Low circulating level of CD133+KDR+cells in patients with systemic sclerosis

M.Y. Mok[1], K.H. Yiu[2], C.Y. Wong[2], J. Qiuwaxi[2], W.H. Lai[2], W.S. Wong[1], H.F. Tse[2], C.S. Lau[1]

[1]*Division of Rheumatology & Immunology, and* [2]*Division of Cardiology, Department of Medicine, Queen Mary Hospital, University of Hong Kong, Hong Kong.*

Mo Yin Mok, FRCP
Kai Hang Yiu, MRCP
Ching Yuen Wong, MBBS
Jianati Qiuwaxi, MD
Wing Hon Lai, MPhil
Woon Sing Wong, FRCP
Hung Fat Tse, FRCP
Chak Sing Lau, FRCP

Please address correspondence and reprint requests to:
Dr Mo Yin Mok,
Division of Rheumatology & Immunology,
Queen Mary Hospital,
University of Hong Kong,
Hong Kong.
E-mail: temy@hkucc.hku.hk

Received on March 25, 2010; accepted in revised form on July 7, 2010.

Clin Exp Rheumatol 2010; 28 (Suppl. 62): S19-S25.

Key words: disease activity, endothelial dysfunction, endothelial progenitor cells, systemic sclerosis

Competing interests: none declared.

ABSTRACT

Background. *Results of previous studies on the level of circulating endothelial progenitor cells (EPCs), which are involved in vascular repair, in scleroderma (SSc) patients have been controversial.*

Objectives. *To enumerate circulating EPC subsets and to examine their relation with endothelial dysfunction, biochemical markers of endothelial injury and vascular outcome in SSc patients.*

Methods. *Enumeration of circulating CD34+KDR+ and CD133+ KDR+ EPCs was performed by flow cytometry. Endothelium-dependent vasodilation was evaluated by changes in flow-mediated dilation (FMD%) in the brachial artery. Serum level of vascular endothelial growth factor (VEGF) was measured by enzyme linked immunosorbent assay.*

Results. *SSc patients (n=52) were found to have significantly lower CD133+KDR+EPCs (3.0 vs. 7.0/μl, p<0.001) as well as FMD% (4.8% vs. 7.8%, p<0.001) compared with age- and sex-matched controls (n=52). Among patients who had no concomitant cardiovascular risk factors (n=28), CD133+KDR+ EPC level was significantly lower than controls (3.8 vs. 7.3/μl, p=0.001) and correlated modestly with FMD% (r=0.29, p=0.03). Disease duration was the only determining factor identified for circulating CD133+KDR+ EPCs (p=0.03) by logistic regression analysis. Levels of serum VEGF (p=0.92) and KDR expression were not different between patients who had early and intermediate/late disease. Circulating CD34+KDR+ EPCs was not different between SSc patients and controls and did not correlate with any clinical or biochemical parameter.*

Conclusion. *Lower circulating CD133+KDR+ EPC subset was found in SSc patients and correlated with impaired endothelium-dependent vasodilation in patients without cardiovascular risk factors suggesting a potential role of deficient EPC recruitment contributing to endothelial dysfunction in this disease.*

Introduction

Systemic sclerosis (SSc) is a connective tissue disease that is characterised by Raynaud's phenomenon (RP), sclerodermatous skin changes and internal organ fibrosis. Microvascular abnormalities are common in SSc (1) and are believed to be the *sequelae* of vascular injury secondary to inflammatory immune processes, ischaemia-reperfusion injury and imbalance between coagulation and fibrinolysis (2). Markers of endothelial activation and damage such as soluble vascular cell adhesion molecule-1 (sVCAM-1) have been found to be elevated in serum of SSc patients (3, 4). Endothelial dysfunction reflected by impaired endothelium-dependent flow mediated dilation (FMD) has previously been demonstrated in SSc patients (5, 6).

Endothelial progenitor cells (EPCs) are bone marrow-derived endothelial precursor cells that contribute to vascular repair (7). This process of rapid endothelialisation of denuded vessels and collateral vessel formation is mediated by vascular endothelial growth factor (VEGF), a potent angiogenic factor that binds to its receptors VEGFR-1 and VEGFR-2 (alternatively known as kinase insert domain receptor (KDR)) on EPCs leading to their mobilisation from bone marrow and subsequent differentiation into mature endothelial cells (8, 9). Deficient recruitment and defective function of EPCs have been reported in cardiovascular conditions including myocardial infarction and associated risk factors (10, 11). However, the role of EPCs in the pathogenesis of

SSc has not been well characterised. There are controversies with regard to increased or decreased circulating EPC levels in SSc patients compared to controls (4, 12-15). In this study, we sought to enumerate circulating EPC subsets and to examine their relationship with endothelial dysfunction evaluated by FMD of the brachial artery, biochemical markers of vascular injury and vascular outcome in our cohort of SSc patients.

