q and fibrin antibodies (Dako Corporation, Carpinteria, CA, USA). Two pathologists, who were blinded to the CEC data, performed histopathological evaluation.

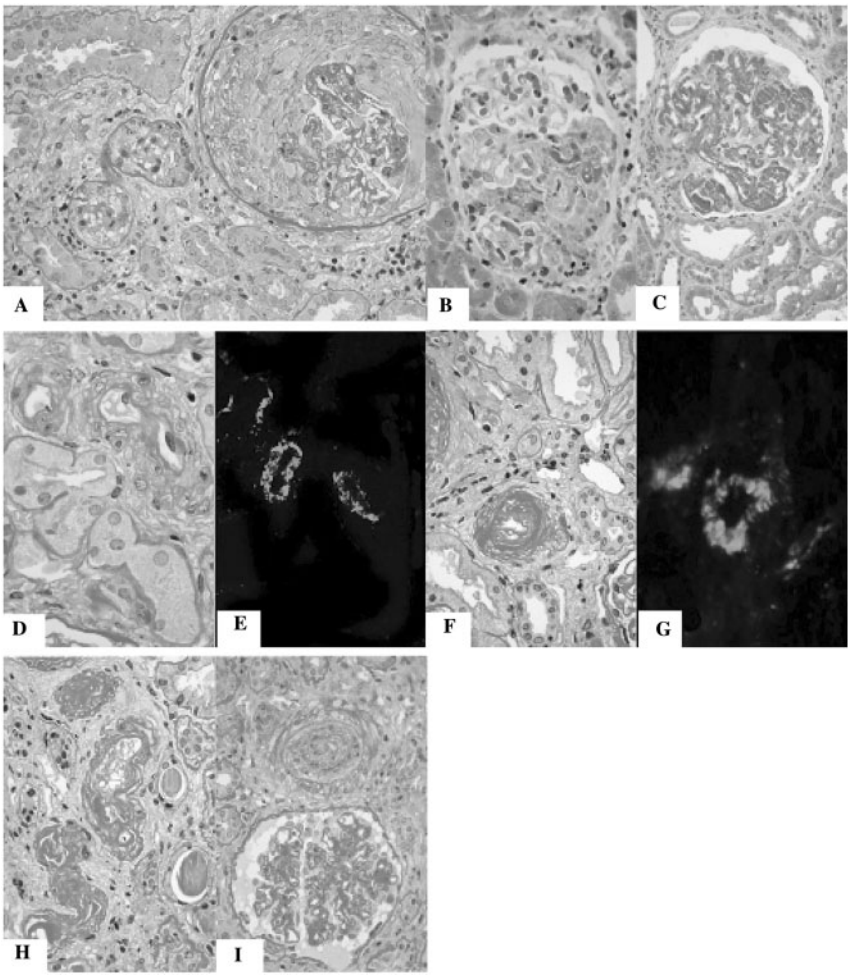

FIG. 1. The pathology of the renal vasculopathies in LN patients. (**A**) Cellular crescent in glomerular (PAS, ×400). (**B** and **C**) Segmental glomerulus necrosis and intracapillary thrombi (Masson's trichrome stain, ×400). (**D**) Segmental PAS strong staining in interstitial vascular (PAS, ×400). (**E**) Vascular immune deposits of IgG [immunofluorescence (IF), ×400]. (**F** and **G**) Non-inflammatory necrotizing vasculopathy (F: PAS; G: IF fibrin, ×400). (**H** and **I**) TMA (H: PAS; I: periodic acid silver methanamine, ×400).

Routine laboratory measures

Urine and blood was collected at the same day the renal biopsy was taken. Urinary protein was detected by the biuret method (normal value <0.5 g/day.) Urinary red blood cells (URBCs) were counted in morning urine, normal value was <10 000 RBC/ml of urine. Serum creatinine, total IgG, complement C3 and C4 were measured by routine laboratory tests. ANAs were determined by indirect immunofluorescence. Anticardiolipin antibodies (ACL-A) and Anti-dsDNA antibodies (Anti-dsDNA) were determined by ELISA (Euroimmun, Luebeck, Germany). Serum cryoglobulin was detected by turbidimetry method, with normal value <193.6 mg/l.

