ORIGINAL ARTICLES

Endothelial dysfunction in ankylosing spondylitis associated with reduced endothelial progenitor cell population

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Abstract

Background: Endothelial dysfunction, and cardiovascular (CV) morbidity and mortality have been documented in patients with ankylosing spondylitis (AS). Endothelial progenitor cells (EPCs) have reparative potential in overcoming the endothelial dysfunction and reducing cardiovascular risk.

Aim: To investigate the relationship between endothelial function and EPCs in patients with AS in a cross-sectional study.

Methods: Circulating EPCs (CD34+/CD133+) were isolated and quantified from peripheral blood samples of AS (n23) and healthy controls (n=20) matched for age and sex. Endothelium-depended vascular function, i.e. flow-mediated dilation (FMD), was assessed for all subjects. Disease activity was evaluated using Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). Functional ability was monitored using Bath Ankylosing Spondylitis Functional Index (BASFI). All subjects were free of any other known traditional CV risk factors.

Results: AS patients had depleted level of circulating %EPCs (0.028 ± 0.001% versus 0.045 ± 0.011%, P <0.001) and reduced FMD% (6.77 ± 2.15 versus 10.06 ± 0.55, P <0.001) than healthy controls. Circulating EPC population significantly positively correlated with FMD% (r 0.538, P = 0.008). Levels of CD34+/CD133+ putative cells showed a significant inverse correlation with disease duration (P = 0.01), BASDAI (P = 0.04), ESR (P = 0.002) and CRP (P = 0.007).

Conclusion: AS patients, free of any other known CV risk factors, demonstrated depleted levels of EPCs and reduced endothelial function. These alterations may cause further deterioration of endothelial function in AS patients. EPC would possibly serve as a novel therapeutic target for preventing cardiovascular risk in AS.

Keywords: Endothelial dysfunction, endothelial progenitor cells, ankylosing spondylitis, cardiovascular risk and inflammation

Introduction

Atherosclerosis is a chronic progressive vascular disease, characterized by plaque formation and damage of the vascular endothelium leading to the endothelial dysfunction.[1] Presence and extent of endothelial dysfunction is strongly associated with cardiovascular (CV) risk in rheumatic diseases.[2] Endothelial progenitor cells (EPCs) are a population of bone marrow-derived stem cells characterized by expression of CD34/CD133 and vascular endothelial growth factor (VEGFR)-2.[3] EPCs contribute to vascular homeostasis and may serve as a circulating pool of cells to improve endothelial dysfunction.[4-6] Patients with ankylosing spondylitis (AS) have higher risk for cardiovascular diseases and the corresponding risks noted as compared to healthy population are as follows:, 58% for valvular heart disease, 37% for ischemic heart disease, and 25% for cerebrovascular disease or stroke.[7] A prominent relation between endothelial dysfunction and depleted level of EPCs has been demonstrated in RA and patients with cardiovascular risk.[8-9] Decreased

levels of EPCs have been demonstrated in rheumatic diseases like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc), anti-neutrophil cytoplasmic autoantibody-associated vasculitis (AAV) and recently in PsA.[10-13]

We hypothesize that the depleted circulating EPCs population might contribute to endothelial dysfunction in patients with AS.[14] To test this hypothesis, we determined the level of EPCs and compared with endothelial function in patients with AS and in healthy subjects matched for age and sex. To avoid the potential confounding effects of other well-characterized traditional CV risk factors, we excluded individuals with obesity, diabetes, hypertension, dyslipidemia or a history of smoking.

Patients and Methods

Twenty three AS patients (mean age 35.81 ± 8.33; 6 female and 17 male) who fulfilled the 1984 modified New York diagnostic criteria for diagnosis of AS were recruited. Twenty age- and sex-matched (mean age 32.93 ± 10.16; 6 female and 14 male) healthy controls were recruited from clinic staff. Characteristics of patients and healthy controls are depicted in table 1.[15] All subjects enrolled in the study signed the informed consent approved by the institutional ethics committee of the Punjabi University Patiala, India. The study complies with the Declaration of Helsinki. All AS patients were on sulfasalazine 1-3 gm/day.