Methods

Patients

The study was approved by the Institutional Review Board of The University of Hong Kong/ Hong Kong West Cluster of the Hospital Authority. A written informed consent was obtained from all participating subjects according to the Declaration of Helsinki. Consecutive SSc patients were recruited from a University affiliated rheumatology clinic. Age- and sex- matched healthy controls were recruited from staff clinic. Subjects who had symptomatic cardiovascular or cerebrovascular diseases were excluded from study. SSc was designated as limited (lSSc) or diffuse (dSSc) depending on the extent of skin involvement according to LeRoy *et al*. (16). Disease duration was measured from onset of first non-RP manifestations. Early disease was referred to as <3years and <5years of disease duration for dSSc and lSSc respectively (13, 17). Baseline demographic data, serological results and medications were documented. Patients who had a history of severe vasospasm leading to the development of ischaemic digital ulcers and/or requiring the use of intravenous iloprost were regarded as having severe RP. Total disease activity score was graded according to the European Scleroderma Activity criteria which includes the total skin score, scleredema, digital necrosis, arthritis, reduced total lung carbon monoxide transfer factor (TLco), ESR>30mm/1st hour, hypocomplementaemia and deterioration in skin, vascular and muscular/articular condition in the month preceding the assessment (18, 19). According to these criteria, disease was considered active if the sum of the scores for these items was ≥3.

Echocardiography and lung function test, blood sampling and ultrasound scan of the brachial artery were carried out on the same day. Fasting venous blood sample was obtained to determine serum glucose and lipid levels, erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), sVCAM-1 and VEGF levels and circulating EPC counts. Lung function parameters measured included total forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1) and TLco. Less than 80% prediction of any of these parameters was considered abnormal. Cardiac involvement was defined as persistently elevated mean pulmonary arterial pressure (PAP) >35 mmHg by echocardiography on at least 2 occasions. Gastrointestinal involvement included clinical dysphagia, reflux oesophagitis requiring the use of proton pump inhibitors or small bowel bacterial overgrowth. Cardiovascular risk factors including current or past history of smoking, hypertension (blood pressure >140/90 or on antihypertensive agents), diabetes mellitus according to WHO definition and hyperlipidaemia (total cholesterol >5.2mmol/l or on anti-lipid agents) were assessed.

Measurement of flow-mediated dilation

Vascular ultrasound was performed with a high-resolution system (Agilent Sonos 5500; Philips, Andover, MA) and a 7.5-MHz linear array transducer by an experienced operator blinded to the studied subjects. All subjects were examined in the fasting state with vasoactive medications, caffeine and nicotine withheld for at least one day before the study. Longitudinal scans of the brachial artery were obtained at rest. FMD was induced by inflation of a pneumatic tourniquet placed on the forearm to a pressure of 250mmHg for 5 minutes. The cuff was then released, and serial imaging was recorded for 5 minutes. FMD% was defined as the percentage change in brachial artery diameter from baseline (20, 21).

Flow cytometry

Circulating EPCs were determined by the expression of surface markers CD34+, CD133+ and KDR+ on mononuclear cells, and their numbers were measured by fluorescence-activated cell analysis of peripheral blood sample processed immediately after venesection with method as described previously (22). In brief, 100μl of peripheral blood and incubated with a phycoerythrin (PE)-conjugated monoclonal antibody against human KDR (Sigma, St Louis, USA) followed by a fluorescein isothiocyanate (FITC) conjugated anti-CD34 and CD133 antibodies (Beckman Coulter, Fullerton, USA). FITC-labelled anti-human CD45 antibody was used for differential gating during flow analysis. FITC-labelled IgG1a (Beckman Coulter) and PE-conjugated IgG2b (Becton Dickinson, Franklin Lakes, USA) served as isotypic controls for colour compensation. Analysis was performed with an automated fluorescence-activated cell counter (Elite, Beckman Coulter) in which 1,000,000 events were counted. The absolute counts and the percentages of all the measured components defined as the absolute cell counts divided by the lymphocyte counts were calculated. The intra-observer variability testing found an intra-class correlation coefficient of 0.9 (p<0.001).

Serum sVCAM-1 and VEGF levels

Plasma sVCAM-1 and VEGF concentrations were measured in SSc patients by commercial enzyme-linked immunosorbent assays (Biosource, USA) according to the manufacturer's recommendations. The intra-assay and inter-assay variability for sVCAM-1 kit were 4.4% and 6.4% respectively whereas those for VEGF kit were 4.7% and 8.1% respectively.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. Continuous variables were checked for normal distribution by histogram and probability plot. Normally distributed data was presented as mean±standard deviation (SD). Serum level of EPCs was expressed in median value as the distribution was highly skewed. Categorical data were presented as frequencies and percentages. Comparison between

Table I. Demographic characteristics of scleroderma patients (n=52).