Isolation and counting of CECs

Isolation of CECs was performed by immunomagnetic separation after an antibody incubation step according to previously reported and validated methodology [20]. Three millilitres of ethylenediaminetetraacetic acid (EDTA) blood from patients with LN and from healthy volunteers after obtaining their informed consent was collected for isolation of CECs. Anti-endothelial cell monoclonal antibody (anti-CD146)-coated M-450 Dynabeads were obtained as recommended by the manufacturer. Coated Dynabeads were stored at 4°C for a maximum of 4 weeks. Blood was obtained by venipuncture. After careful rotation of the tube, 1 ml blood was mixed with 1 ml isolation buffer (phosphate-buffered saline, 0.1%BSA, 0.1% sodium azide and 0.6% sodium citrate) at 4°C. Next, the sample was mixed in a head-over-head mixer for 30 min at 4°C and separated using a Dynal MPC-1 magnetic particle concentrator (Dynal). Then the sample was washed with buffer four times inside the magnet at 4°C. Between each washing procedure, the sample was flushed ten times with buffer in a 100 μl pipette. The cell-bead suspension was finally dissolved in 200 μl buffer. Cells were counted with a Nageotte chamber. Endothelial cells were larger than other blood cells, had a well-delineated round or oval cell shape and carried more than five beads (Fig. 2). Various concentrations of fresh human umbilical vein endothelial cells were diluted in blood of healthy volunteers to serve as positive controls.

Statistical analysis

Statistical calculations were performed with Statistical Package for the Social Sciences 10.0 for Windows (Beijing, China). URBC count and cryoglobulin were presented as median. Other data were presented as mean ± S.E.M., with n, the number of humans. Groups were compared by analysis of variance (ANOVA), and individual groups were compared by the Kruskal–Wallis test for unpaired analysis. Spearman's rank correlation was used to assess the correlations between the variables. $P < 0.05$ was considered statistically significant.

Results

Characteristics of LN patients

The age, sex, SLEDAI score, serum creatinine and urea concentrations of LN patients were summarized in Table 2. The creatinine concentrations of LN with VLS and LN without VLS were 170.4 ± 23.8 and 60.9 ± 7.1 μmol/l, respectively ($P < 0.01$). The mean blood pressure and pulse pressure of patients with VLS were significantly higher than those of patients without VLS ($P < 0.01$). Fibrin was found in 18 patients with VLS and occurred only in 1 patient without VLS ($P < 0.01$). The frequencies of crescent formation (93.3% *vs* 3%) were significantly higher in the patients with VLS than those without VLS. The percentage of crescent was higher (35.2 ± 27.4%) in patients with VLS than in those without VLS (1 ± 2.6%) ($P < 0.01$). There were no significant differences in the level of anticardiolipin antibody, cryoglobulinaemia, complement components, haematuria and proteinuria between LN patients with and without VLS.

Quantification of CECs

As shown in Fig. 3, the numbers of CECs in control and LN without VLS were 14.7 ± 2.0 and 20.7 ± 2.9 cells/ml, respectively. Although the CECs in LN without VLS were increased compared with control, the difference was not significant. However, the number of CECs in LN with VLS (62.0 ± 7.3 cells/ml) was significantly higher in LN with VLS than in LN without VLS and control subjects ($P < 0.01$).

Relationship between CECs and LN with TMA

In order to further analyse the relationship between CEC level and severity degree of VLS in LN patients, we subdivided the patients with VLS into two groups: patients with TMA ($n = 8$) and without TMA ($n = 22$). As shown in Fig. 4, CECs of the two groups were significantly increased than that of the control. CECs of the patients with TMA (103.4 ± 15.8 cells/ml) were significantly higher than those without TMA (47.6 ± 5.8 cells/ml) ($P < 0.01$). Serum creatinine levels of patients with TMA (320 ± 151.16 mg/dl) were significantly higher than those without TMA (118.46 ± 74.26 mg/dl) ($P < 0.01$).