Patients who had obesity, diabetes, hypertension, dyslipidemia or a history of smoking were excluded. Another exclusion criterion was subjects who had chronic disease (chronic obstructive pulmonary disease, coronary artery diseases, stroke, liver cirrhosis, thyroid dysfunction, multiple sclerosis, human immunodeficiency virus, chronic renal failure and/or psychiatric disorders) or rheumatic diseases other than AS. Subjects who were taking any type of medication likely to affect vascular function receiving lipid lowering therapy, antihypertensive or antiaggregant drugs, nitrates, peroxisome proliferator-activated receptor

Table 1: Demographic, clinical and laboratory features of 20 matched controls and 23 patients with AS

Variables	Healthy controls (n=20)	Ankylosing spondylitis (n=23)	P-value
Sex (F:M)	6:14	6:17	-
Age (years)	32.9 ± 10.1	35.8 ± 8.3	0.27
Height (cm)	170 ± 8.5	159 ± 4.9	0.08
Body Weight (Kg)	65.1 ± 12.5	63.0 ± 12.5	0.12
BMI (kg/m2)	22.4 ± 3.0	23.9 ± 5.6	0.23
Systolic BP (mm Hg)	116.6 ± 9.7	119.5 ± 10.1	0.36
Diastolic BP (mm Hg)	74.8 ± 7.1	73.9 ± 11.6	0.43
Disease Duration (years)	-	8.8 ± 5.8	-
BASDAI	-	3.05 ± 2.23	-
BASFI	-	2.98 ± 2.79	-
ESR (mm 1sthr)	16.68 ± 4.54	26.04 ± 10.36	0.003*
CRP (mg/dl)	3.93 ± 1.10	12.76 ± 15.18	0.001*
Total cholesterol (mg/dl)	187.2 ± 24.3	198.6 ± 32.4	0.26
HDL cholesterol (mg/dl)	53.9 ± 11.6	61.5± 10.7	0.33
LDL cholesterol (mg/dl)	114.2 ± 22.3	119.7 ± 26.5	0.28
Triglycerides (mg/dl)	117.2 ± 16.1	108.0 ± 13.6	0.18
Fasting serum glucose (mg/dl)	96.1 ± 9.2	103.4 ± 13.4	0.43

F: female, M: male, BMI: body mass index, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, BASFI: Bath Ankylosing Spondylitis Functional Index, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, HDL: high density lipoprotein, LDL: low density lipoprotein, Data are presented as mean ± SD. *P <0.05

alpha agonists, angiotensin converting enzyme inhibitor, angiotensin receptor blocker or long-term systemic steroids were also excluded. Patients with a history using anti-TNF-α medications were also excluded.

All patients underwent clinical and biochemical assessment at the time of recruitment. Clinical, biochemical and vascular assessment (FMD, CIMT and EPC) were also carried out on the same day of recruitment after overnight fasting.

Assessment of EPC population through flow cytometry analysis

Peripheral blood samples were collected in the morning after overnight fasting for routine analysis. EPCs were quantified by fluorescence-activated cell sorting (FACS) analysis by caliber flow cytometry (Canto II; BD Biosciences, San Jose, CA). FACS analysis was performed using the following three markers:

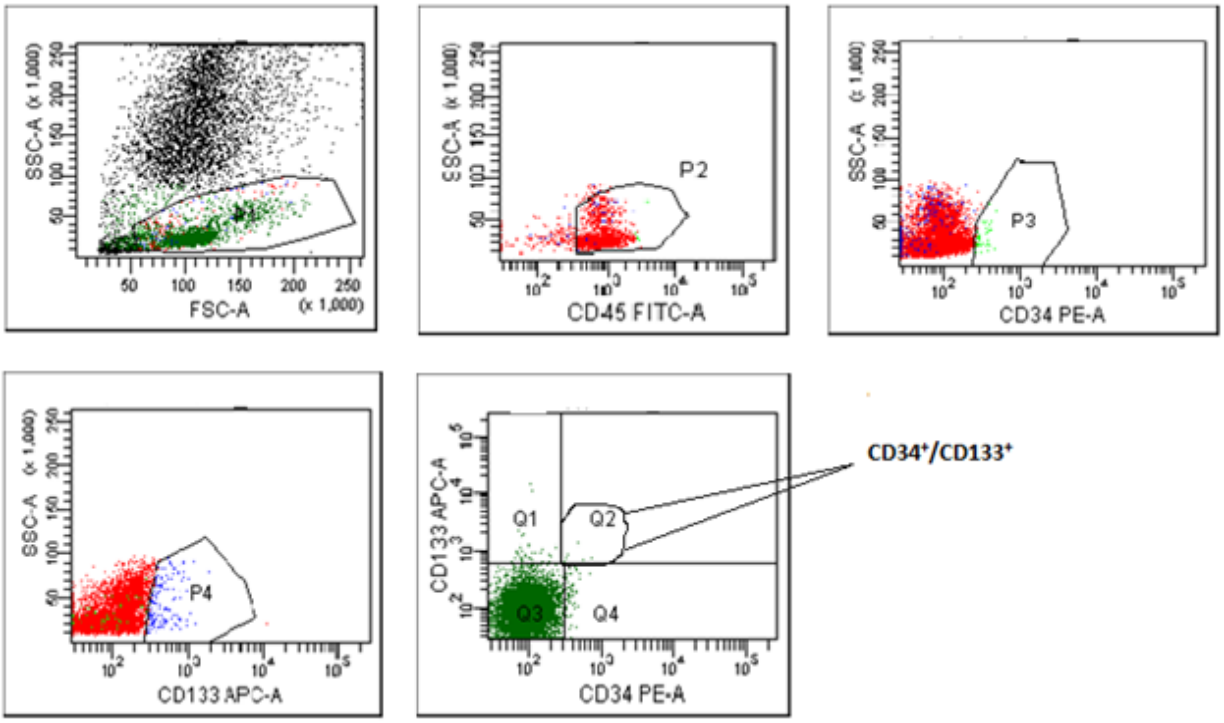
1. Fluorescein isothiocyanate (FITC) anti-CD45 (BD Sciences, San Jose, CA)
2. Phycoerythrin (PE) anti-CD34 (BD Sciences, San Jose, CA)
3. Allophycocyanin (APC) anti-CD133 (MiltenyiBiotec, BergischGladbach, Germany)