Demographic characteristics	Mean ± SD (range)	Number (Percentage)
Age at study (years)	51.9 ± 12.5 (25-81)	
Age at onset (years)	40.6 ± 13.3 (15-71)	
Disease duration (years)	12.2 ± 9.3 (1-40)	
Number of patients with early disease		11/52 (21.2%) lSSc (n=7) dSSc (n=4)
Female : Male		49:3
SSc subset (diffuse : limited)		11:41
Symptomatology / organ involvement		
Total disease activity score, n>3	2.8 ± 1.7 (0-6.5)	22/52 (42.3%)
Interstitial lung disease		13/52 (25.0%)
Elevated Pulmonary arterial pressure		14/52 (26.9%)
Gastrointestinal involvement		21/52 (40.4%)
Severe Raynaud's phenomenon		19/52 (36.5%)
Treatment at the time of study		
Prednisolone	6.8 ± 3.1mg/day*	18/52 (34.6%)
Penicillamine		19/52 (36.5%)
Calcium channel blocker		35/52 (67.3%)
Angiotensin converting enzyme inhibitor		5/52 (9.6%)
Statins		1/52 (1.9%)
Risk factors of cardiovascular disease		
Ever Smoker		6 (11.5%)
Hypertension		4 (7.7%)
Hyperlipidaemia		14 (28.0%)
Diabetes mellitus		6 (11.8%)

*daily dose among prednisolone users.

groups was done by unpaired t test for normally distributed data or non-parametric Mann-Whitney test for non-normally distributed data. Correlations between clinical parameters were evaluated using Spearman's rank correlation tests. Linear regression analysis was used to evaluate predictive factors for CD133+KDR+ and CD34+KDR+ EPCs in SSc patients. Variables identified in univariate analysis with $p<0.1$ were analysed as independent variables with CD133+KDR+ or CD34+KDR+ EPCs as dependent variable. Bonferroni's correction was applied for multiple comparisons. *P*-values <0.05 were considered significant in this study.

Results

Clinical characteristics

Fifty-two SSc patients and 52 age- and sex-matched healthy controls (49 female and 3 male) were recruited. There were 41 lSSc and 11 dSSc patients. The mean±SD age of these patients was 51.9±12.5 (range 25-81) years compared to 53.1±10.2 (range 30-78) years for controls ($p=0.61$). Table I shows the clinical characteristics of these patients. The median disease duration of lSSc patients was similar to dSSc patients (9.0 *vs*. 7.0 years) ($p=0.13$). Eleven (21.2%) patients (7 lSSc and 4 dSSc) had early disease. The mean±SD disease activity score of SSc patients was 2.8±1.7 (range 0-6.5). Some patients had total skin score>20 (21.2%, n=11), scleredema (76.9%, n=40), digital necrosis (30.8%, n=16), arthritis (11.5%, n=6), decreased TLCO (25.0%, n=13), ESR >30mm/1st hour (51.9%, n=27), hypocomplementaemia (3.8%, n=2) and deterioration in skin (11.5%, n=6), vascular (13.5%, n=7) and muscular/articular (11.5%, n=6) conditions. Some patients were receiving low dose maintenance dose of prednisolone (6.8±3.1mg/day) for the treatment of interstitial lung disease (n=18) and calcium channel blocker for RP (n=35).

Depletion of circulating EPCs in SSc

SSc patients tended to have lower lymphocyte count (1.5±0.7 *vs*. 1.7±0.6 x10^9/l, $p=0.07$) than controls. There were significantly lower median circulating CD133+KDR+ percentage (0.23 *vs*. 0.40%, $p<0.001$) and count (3.0 *vs*. 7.0/μl, $p<0.001$) in SSc patients compared to normal. There was a trend of lower CD34+KDR+ (11.9 *vs*. 14.6/μl, $p=0.06$) EPC count with similar percentages (0.95 *vs*. 0.89%, $p=0.53$) in SSc patients compared to controls. The

Table II. Summary of endothelial dependent vasodilation and circulating EPC level in Scleroderma subsets and patients with different clinical features.

	*Number of circulating CD133+KDR+EPC / CD34+KDR+ EPC (/μl)		*p*-value	*FMD (%)		*p*-value
SSc subsets	Patient	Control**		Patient	Control**	
All SSc	3.0 / 11.9	7.0 / 14.6	<0.001 / 0.06	4.8	7.6	<0.001
lSSc (n=41)	2.3 / 11.0	7.3 / 13.0	<0.001 / 0.14	4.9	8.0	<0.001
dSSc (n=11)	4.6 / 15.0	5.8 / 18.0	0.10 / 0.24	4.1	6.6	0.06
Clinical features	Present	Absent		Present	Absent	
Early disease (n=11)	4.6 / 15.0	3.0 / 11.3	0.58 / 0.54	4.6	4.9	0.79
Elevated PAP (n=14)	3.8 / 8.6	3.0 / 13.5	0.24 / 0.08	4.6	4.8	0.85
Ischaemic digital necrosis	(n=16) 3.0 / 15.0	3.0 / 11.0	0.82/ 0.29	5.2	3.9	0.39

