

ORIGINAL ARTICLES

Endothelial progenitor cells as cardiovascular surrogate markers in seropositive rheumatoid arthritis

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Abstract

Background

Bone marrow-derived endothelial progenitor cells (EPCs) confer protection against atherosclerotic vascular damage, thereby reducing the cardiovascular (CV) risk. Hence, the depletion of EPCs in rheumatoid arthritis (RA) is associated with enhanced CV risk. Patients with seropositive RA have increased risk of CV morbidity and mortality. The aim of this study was to investigate the endothelial progenitor cell population in seropositive and seronegative RA patients and its potential relationships with disease variables and proinflammatory cytokines.

Methods

Forty adult RA patients were classified into seropositive (n = 20) and seronegative (n = 20) groups based on the presence of rheumatoid factor (RF). EPCs (CD34+/CD133+) quantified by flow cytometry and proinflammatory cytokines (TNF- α , IL-6 and IL-1) were measured using standard ELISA kits, and disease activity by DAS28, CRP, and ESR.

Results

EPCs were significantly reduced in total RA and seropositive patients compared to seronegative subjects (P <0.05). Levels of proinflammatory cytokines (TNF- α , IL-6, and IL-1) were significantly (P = 0.01) higher in seropositive patients as compared to seronegative but in total RA patients level of IL-6 and IL-1 were significantly (P <0.05) higher as compared to seronegative patients. Significant negative correlation was observed between percentage of EPCs and IL-1, TNF- α , and disease activity in seropositive patients, between RF and TNF- α in seropositive patients, and between EPCs and DAS 28 in total RA patients.

Conclusion

EPC depletion is inversely correlated with disease activity, RF, and proinflammatory cytokines in seropositive patients, suggesting the pivotal role of inflammation in depletion of EPCs. EPC may serve as a probable therapeutic target for preventing CV disease in seropositive RA.

Key words: Endothelial Progenitor Cells, Proinflammatory cytokines, Rheumatoid Arthritis, Seropositivity.

Introduction

Endothelial progenitor cells (EPCs) are a population of circulating bone marrow-derived cells involved in tissue development, vascular repair and atherosclerosis, and are estimated to constitute about 0.01% of peripheral

mononuclear cells.¹⁻³ They are characterized by the ability to migrate into areas of increased angiogenesis and differentiate into mature endothelial cells. EPCs participate in ongoing endothelial repair, while an imbalance between endothelial damage and repair may lead to an increase in

cardiovascular (CV) events.⁴ Recent studies have identified EPCs as a better predictor of endothelial function and CV health.⁵ EPCs have been identified in synovial tissue of rheumatoid arthritis patients where they participate in angiogenesis.⁶ Reduced numbers and/or altered functions of EPCs have been recently demonstrated in patients with inflammatory rheumatic disorders like rheumatoid arthritis (RA), systemic sclerosis, systemic lupus erythematosus (SLE) and vasculitides associated with increased CV morbidity and mortality.⁷⁻¹⁰ Higher levels of serum IL-6 and TNF- α are associated with reduced number of EPCs and enhanced CV risk in RA patients.^{7, 11} CV disease continues to be the leading cause of morbidity and mortality in RA.¹² The mortality rate is higher in seropositive RA patients.¹³ However, the association between EPCs and proinflammatory cytokines has not yet investigated in seropositive RA patients. In view of these observations, we have compared the levels of the EPCs and proinflammatory cytokines in seropositive and seronegative RA patients.

Materials and methods

Patients

A total of 52 RA patients fulfilling the 2010 Rheumatoid Arthritis Classification Criteria for diagnosis and classification of RA were recruited.¹⁴ Of these, 12 were excluded: 8 were current smokers, 3 with diabetes mellitus and 1 had hypertension treated with propranolol and diuretics. Thus, 40 patients were included in the study. These patients were subgrouped on the basis of rheumatoid factor (RF) into seropositive (n = 20: 4 male, 16 female; mean age 41.35 ± 1.6 years, range 21-55) and seronegative (n = 20: 6 male, 14 female; mean age 40.80 ± 1.33 years, range 24-56) groups with active RA, defined by the presence of modified disease activity score (DAS28 >3.2). Exclusion criteria included: presence of conventional risk factors including hypertension, chronic kidney disease, diabetes mellitus, dyslipidemia, obesity, a past medical history of coronary artery disease and smokers, and receiving medications affecting EPC count such as statins or angiotensin receptor blockers/angiotensin converting enzyme inhibitors or any other CV medications.