Peripheral blood in EDTA (200µL) was labeled with a panel of above mentioned antibodies and incubated for 1h at room temperature. After 1h incubation, red blood cells were lysed with ammonium chloride for 15 minutes at room temperature. Thereafter, cells were washed and resuspended in 500 µl phosphate buffered saline (PBS; Seromed). Appropriate analysis gates were used to enumerate total EPCs and to exclude debris (Fig. 1). Putative EPCs were defined as positive for anti-CD34 and anti-CD133. Cells stained with FITC, PE, and APC isotypic controls were used as negative controls. Around 100,000-250,000 cells per sample were acquired. Data were analyzed with Cell Quest software (Becton Dickinson). Results are expressed as % cells gated.[16-17]

Assessment of endothelial function

Subjects were studied in the morning, after fasting for at least 8 h, in a quiet room with controlled temperature with the subjects lying supine and their arms in a comfortable position. Flow-mediated dilation (FMD) was assessed

Fig.1: Fluorescence-activated cell sorting (FACS) analysis without the use of a viability marker



The first region (P1) sorts the immature mononuclear cells, the second (P2) identifies CD45 positive cells. The third (P3) identifies CD34 positive cells and the forth (P4) identifies CD133 positive cells. The fifth dotblot gates in quadrant 2 (Q2) identifies the cells positive for CD34 and CD133. Percentage of EPC cells was calculated as double positive cells on dotblot of CD34+ and CD133+.

using AngioDefenderTM (Everist Genomics, Ann Arbor, MI, United States) as per published guideline (www.everisthealth.com). The AngioDefender procedure is non-invasive and it neither employs ultrasound nor Doppler flow analysis. AngioDefender device runs through a series of inflations and deflations to analyze the endothelium's response to increased blood flow. The AngioDefender device uses a novel, proprietary software algorithm to analyze pulse wave data collected before and after brachial artery (BA) occlusion by an upper arm sphygmomanometric cuff. At the end of the AngioDefender testing procedure (~15 min), the maximal relative post-occlusion change in the diameter of the BA relative to baseline is calculated and expressed as a percentage of flow-mediated dilation (% FMD).[18] Mean of the two measures were considered for analysis. The intra- and inter-observer variability coefficients for the measurements of FMD were 1.50% and 2.25% respectively.

Clinical and biochemical assessment

Disease severity and functional ability were evaluated using Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Bath Ankylosing Spondylitis Functional Index (BASFI) respectively.[19, 20] Biochemical analysis included a complete blood count, liver function tests, renal function test, fasting blood sugar, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides (TG). Erythrocyte sedimentation rate (ESR) was measured using Westergren method, C-reactive protein (CRP) level using standard commercial kits and urine analysis to detect proteinuria, hematuria, and cellular casts.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Patients and control subjects were compared using unpaired Student's t-test for continuous variables and using chi-squared test for categorical variables. Spearman correlation coefficients were calculated for AS patients to study the relationship between EPC and FMD and disease variables. Statistical significance was assumed when a null hypothesis could be rejected at $P < 0.05$. Statistical analysis was performed using Sigmastat 5.5 for Windows 7.