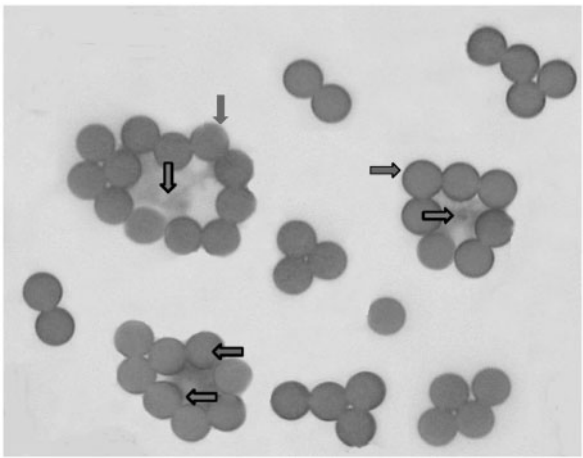

FIG. 2. CECs detected by magnetic beads. (HE, ×400, blue arrow: magnetic beads; red arrow: CECs). Colour figure online.

TABLE 2. Clinical features of patients with LN

	LN without VLS ($n = 30$)	LN with VLS ($n = 30$)
Age, mean ± S.D. (yr)	28.9 ± 10.9	29.0 ± 10.3
Male/female	1/29	2/28
SLEDAI, mean ± S.D.	16.8 ± 4.7	17.6 ± 6.4
Scr, mean ± S.D. (μmol/l)	60.9 ± 7.1	170.4 ± 23.8**
Hb, mean ± S.D.(g/l)	92.1 ± 5.7	73.8 ± 13.0
Serum albumin, mean ± S.D. (g/l)	31.1 ± 1.6	27.7 ± 1.3
Proteinuria, mean ± S.D. (g/day)	2.53 ± 0.59	4.24 ± 0.47
Urine RBC count, (range) (10^4/ml)	220 (1–950)	483 (1–2100)
Anti-ds-DNA+ (%)	47.6	100**
Anti-ANA+ (%)	64.50	100**
ACL+ (%)	33.3	29
C3, mean ± S.D. (g/l)	0.67 ± 0.12	0.47 ± 0.24
C4, mean ± S.D. (g/l)	0.18 ± 0.04	0.16 ± 0.08
CD4+/CD8+, mean ± S.D.	0.89 ± 0.41	1.02 ± 0.11
Cryoglobulin, (range) (mg/l)	286 (0–1051)	206 (109–915)
Mean blood pressure, mean ± S.D. (mmHg)	88 ± 9	106 ± 15**
Pulse pressure, mean ± S.D. (mmHg)	41 ± 9	51 ± 10**
Frequency of fibrin+ (%)	3.3	60**
Frequency of crescent formation (%)	3.3	93.3**

**$P < 0.01$.

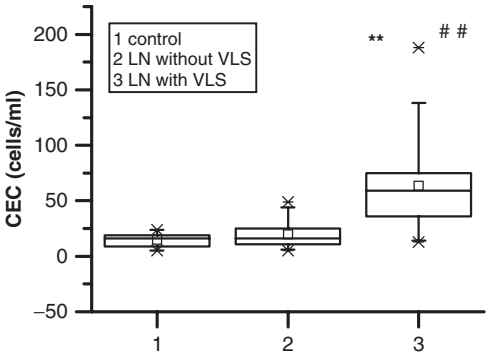

FIG. 3. Comparison of CEC in LN patients and controls (The CEC numbers were presented as mean ± S.E.M. $^{**}P < 0.01$ *vs* control, $^{\#\#}P < 0.01$ *vs* LN without VLS).