Patients were on stable doses of DMARDs for at least 3 months before entering the study. Oral glucocorticoids (≤ 10 mg/day prednisone or equivalent) and non-steroidal anti-inflammatory drugs (NSAIDs) including cyclooxygenase-2 inhibitors were permitted if the doses were stable for ≥ 6 weeks. NSAIDs were discontinued at least 10 days before

vascular examination.

The study protocol was approved by the regional ethical research committee and was performed in accordance with the declaration of Helsinki and the code of Good Clinical Practice. All the patients provided written informed consent to participate after a full explanation of the study.

Assessment

Blood samples were drawn in the morning from all the subjects, after an overnight fasting, and the following variables were determined: complete blood count, rheumatoid factor (RF) titer determined by immunoturbidimetric method on Microlab Merck 300 analyzer (positive if patient had >20 IU), and fasting blood sugar by conventional methods using standard commercial kits.

Assessment of EPCs by flow cytometry

EPCs were quantified by fluorescence-activated cell sorting (FACS) analysis using Calibur flow cytometer (Canto III, BD Biosciences). Three-color analysis was performed using CD45 FITC (BD Sciences), CD34 PE (BD Sciences), and CD133 APC (Miltenyi Biotec) antibodies. Data were analyzed using CellQuest software (Becton Dickinson). Results are expressed as % cells gated.¹⁵

Assessment of inflammatory disease activity

The following measures were employed for the clinical evaluation of inflammation:

- Estimation of proinflammatory cytokines, i.e. TNF- α , IL-6 and IL-1, was done using standard ELISA kits (Diaclone SAS, France).
- Disease activity score of 28 joints (DAS28) was used to assess disease activity of 28 joints by a composite measure, which is a linear sum of four parameters including tender joint count (TJC), swollen joint count (SJC), patient global assessment of general health on a visual analogue scale (VAS), and ESR.
- ESR was measured by Westergren method and C-reactive protein (CRP) level was determined using standard commercial kits.

Statistical analysis

Test values are reported as mean \pm SEM. Spearman analysis was used to find the relationship between EPCs and proinflammatory cytokines. P value <0.05 was considered to indicate significant difference. Statistical

analysis was done using the Prism Software 5.04 version for Windows 8.0.

Results

Patient profile

The baseline demographic and clinical characteristics of the total RA patients, seropositive and seronegative RA are presented in table 1. The patients included in this study were all adults and were subgrouped by RF into seropositive (n = 20) and seronegative (n = 20) groups with established active disease.

Endothelial progenitor cells (EPCs)

On analysis of EPCs, it was found that the number of circulating EPCs, analyzed by fluorescence-activated cell sorting (FACS) analysis i.e. CD34/CD133-positive cells, were significantly decreased in total RA (CD34+/CD133+:0.019±0.001%) and seropositive (CD34+/CD133+: 0.018±0.001%) patients as compared to

seronegative (CD34+/CD133+: 0.022±0.001%) (P <0.05) RA patients. But the EPC population was reduced to a greater extent in seropositive patients (Table 1).

Inflammatory cytokines

Levels of proinflammatory cytokines i.e. TNF-α (P = 0.01), IL-6 (P = 0.04), and IL-1 (P = 0.04) were significantly higher in seropositive patients as compared to seronegative patients (Table 1). But in total RA patients, only IL-6 and IL-1 were significantly higher as compared to seronegative patients, suggesting that higher levels of proinflammatory cytokines are associated with reduced number of EPCs in seropositive RA patients as compared to total RA and seronegative patients.