*Median value; **Age- and sex- matched to scleroderma patients; dSSc: diffuse scleroderma; EPC: endothelial progenitor cells; FMD: flow mediated dilation; FVC: forced vital capacity; lSSc: limited scleroderma; PAP: pulmonary arterial pressure; SSc: scleroderma

lSSc subset had significantly lower CD133+KDR+ (2.3 *vs*. 7.3/μl, $p<0.001$) but not CD34+KDR+ (11.0 *vs*. 13.0/μl, $p=0.14$) EPCs than healthy subjects. Circulating CD133+KDR+ (4.6 *vs*. 5.8/μl, $p=0.10$) and CD34+KDR+ (15.0 *vs*. 18.0/μl, $p=0.24$) EPCs were not different between dSSc patients and controls. There was no difference in CD34+KDR+ ($p=0.61$) but a trend of lower CD133+KDR+ ($p=0.08$) EPCs in lSSc patients compared to dSSc patients.

Impaired FMD% in SSc

Figure 1 shows the median FMD% of all SSc patients and the SSc subsets. The median FMD% of the brachial artery of SSc patients was significantly lower compared to controls (4.8 *vs*. 7.6%, $p<0.001$) suggesting impaired endothelium-dependent vasodilation. The FMD% of lSSc patients was significantly lower (4.9 *vs*. 8.0% $p<0.001$) but only a trend of lower FMD% was observed for dSSc patients (4.1 *vs*. 6.6%, $p=0.06$) compared to controls. The FMD% was not different between patients who had early and intermediate/late disease (4.9% *vs*. 4.6%, $p=0.79$) and was not related to medication use.

Elevated sVCAM-1 level in lSSc patients

lSSc patients were found to have significantly higher serum sVCAM-1 level than dSSc patients (25.4±13.1 *vs*. 14.8±8.6ng/ml, $p=0.01$) suggesting a higher state of endothelial activation. This discrepancy was not related to the different disease activity score of these subsets ($p=0.81$). Serum VEGF levels were not different between SSc subsets and between patients with early and intermediate/late disease.

Correlations between EPC count, endothelial dysfunction and markers of endothelial injury

Age was not shown to correlate with CD133+KDR+($p=0.27$) or CD34+KDR+($p=0.97$) EPCs. Circulating CD133+KDR+ ($r=-0.28$, $p=0.04$) but not CD34+KDR+ ($p=0.59$) EPCs in SSc patients were found to correlate modestly and inversely with disease duration (Fig. 2). The levels of expression of KDR on CD34+ ($p=0.38$) and CD133+ ($p=0.27$) cells between patients who had early and intermediate/late disease were similar. There were no significant correlations between the EPC subsets, FMD%, VEGF and sVCAM-1 levels. The FMD% of the brachial artery was found to correlate significantly with serum VEGF level in dSSc patients ($r^2=0.75$, $p=0.03$) (Fig.