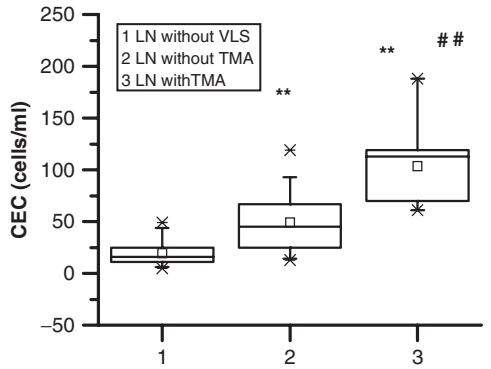

FIG. 4. Comparison of CEC in LN patients with and without TMA (The CEC numbers were presented as mean ± S.E.M. $^{**}P < 0.01$ *vs* LN without VLS, $^{\#\#}P < 0.01$ *vs* LN without TMA).

Relationship between CECs and clinical parameters

In LN patients with VLS, CECs showed a positive correlation with serum creatinine ($r = 0.503$, $P < 0.01$) and pulse pressure ($r = 0.423$, $P < 0.05$). The CECs also showed a positive correlation with serum creatinine ($r = 0.891$, $P < 0.01$) and pulse pressure ($r = 0.683$, $P < 0.05$) in patients with TMA. However, there was no correlation between CECs and SLEDAI, C3, C4, anti-dsDNA, URBCs, proteinuria and crescents (Table 3).

TABLE 3. Relationship between CECs and clinical parameters

	Numbers of CEC	
	Patients with VLS	Patients with TMA
Creatinine	$r = 0.503$ $P = 0.003^{**}$	$r = 0.891$ $P = 0.001^{**}$
Mean blood pressure (mmHg)	$r = 0.048$ $P = 0.801$	$r = -0.451$ $P = 0.164$
Pulse pressure (mmHg)	$r = 0.423$ $P = 0.02^{*}$	$r = 0.683$ $P = 0.021^{*}$
Crescents	$r = 0.069$ $P = 0.717$	$r = -0.190$ $P = 0.651$
Proteinuria	$r = 0.188$ $P = 0.321$	$r = 0.548$ $P = 0.160$
SLEDAI	$r = 0.430$ $P = 0.110$	$r = -0.200$ $P = 0.704$
C3	$r = -0.121$ $P = 0.525$	$r = -0.108$ $P = 0.799$
C4	$r = -0.156$ $P = 0.411$	$r = 0.323$ $P = 0.435$
Anti-dsDNA	$r = -0.255$ $P = 0.174$	$r = 0.262$ $P = 0.531$
Urine RBCs	$r = 0.108$ $P = 0.570$	$r = -0.429$ $P = 0.289$

$^{*}P < 0.05$ and $^{**}P < 0.01$ were considered to be statistically significant.

Discussion

Lupus nephritis is a major cause of morbidity and mortality in SLE. It is the most frequent secondary glomerular disease. A variety of histopathological lesions of renal vessels as well as a number of distinct clinical syndromes related to vascular damage may occur in SLE. These renal vasculopathies include vascular immune complex deposition, non-inflammatory necrotizing vasculopathy, TMA and true renal vasculitis. All these vascular changes in SLE indicate poor prognosis and higher risk of progression to end-stage kidney disease [1, 21].

Glucocorticoid combined with intermittent intravenous cyclophosphamide pulse has been used as the standard therapy for LN. Although many clinical trials demonstrated that long-term cyclophosphamide therapy improved renal outcome, it is still unfavourable to a significant proportion of patients with LN. MMF is a selective inhibitor for B and T lymphocytes [22]. Hu *et al.* [23] performed a follow-up study, and reported that MMF was more effective in controlling the clinical activity of diffuse proliferative lupus nephritis and renal VLS. The above data support the fact that MMF is more suitable in treatment for lupus nephritis with renal VLS. Therefore, measuring the numbers of CECs might be a useful tool in diagnosing the disease and justifies therapy efficacy in LN patients.

CECs were first found in the blood over 30 yrs ago. In the past 30 yrs, the numbers of CECs have been measured in both normal individuals and patients with various pathological conditions [24, 25]. However, these reports are diversified, not only due to different diseases studied, but also because of different methods of isolation and detection [16, 26]. In 1991, George *et al.* [27] unequivocally demonstrated CEC in whole blood using an endothelial cell-specific antibody. Subsequently, a number of different laboratories have identified CEC in whole blood using endothelial cell-specific monoclonal antibodies. A lot of studies showed that patients with SLE had higher numbers of CECs [16, 28, 29]. However, to our knowledge, there is no report of CECs in LN patients. Therefore, this is the first study on CECs in LN with VLS.