Univariate analysis of EPCs with proinflammatory cytokines and disease activity measures in total RA, seropositive and seronegative patients

Univariate regression analysis was performed to determine

Table 1: The demographic and clinical characteristics of the seropositive and seronegative RA patients

Patient characteristics	RA (n = 40)	Seropositive RA (n = 20)	Seronegative RA (n = 20)
Sex (F:M)	30:10	16:4	14:6
Age (years)	41.05±1.4 (Range:21-56)	41.35 ± 1.6 (Range:21-55)	40.80±1.33 (Range:24-56)
Disease duration (years)	6.5±0.92	7.5±1.0	6.0±0.64
Height (cm)	160.05±4.5	159.4±4.9	160.7±4.95
Body weight (Kg)	63.60±12.56	64.07±12.58	63.13±12.51
BMI (kg/m ²)	24.58±3.80	24.43 ±5.2	24.73±2.91
ESR (mm 1st hr)	34.5±1.2*	40.30±1.7*	28.55±1.43
CRP (mg/dl)	10.4±2.06*	14.35±3.6*	6.31±0.52
DAS 28 score	3.7±0.15	3.83±0.17	3.59±0.12
TNF-α (pg/ml)	6.04±0.27	6.76±0.32*	5.72±0.25
IL-6 (pg/ml)	12.51±1.98*	14.97±2.03*	10.05±1.89
IL-1 (pg/ml)	145±19.1*	170.1±19.8*	120.90±18.6
EPC (%)	0.019±0.001*	0.018 ± 0.001*	0.022 ± 0.001
RF (IU/ml)	49.5±1.08*	68.7±1.85*	18.7±0.32

F- female, M- male, BMI- body mass index, ESR- erythrocyte sedimentation rate, CRP- C-reactive protein, DAS 28 - disease activity score of 28 joints, TNF-α -Tumor necrosis factor, IL-6- Interleukin-6, IL-1- Interleukin-1, EPC- Endothelial progenitor cell, RF- Rheumatoid factor, *P<0.05- Statistically significant versus seronegative

Table 2: Univariate analysis of EPCs with selected variables in seropositive and seronegative RA patients

Variables	EPCs in total RA		EPCs in seropositive RA		EPCs in seronegative RA	
	r	P value	r	P value	r	P value
Disease duration	-0.28	0.22	-0.30	0.18	-0.26	0.33
DAS 28	-0.44	0.04*	-0.46	0.03*	-0.05	0.81
ESR	-0.39	0.08	-0.38	0.09	-0.16	0.49
CRP	-0.28	0.18	-0.11	0.96	-0.29	0.20
IL-6	-0.22	0.21	-0.24	0.29	-0.02	0.92
IL-1	-0.37	0.10	-0.45	0.03*	-0.19	0.40
TNF-α	-0.22	0.18	-0.48	0.02*	-0.41	0.06
RF	-0.38	0.13	-0.46	0.02*	-0.21	0.18

DAS 28- Disease activity score of 28 joints, ESR- Erythrocyte sedimentation rate, CRP- C-reactive protein, IL-6- Interleukin -6, IL-1- Interleukin-1, TNF- α - Tumor necrosis factor, EPC- Endothelial progenitor cell, RF- Rheumatoid factor, *P <0.05, statistically significant

whether an association existed between EPCs and the inflammatory disease activity measures (DAS-28, ESR, and CRP), serological measure (RF titer), proinflammatory cytokines (IL-1, IL-6 and TNF- α) and levels of EPCs (Table 2). Among the markers analyzed, EPCs revealed a significant inverse correlation with DAS28, IL-1, TNF- α , and RF in seropositive patients and with DAS28 in total RA as compared to seronegative patients. In addition, another significant correlation was found between RF and TNF- α in seropositive RA patients.

Discussion

The present study is novel for several reasons and it is potentially the first one to determine the level of EPCs and to correlate them with disease activity, proinflammatory cytokines and RF in total RA, seropositive, and seronegative RA patients. Levels of EPCs were significantly reduced in seropositive and total RA patients as compared to seronegative patients in active RA. The decrease in the number of EPCs in RA patients is similar to that seen in other diseases with inflammatory component like SLE and chronic renal failure.^{9, 16} In this study an inverse correlation has been observed between levels of EPCs and inflammatory disease activity index (DAS28) and proinflammatory cytokines i.e. IL-1 and TNF- α and RF that underlines the link between these biomarkers in seropositive RA patients.