Results

Demographic and clinical features of healthy controls and AS patients

The demographic and clinical characteristics of matched healthy controls and AS patients are summarized in table 1. Patients and healthy controls were not significantly different with regard to demographic characteristics (age, sex, and BMI). The corresponding values of ESR and CRP were significantly higher in AS patients (26.04 ± 10.36 and 12.76 ± 15.18) as compared to healthy controls (16.68 ± 4.54 and 3.93 ± 1.10).

Percentage EPC and FMD in AS patients and healthy controls

The population of circulating EPCs (CD34+/CD133+) was significantly lower in AS patients compared to healthy controls, (0.028 ± 0.009 versus 0.045 ± 0.011, $P < 0.001$). Reduced FMD (6.77 ± 2.15 versus 10.06 ± 0.55, $P < 0.001$) was also noted in the former group.

Spearman correlation analysis of EPC population with endothelial function, biomarkers of inflammation, and clinical characteristics of AS patients

In our study, EPC population in AS patients was positively associated with endothelial function ($r = 0.538$, $P = 0.008$) (Fig. 2), while ESR ($r = -0.592$, $P = 0.002$) and CRP ($r = -0.654$, $P = 0.007$), BASDAI ($r = -0.393$, $P = 0.04$) and disease duration ($r = -0.519$, $P = 0.01$) were negatively correlated with EPC population (Table 2). In AS patients, there is no statistically significant correlation between percentages of EPC population and age, BMI, functional activity of the disease (Table 2), and serum lipids (P 0.05, data not shown).

Discussion

The present study shows that AS patients, without clinically evident cardiovascular (CV) disease or associated risk factors, demonstrated a reduction in level of circulating EPCs (CD34+ CD133+) when compared to healthy subjects of same age and sex. The main and novel finding is that circulating EPC population depletion in AS is correlated with endothelial dysfunction.

AS is characterized by chronic inflammation and endothelial dysfunction, contributing to accelerated atherosclerosis and increased risk of CV morbidity and mortality.[21] The bone marrow-derived EPCs are important in repair process of endothelial damage and conferring protection against atherosclerotic vascular disease.[5-6] EPCs are better predictor of endothelial dysfunction and CV events than conventional CV risk factors.[22] EPC depletion has been reported in rheumatic disease patients and more recently in AS.[10-14] To the best of our knowledge, this is the first study to report potential association between the EPC

Fig. 2: EPC percentage in 23 AS patients showed a strong positive correlation with FMD% score