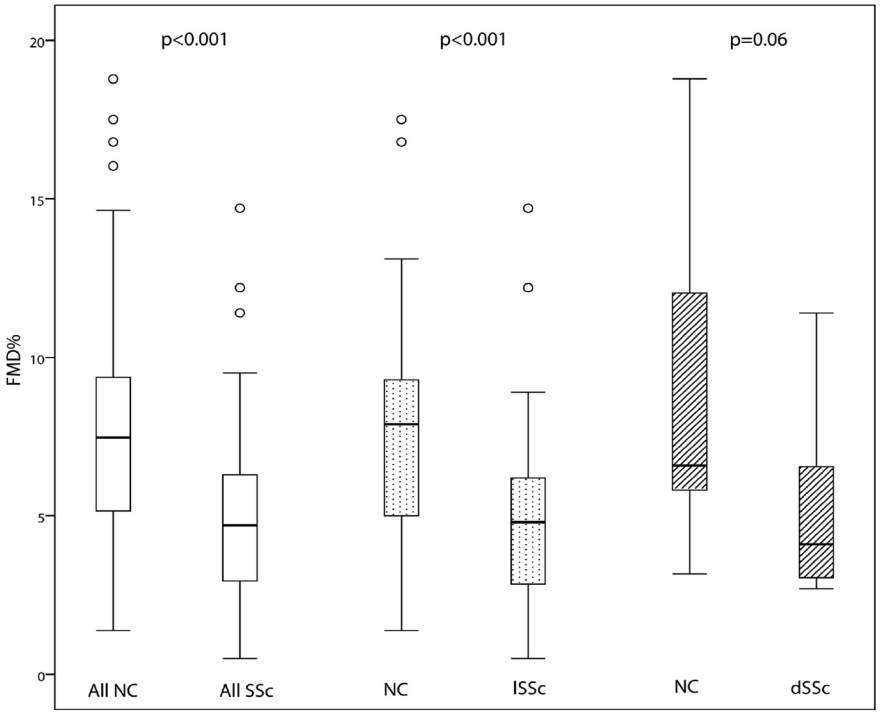

Fig. 1. Box plots showing flow-mediated dilation (FMD%) of the brachial artery in in age- and sex-matched healthy control subjects and all SSc patients and the SSc subsets.
dSSc: diffuse scleroderma; FMD%: percentage change in flow mediated dilation; lSSc: limited scleroderma: NC: normal control; SSc: scleroderma.

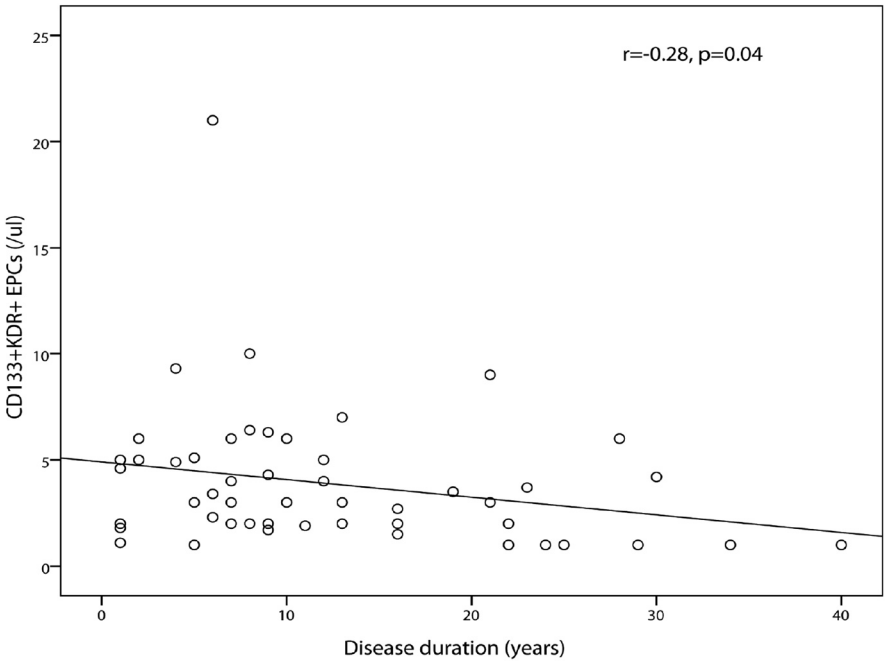

Fig. 2. Correlation between CD133+VEGFR2+ EPC count with duration from first symptoms in all SSc patients.

3) but not in all SSc patients (p=0.77) or the lSSc subset (p=0.22).

Correlation of EPCs with clinical and vascular outcomes

Total disease activity score was shown to correlate modestly and inversely with circulating CD133+KDR+ (r=-0.29, p=0.04) but not ESR (p=0.91) or CRP (p=0.63) in SSc patients, particularly the lSSc subset (r=-0.35, p=0.03). The EPC subsets were comparable between patients with (n=14) and without elevated PAP and between patients who had digital necrosis secondary to severe RP compared to those without. The EPC subsets were not found to be related to medications but higher CD133+KDR+ (4.0 *vs*. 2.3/μl, p=0.005) EPCs were found in patients on penicillamine who tended to have shorter median disease duration (8.5 *vs*. 11.5 years, p=0.09), compared to whose not on this medication.

EPCs in SSc patients without cardiovascular comorbidities

Twenty-eight SSc patients (7 dSSc and 21 lSSc) and their age- and sex-matched controls (n=28) were further analysed after elimination of those who had concomitant cardiovascular risk factors. These SSc patients were found to have significantly lower CD133+KDR+ EPCs (3.8 *vs*. 7.3/μl, p=0.001) but similar CD34+KDR+ EPCs (p=0.43) compared to controls. FMD% was shown to correlate modestly with CD133+KDR+ (r=0.29, p=0.03) but not CD34+KDR+ (p=0.84) EPCs among these patients. The EPC subsets were not found to correlate with total disease activity score, ESR or CRP. The EPC subsets were not different between patients who had low TL_{CO}, digital necrosis secondary to severe RP and elevated PAP compared to those who did not have these manifestations.

Multiple logistic regression analysis

Using CD133+KDR+ EPC count as dependent variable, variables identified in univariate analysis with p-value <0.1 including penicillamine, total disease activity, low FVC, disease duration and SSc subset were analysed as independent variables in multiple logistic regression analysis. Disease duration was the only factor found to be associated with EPC counts (p=0.03).