Endothelial contribution to human vascular disorders is difficult to investigate, due to the paucity of non-invasive methods and of specific endothelial markers. CECs might be used as a surrogate marker for the study of VLS. Our study showed that normal adults have a small number of CECs in peripheral blood (14.7 ± 2.0 cells/ml). The number of CECs in LN patients without VLS was 20.7 ± 2.9 cells/ml. Although the number of CECs in LN patients without VLS was increased in contrast to that of control, the difference was not statistically significant ($P = 0.78$). This is in contrast to the results of Clancy *et al.* [16]. Their study demonstrated that elevated levels of CEC were observed in patients with active SLE [16]. One possible explanation for the discrepancy is that the patients in our study included a group of LN patients in remission. The CEC numbers in LN patients with VLS (62.0 ± 7.3 cells/ml) were significantly increased compared with those of LN without VLS and control subjects ($P < 0.01$).

Renal TMA is an uncommon vascular complication of SLE. Renal TMA in SLE has been shown to have deleterious effects on long-term renal function and overall survival of patients. Microvascular endothelial damage, during which the endothelium is usually swollen or denuded, is the histopathological hallmark of TMA. Sometimes, there is marked luminal narrowing or total occlusion by intraluminal, subendothelial or medial accumulation of eosinophilic, fuchsinophilic material [30]. Therefore, in analogy to vasculitis, damaged endothelial cells undergo detachment from the basement membrane and become detectable in peripheral

blood in patients with TMA. To evaluate the relationship between increased CECs in LN and the degree of VLS, the patients with VLS were divided into two groups: patients with TMA ($n = 8$) and without TMA ($n = 22$). The results showed that CEC number of both these groups were significantly higher than those of the control. The numbers of CECs in the patients with TMA increased significantly higher than those without TMA ($P < 0.01$). The results suggested that CECs might reflect the severity of vascular damage in LN patients.

In the study of Erdbruegger *et al*. [31], the authors described CECs as a novel marker of endothelial damage in patients with TMA and found that cell numbers were markedly elevated in patients compared with healthy controls. Their results also showed that CEC numbers decreased significantly after four treatments of plasma exchange in patients, who improved clinically. These findings suggested that number of CECs could be used to evaluate therapy effect.

Previous studies have demonstrated that the functional status of CEC was different between patients and controls. Study of Clancy *et al*. [16] suggested that the activated phenotype of CEC might be capable of further potentiating vascular injury by the production of inflammatory and pro-thrombotic mediators and engaging in heterotypic aggregation with neutrophils or platelets. Mutin [25] also reported that CECs were not apoptotic. Whether the increased CECs in our patients with VLS were in activated state or apoptotic remains to be elucidated.

Significant correlation between CECs and blood pressure was found in some studies [32]. However, several studies have shown increased numbers of CECs in cardiovascular diseases and its risk factors, such as unstable angina, acute myocardial infarction and stroke, but no change in essential hypertension [33]. In the present study, there were no significant correlations between CECs and systolic blood pressure, diastolic blood pressure and mean blood pressure. Therefore, we did not think that elevated CECs were secondary to high blood pressure.

Our study suggested that numbers of CECs were indicative of the presence and severity of endothelial injury. Monitoring the CECs might provide a new evidence to guide the diagnostic work-up (reference range of controls: 14.7 ± 12.5, CEC value above the reference range: LN without VLS 20% *vs* LN with VLS 76.7%), assess the prognosis and evaluate treatment options. Prospective studies will be conducted to apply CEC number as a biomarker to evaluate the diagnosis, prognosis and therapeutic effect of medicine for LN patients with renal VLS.

Rheumatology key messages

- CECs may be used as a potential marker for vasculopathy in lupus nephritis.
- Dynamic observations of CEC number can provide evidence for disease diagnoses.
- CECs serve as the parameter of pathological changes and therapy efficacy in LN patients.

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