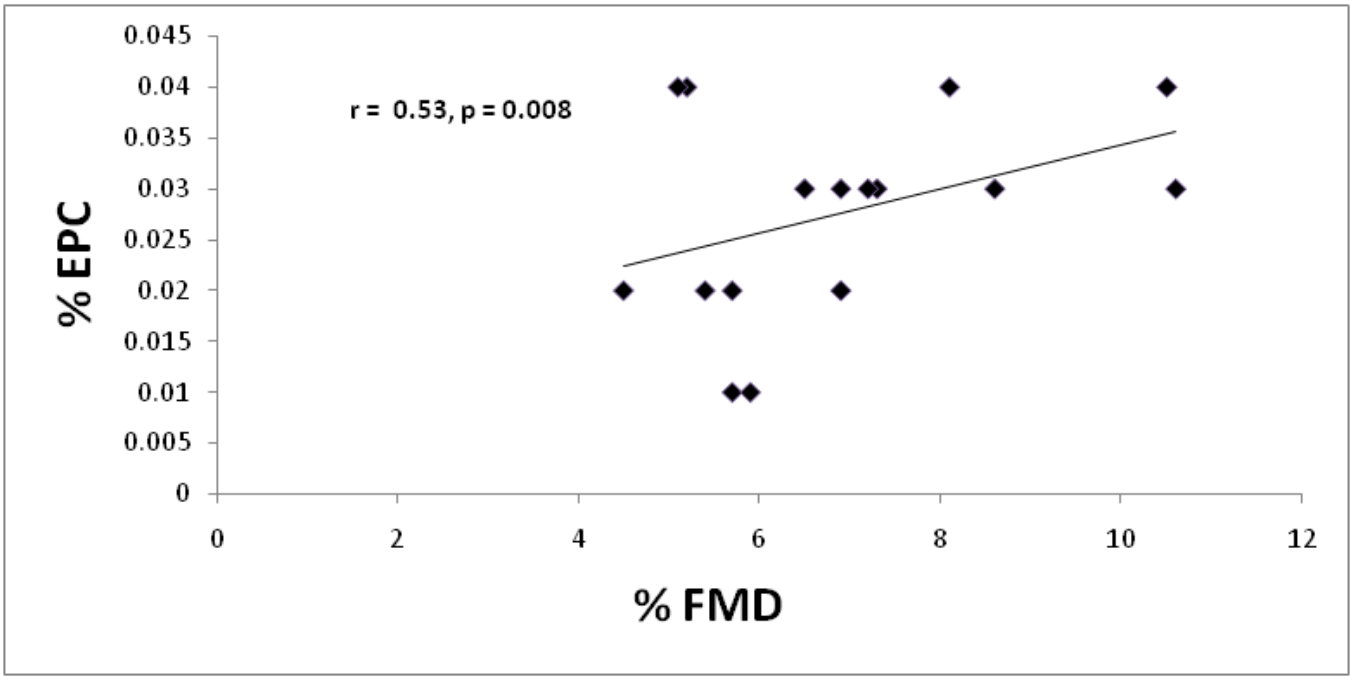


Table 2: Spearmen correlation analyses between EPC and clinical characteristics of AS patients

Variables	Correlation Coefficient	P-value
Age	-0.194	0.374
Disease duration	-0.519	0.01*
BMI	-0.167	0.281
ESR	-0.592	0.002*
CRP	-0.654	0.007*
BASDAI	-0.393	0.04*
BASFI	-0.335	0.118
FMD	0.538	0.008*

BMI: body mass index, ESR: erythrocyte sedimentation rate, CRP: C reactive protein, BASFI: Bath Ankylosing Spondylitis Functional Index, BASDAI: Bath Ankylosing Spondylitis Disease Activity Index, FMD: flow mediated vasodilatation

*Correlation is significant at the P<0.05 level (2-tailed)

and endothelial function in AS patients.

In this study, only three markers were used to determine circulating EPCs by flow cytometry because 200µl of peripheral blood is not sufficient to analyze other markers through direct analysis.[23] Vascular endothelial growth factor receptor-2 (VEGFR2) combined with CD34 and CD133 was not used to quantify EPCs because VEGFR2 is the surface marker for both EPCs and circulating endothelial cells (CECs).[23] CD133 is the sole surface marker of circulating EPCs and not of matured endothelial cells.[23] The result of significantly reduced putative EPCs

(CD34+/CD133+) in patients with AS adds to our previous finding showing a reduced circulating EPC population in AS patients.[14] An interesting observation of this study is a significant strong correlation between percentage EPC and endothelial function over the entire study group. These results indicate that the integrative regenerative capacity of circulating EPC may be relevant for this important state of vascular dysfunction and subsequent development of atherosclerosis. A recent study demonstrates a close tripartite relationship between bone erosion, circulating EPC counts, and FMD in patients with RA.[24] Herbrig *et al* have reported that endothelial dysfunction indicated by flow-mediated vasodilation correlated with lower number and abnormal function of circulating EPC in RA.[17] Endothelial function also closely correlates with the number of EPCs in coronary artery disease patients.[25] Collectively these data suggest that EPC provide a new mechanistic insight and a better predictor of vascular injury in patients with rheumatic disorders, which is independent of traditional CV risk factors.

ESR and CRP were high in AS patients, indicating the presence of inflammation. A significant inverse correlation between EPC and these biomarkers observed, suggesting that a systemic inflammatory state stimulates EPC mobilization in AS patients. A relationship between CRP (one of the best surrogate parameters of systemic inflammation) levels and circulating EPCs has been documented in patients with RA and coronary arteries disease patients.[8, 26, 27] CRP has the potential to contribute to vascular damage by acting on EPC apoptosis, proliferation, and differentiation.[28, 29]

EPC population also negatively correlated with disease duration and disease activity. These correlations suggest that EPC in AS is significantly affected by chronic inflammatory disease state. In our recent published study, we have reported a significant correlation between disease duration and disease activity.[14] Previously, Grisar *et al*. have found that lower level of hemangioblastic EPCs in RA negatively correlate with DAS28.[8] Another study in RA has shown a significant inverse correlation between the change in DAS28 and the change in EPC colony-forming units after anti-TNF-α treatment.[30]

Collectively, these results suggest that circulating EPC population is significantly impaired in the presence of disease activity and high inflammatory stimulation. Our data indicates that the loss of circulating EPCs in the circulation of AS patients may be involved in the pathogenesis of endothelial dysfunction, atherosclerosis, and increased CV morbidity and mortality. EPCs may improve risk stratification and offer novel tools for monitoring disease progression and response to therapy. They may also provide novel therapeutic target for CV risk in AS.

Competing interests
The authors declare that they have no competing interests.

Declaration of Interest
None

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