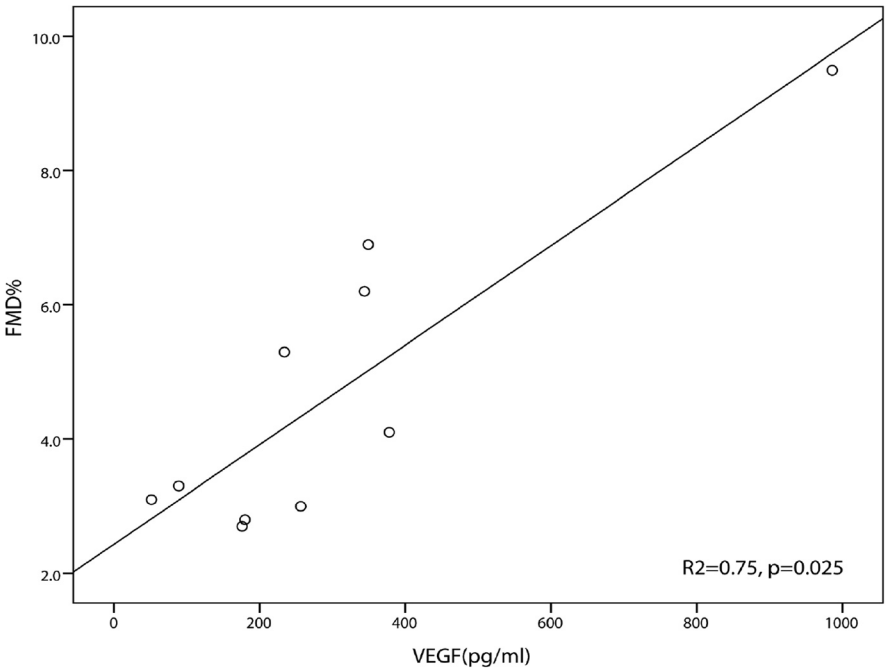

Fig. 3. Correlation between FMD% and VEGF in dSSc patients.

Discussion

Our study showed that the CD133+KDR+ EPC subset was significantly lower in SSc patients compared to age- and sex- matched controls. This was further confirmed by analysing only those patients who did not have concomitant cardiovascular risk factors. However, the CD34+KDR+EPC subset was only marginally lower than control subjects. Disease duration was the only significant determining factor for CD133+KDR+EPC count found in multiple logistic regression analysis in an inverse relationship. Indeed, previous studies have demonstrated higher EPC level in early SSc compared to long-standing disease (4, 12, 13, 15). Endothelial repair has been suggested to be more effective in early SSc disease (23). Reduced level of angiogenic factors (24, 25), impaired expression of VEGFR-1 by EPCs (26) have also been reported in SSc patients with long standing disease. However, our patients with early and intermediate/late disease had similar levels of serum VEGF and KDR expression on EPCs suggesting alternative mechanisms. Bone marrow derived mesenchymal stem cells have been reported to be decreased with impaired function in these patients (14, 17, 27).

We found significant correlation between circulating CD133+KDR+EPCs and FMD% among SSc patients who had no concomitant cardiovascular factors suggesting a potential role of deficient EPC recruitment in endothelial dysfunction. Endothelial dysfunction has been associated with cardiovascular risk factors (28) as well as SSc disease (5, 6, 29, 30). Elevated serum pro-inflammatory nitric oxide metabolites in SSc patients have also been suggested to contribute to endothelial dysfunction by oxidative stress (31, 32). Impaired endothelium-dependent vasodilation was predominantly found in our lSSc patients. This may be related to the small sample size of dSSc subjects or a lower state of endothelial activation reflected by lower serum sVCAM-1 in these patients. Nevertheless, the correlation between impaired endothelium-dependent vasodilation and VEGF level in the dSSc subset suggested endothelial dysfunction may account for the deficient release of angiogenic factors in some patients.

The association between EPCs and disease activity (4, 13, 14) or severity (15) remains controversial. We only found a weak correlation between CD133+KDR+ EPCs with total disease activity. EPC subsets were not shown to be related to vascular outcomes including elevated PAP or digital necrosis secondary to severe RP in our SSc patients. Furthermore, discrepancies have also been reported in EPC levels in SSc patients (12, 13, 15, 27). This may chiefly be accounted by variations in definition of EPCs together with different composition of cohorts comprising of patients with variable disease duration. Indeed, CD133+KDR+ and CD34+KDR+EPCs represent subgroups of putative EPCs in the circulation. More mature EPCs are negative for CD133 but positive for CD34 and KDR (33, 34). The CD34+EPCs population we measured may have included CD34 expressing mature endothelial cells derived by shedding. Thus, recently, the EULAR Scleroderma Trials and Research (EUSTAR) group recommended the use of three colour staining involving CD133, KDR and CD34 by flow cytometry in combination with a viability marker to define EPCs in peripheral blood (35). Since our research work was performed before the issue of the EUSTAR recommendation, further work is required using EPCs as defined by EUSTAR needs to be carried out. Our study has, nevertheless, raised a concern on the practicality of using EPCs as vascular biomarkers in clinical use in SSc because of their susceptibility to influence from concomitant cardiovascular risk factors. In addition, circulating EPC levels have also been found to vary during different phases of menstrual cycle (36). This may add to difficulties in studying EPCs in SSc patients as irregular menstrual cycle has been reported to be common among female patients (37).