RA patients generally produce autoantibodies against

various 'self' substances such as IgG, RF, type II collagen (IIC), and nuclear antigens, suggesting an autoimmune nature of the disease.¹⁷ Moreover, various proinflammatory cytokines, including IL-1, IL-6, and TNF- α , are overexpressed in the joints of RA patients. As these cytokines can induce inflammation, promote synovial cell growth, and induce differentiation of osteoclasts, it is suspected that they may play an important role in the development of the disease.¹⁸ In this study, there was no statistically significant difference between the three groups in relation to age, disease duration, and DAS 28. In the present study, total RA and seropositive patients have shown increased level of ESR, CRP, and proinflammatory cytokines as compared to seronegative patients in active RA but the level was much higher in seropositive patients, which suggests that autoantibodies like RF play an important role in modulating the level of proinflammatory cytokines in RA. Results of a previous study have shown an increased level of ESR and CRP in seropositive RA patients as compared to seronegative patients.¹⁹

Recent evidence indicates that there is a close relation between inflammation and morphologic features of rapidly progressive carotid atherosclerosis, which suggests that elevations in inflammatory biomarkers might help in predicting the presence of atherosclerosis.²⁰ In RA, increased levels of circulating inflammatory mediators may cause activation and damage of endothelial cells, which contributes to endothelial dysfunction.²¹ Homing of EPCs

occurs at sites of endothelial damage, including inflamed synovium. Pools of bone marrow EPCs may get depleted over time. EPC mobilization from the bone marrow is also impaired owing to dysfunction of the bone marrow, in which impaired stromal cell function occurs.²² Previous study has shown an inverse relationship between EPC and IL-6 in RA patients.⁶ However, EPC number with respect to proinflammatory cytokines (IL-1 and TNF- α) in seropositive RA has not been studied to date. The present finding of a negative correlation between the EPCs and IL-1 and TNF- α level in seropositive RA may support the hypothesis that the individual microinflammatory state is related to the number of circulating EPC, thereby suggesting a potential role for these cytokines in EPC biology in seropositive RA as compared to seronegative and total RA patients. This is the first study that shows an inverse relationship between EPC and IL-1 and TNF- α in seropositive RA patients. The results are consistent with a previous study that has shown an inverse relationship between EPC and TNF- α in Kawasaki disease.²³

On the other hand, a significant correlation was found between RF and EPCs and TNF- α , which suggests that an enhanced inflammatory process may promote the development of endothelial dysfunction in seropositive RA patients as EPCs are reliable marker of endothelial damage and RF identified as an independent predictor of both endothelial dysfunction and CV disease.^{24, 25} Preliminary evidence indicates that CV disease-associated mortality risk is increased in both men and women with seropositive RA²⁶ and RF-positive inflammatory arthritis exhibit evidence of abnormal endothelial function, which is considered as a good predictor of future development of atherosclerosis.²⁷ In a recent study of RA, EPC populations have been found to be negatively correlated with RF and the present study results conform with the observation of increased risk of CVD in seropositive RA patients as compared to seronegative patients.²⁸

In the present study, the inflammatory disease activity (DAS 28) was comparable in all the three groups. The current findings of a negative correlation between the number of EPC and inflammatory disease activity measures like DAS 28 in seropositive and total RA patients may support the hypothesis that the severity of the disease in seropositive RA is associated with higher proinflammatory cytokine concentration, which may diminishes the EPC level. In line with the present study results, Grisar *et al.* (2005) recently demonstrated that EPCs were negatively correlated with

DAS 28 score in patients with RA.¹¹ Hence, more severe disease in seropositive patients with high proinflammatory cytokines and reduced EPC concentration suggests negative impact of disease severity on EPC biology.

The limitation of this study was the small sample size, yet we could achieve strong association between EPCs, proinflammatory cytokines and RF in seropositive patients. The correlations achieved in the present study are significant, but further studies with larger patient groups may provide a mechanistic explanation regarding EPCs and proinflammatory cytokines in seropositive RA patients.

Conclusion

To the best of our knowledge this is the first study that shows inverse correlation between EPCs and proinflammatory cytokines and RF in seropositive patients as compared to total RA and seronegative patients. These results suggest that EPC biology play a major role in seropositive patients as compared to seronegative patients and EPCs are better predictors of endothelial function and CV health.⁵ Thus, low peripheral EPC levels and high proinflammatory cytokines levels may significantly contribute to CV morbidity and mortality in seropositive patients. EPCs and proinflammatory cytokines are emerging as useful potential biomarkers in inflammatory rheumatic diseases for assessing CV morbidity and mortality and response to therapy. It will also be interesting to study the impact of different therapies especially anticytokine therapy with biological DMARD used in the management of RA on EPC biology in both seropositive and seronegative disease.

Competing interests

The authors declare that they have no competing interests.

Disclosure

None

Citation

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