In conclusion, we found decreased circulating CD133+KDR+EPCs in association with impaired endothelium-dependent vasodilation in SSc patients suggesting defective mechanism for vascular repair in this disease. Our study was limited by the small sample size with multiple comparisons for different outcomes. Studies into the role of EPCs in the pathogenesis of SSc may have treatment implications as direct infusion of EPCs (38) or mobilization of EPCs by statins (39) or granulocyte colony-stimulatory factor (40, 41) may offer potential treatment options for vasculopathy associated with this disease. Future studies with larger patient cohorts are warranted to examine for subset difference in circulating EPC counts, their relation with disease activity and various vascular outcomes in SSc patients.

References

1. KAHALEH MB: Raynaud phenomenon and the vascular disease in scleroderma. *Curr Opin Rheumatol* 2004; 16: 718-22.
2. LEROY EC: Systemic sclerosis. A vascular perspective. *Rheum Dis Clin North Am* 1996; 22: 675-94.
3. ANDERSEN GN, MINCHEVA-NILSSON L, KAZZAM E *et al*.: Assessment of vascular function in systemic sclerosis: indications of the development of nitrate tolerance as a result of enhanced endothelial nitric oxide production. *Arthritis Rheum* 2002; 46: 1324-32.
4. ALLANORE Y, BATTEUX F, AVOUAC J, ASSOUS N, WEILL B, KAHAN A: Levels of circulating endothelial progenitor cells in systemic sclerosis. *Clin Exp Rheumatol* 2007; 25: 60-6.
5. FREEDMAN RR, GIRGIS R, MAYES MD: Endothelial and adrenergic dysfunction in Raynaud's phenomenon and scleroderma. *J Rheumatol* 1999; 26: 2386-8.
6. LA CIVITA L, ROSSI M, VAGHEGGINI G *et al*.: Microvascular involvement in systemic sclerosis: laser Doppler evaluation of reactivity to acetylcholine and sodium nitroprusside by iontophoresis. *Ann Rheum Dis* 1998; 57: 52-5.
7. SHANTSILA E, WATSON T, TSE HF, LIP GY: New insights on endothelial progenitor cell subpopulations and their angiogenic properties. *J Am Coll Cardiol* 2008; 51: 669-71.
8. TAKAHASHI T, KALKA C, MASUDA H *et al*.: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999; 5: 434-8.
9. HRISTOV M, WEBER C: Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *J Cell Mol Med* 2004; 8: 498-508.
10. SHINTANI S, MUROHARA T, IKEDA H *et al*.: Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001; 103: 2776-9.
11. WERNER N, KOSIOL S, SCHIEGL T *et al*.: Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005; 353: 999-1007.
12. NEVSKAYA T, BYKOVSKAIA S, LYSSUK E *et al*.: Circulating endothelial progenitor cells in systemic sclerosis: relation to impaired angiogenesis and cardiovascular manifestations. *Clin Exp Rheumatol* 2008; 26: 421-9.
13. DEL PAPA N, COLOMBO G, FRACCHIOLLA N *et al*.: Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. *Arthritis Rheum* 2004; 50: 1296-304.
14. KUWANA M, OKAZAKI Y, YASUOKA H, KAWAKAMI Y, IKEDA Y: Defective vasculogenesis in systemic sclerosis. *Lancet* 2004; 364: 603-10.
15. AVOUAC J, JUIN F, WIPFF J *et al*.: Circulating endothelial progenitor cells in systemic sclerosis: association with disease severity. *Ann Rheum Dis* 2008; 67: 1455-60.
16. LEROY EC, BLACK C, FLEISCHMAJER R *et al*.: Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988; 15: 202-5.
17. DEL PAPA N, QUIRICI N, SOLIGO D *et al*.: Bone marrow endothelial progenitors are defective in systemic sclerosis. *Arthritis Rheum* 2006; 54: 2605-15.
18. VALENTINI G, DELLA ROSSA A, BOMBARDIERI S *et al*.: European multicentre study to define disease activity criteria for systemic sclerosis. II. Identification of disease activity variables and development of preliminary activity indexes. *Ann Rheum Dis* 2001; 60: 592-8.
19. VALENTINI G, BENCIVELLI W, BOMBARDIERI S *et al*.: European Scleroderma Study Group to define disease activity criteria for systemic sclerosis. III. Assessment of the construct validity of the preliminary activity criteria. *Ann Rheum Dis* 2003; 62: 901-3.
20. CORRETTI MC, ANDERSON TJ, BENJAMIN EJ *et al*.: Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 2002; 39: 257-65.
21. IP MS, TSE HF, LAM B, TSANG KW, LAM WK: Endothelial function in obstructive sleep apnea and response to treatment. *Am J Respir Crit Care Med* 2004; 169: 348-53.
22. LAU KK, CHAN YH, YIU KH *et al*.: Burden of carotid atherosclerosis in patients with stroke: relationships with circulating endothelial progenitor cells and hypertension. *J Hum Hypertens* 2007; 21: 445-51.
23. LUTTUN A, CARMELIET G, CARMELIET P: Vascular progenitors: from biology to treatment. *Trends Cardiovasc Med* 2002; 12: 88-96.
24. DISTLER O, DEL ROSSO A, GIACOMELLI R *et al*.: Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Res* 2002; 4: R11.
25. CHOI JJ, MIN DJ, CHO ML *et al*.: Elevated vascular endothelial growth factor in systemic sclerosis. *J Rheumatol* 2003; 30: 1529-33.
26. AVOUAC J, WIPFF J, GOLDMAN O *et al*.: Angiogenesis in systemic sclerosis: impaired expression of vascular endothelial growth factor receptor 1 in endothelial progenitor-derived cells under hypoxic conditions. *Arthritis Rheum* 2008; 58: 3550-61.
27. CIPRIANI P, GUIDUCCI S, MINIATI I *et al*.:

Impairment of endothelial cell differentiation from bone marrow-derived mesenchymal stem cells: new insight into the pathogenesis of systemic sclerosis. *Arthritis Rheum* 2007; 56: 1994-2004.

28. DREXLER H, HORNIG B: Endothelial dysfunction in human disease. *J Mol Cell Cardiol* 1999; 31: 51-60.
29. LEKAKIS J, PAPAMICHAEL C, MAVRIKAKIS M, VOUTSAS A, STAMATELOPOULOS S: Effect of long-term estrogen therapy on brachial arterial endothelium-dependent vasodilation in women with Raynaud's phenomenon secondary to systemic sclerosis. *Am J Cardiol* 1998; 82: 1555-7, A8.
30. LEKAKIS J, MAVRIKAKIS M, PAPAMICHAEL C *et al*.: Short-term estrogen administration improves abnormal endothelial function in women with systemic sclerosis and Raynaud's phenomenon. *Am Heart J* 1998; 136: 905-12.
31. MOK MY, FUNG PC, OOI C *et al*.: Serum nitric oxide metabolites and disease activity in patients with systemic sclerosis. *Clin Rheumatol* 2008; 27: 315-22.
32. ANDERSEN GN, CAIDAHL K, KAZZAM E *et al*.: Correlation between increased nitric oxide production and markers of endothelial activation in systemic sclerosis: findings with the soluble adhesion molecules E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1. *Arthritis Rheum* 2000; 43: 1085-93.
33. GEHLING UM, ERGUN S, SCHUMACHER U *et al*.: *In vitro* differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* 2000; 95: 3106-12.
34. FRIEDRICH EB, WALENTA K, SCHARLAU J, NICKENIG G, WERNER N: CD34-/CD133+/VEGFR-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circulation Res* 2006; 98: e20-5.
35. DISTLER JH, ALLANORE Y, AVOUAC J *et al*.: EULAR Scleroderma Trials and Research group statement and recommendations on endothelial precursor cells. *Ann Rheum Dis* 2009; 68: 163-8.
36. ROBB AO, MILLS NL, SMITH IB *et al*.: Influence of menstrual cycle on circulating endothelial progenitor cells. *Hum Reprod* (Oxford) 2009; 24: 619-25.
37. BHADAURIA S, MOSER DK, CLEMENTS PJ *et al*.: Genital tract abnormalities and female sexual function impairment in systemic sclerosis. *Am J Obstet Gynecol* 1995; 172: 580-7.
38. NEVSKAYA T, ANANIEVA L, BYKOVSKAIA S *et al*.: Autologous progenitor cell implantation as a novel therapeutic intervention for ischaemic digits in systemic sclerosis. *Rheumatology* (Oxford) 2009; 48: 61-4.
39. KUWANA M: Potential benefit of statins for vascular disease in systemic sclerosis. *Curr Opin Rheumatol* 2006; 18: 594-600.
40. HUANG P, LI S, HAN M, XIAO Z, YANG R, HAN ZC: Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. *Diabetes Care* 2005; 28: 2155-60.
41. KANG HJ, KIM HS, ZHANG SY *et al*.: Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. *Lancet* 2004; 363: 751-6.