Endothelial progenitor cells and colony-forming units in rheumatoid arthritis: association with clinical characteristics

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Objective. To compare levels of a range of endothelial progenitor cells (EPCs) and endothelial colony-forming units (CFUs) in control participants and RA patients, in addition to verifying whether levels of EPCs or CFUs are associated with clinical characteristics in RA patients.

Methods. Peripheral blood mononuclear cells (PBMCs) from 36 RA patients and 30 control participants were analysed by flow cytometry for EPCs defined by the expression of CD34/CD133, CD34/CD117, CD34/CD31, CD34/KDR and CD34/CD133/KDR. Endothelial cell colonies derived from culture of PBMCs were also assessed by CFU assay.

Results. No differences in levels of EPCs were observed in RA patients compared with controls. However, levels of EPCs were negatively associated with prognostic markers of poor disease status, but not cardiovascular (CV)-related risk factors. Furthermore, the majority of EPCs examined were negatively correlated with levels of RF. In contrast, CFU number was significantly reduced in RA patients compared with controls and was negatively associated with CV risk factors only.

Conclusion. These findings indicate that more informative than comparing changes in absolute levels of EPCs, the examination of their relationship with clinical characteristics of RA patients can reveal significant associations, which may provide important clinical insights.

Key words: Endothelial progenitor cells, Rheumatoid arthritis, Disease severity, Peripheral blood mononuclear cells, Rheumatoid factor, Prognostic markers, Flow cytometry, Colony-forming unit, Haematopoietic, Stem cell.

Introduction

RA is associated with increased cardiovascular (CV) morbidity and mortality that cannot be explained by traditional CV risk factors alone [1]. RA is also associated with enhanced angiogenesis [2]. In fact, the inflamed synovial membrane is characterized by intense vascularization, which is essential for synovial expansion and pannus formation [3]. Circulating endothelial progenitor cells (EPCs) represent a population of bone marrow-derived cells capable of repairing damaged vascular endothelium and initiating angiogenesis [4].

EPCs are primarily identified by the expression of combinations of cell-surface antigenic markers, including haematopoietic stem cell markers CD34, CD117, CD133 and/or the endothelial cell marker KDR (VEGF-R2/kinase insert domain receptor) in different combinations [4–7]. However, the precise origin, phenotypic identification and differentiative potential of EPCs, is still debated [8].

Changes in the absolute numbers of circulating EPCs have been reported in different pathological conditions and they have been sometimes linked to increased disease status and/or disease severity [5–7, 9]; however, data reported are not always consistent, due to the difficulty in accurately quantifying these cell populations. In RA, reduced levels of lymphocyte-derived EPCs have been detected in patients with active disease compared with controls [5]. Although another group could not confirm this reduction, they did show that RA patients presented with endothelial dysfunction that was negatively associated with number and function of PBMC-derived endothelial cell colonies in culture [6].

EPCs may serve as useful biomarkers of disease severity in several pathological conditions with CV involvement [5, 7, 9]. Furthermore, analysis of a range of putative EPC markers has revealed evidence of a close association between clinical characteristics and levels of EPCs in diabetic patients [7]. In the present study, we have extended this analysis to RA patients and have examined levels of cells expressing a range of EPC markers and endothelial colony formation in RA patients and their association between clinical characteristics of RA patients.

Patients and methods

Patients and controls

Blood samples were collected from patients referred to the Rheumatology Unit at the University Hospital of Siena. Thirty-six patients were diagnosed with RA according to the 1987 ACR revised criteria [10]. Inclusion criteria included patients presenting with moderate to high disease activity, reflected by a 28-joint disease activity score of ≥3.2 (DAS-28) [5, 6, 11]. All patients were positive for RF. Exclusion criteria included acute macro- or microvascular events, diabetes and statin treatment. Thirty volunteers without diabetes, CV, inflammatory or autoimmune disease served as healthy controls. This group was matched for age, gender, BMI and smoking. Ethics committee approval was taken and written informed consent was obtained from all subjects. Control and patient characteristics are summarized in the Supplementary Table 1 (available as supplementary data at Rheumatology Online).

Clinical characteristics and threshold values

To examine the impact of clinical characteristics associated with RA upon the level of EPCs and number of colony-forming units (CFUs), multivariate regression was performed. Threshold values were based upon medical guidelines or as indicated elsewhere [7, 9–14]. Threshold values included age < or ≥65 yrs; disease duration < or ≥10 yrs; BMI < or ≥30 kg/m2; RF < or ≥50 IU/ml; CRP < or ≥2.5 mg/dl; ESR < or ≥20 mm/1st h for men and < or ≥30 mm/1st h for women and DAS-28, < or ≥5.1. Male gender, previous or current smoker and presence of hypertension were considered as CV risk factors.

PBMC isolation and CFU assay

PBMCs were isolated from peripheral blood samples as previously described [7]. CFU assay was performed according to the method...
by Grisar et al. [5], with minor modifications. PBMCs (2 x 10^6) were plated on fibronectin precoated 24 well plates and cultured in endothelial growth medium (Cambrex, Milan, Italy) containing 5% FCS and penicillin (100 U/ml) streptomycin (100 g/ml, Gibco, Milan, Italy). CFUs were counted after 1 week. Characterization of CFUs for the expression of endothelial and progenitor surface markers, was performed by IF imaging.

IF imaging of CFUs

CFUs cultured in 24 well plates on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature (RT) then washed for 30 min (3 x 10 min) with PBS. CFUs were blocked with 5% goat serum/0.2% BSA in PBS (PBS/BSA) for 1 h at RT, then incubated with primary antibody in 2% BSA/PBS overnight at 4°C. Primary antibodies were as described for flow cytometry and included vWF (Sigma-Aldrich, Milan, Italy) (diluted 1:50, followed by secondary antibody at 1:1000 dilution (BD Biosciences, Franklin Lakes, NJ, USA), CD31 (diluted 1:100), acetylated low-density lipoprotein (AcLDL) (10 μg/ml for 2 h at 37°C), lectin (10 μg/ml for 1 h at 37°C), CD34 (diluted 1:30), CD117 (diluted 1:100) and CD133 (diluted 1:30). Following incubation with primary antibody, CFUs were washed with 0.2% BSA/PBS then incubated with secondary antibody diluted (as required) in 2% BSA/PBS for 1 h at RT and again washed with 0.2% BSA/PBS. CFUs were washed a second time with PBS (3 x 5 min), PBS decanted and one drop of Mowiol containing DABCO (Sigma-Aldrich) was added prior to covering with a coverslip. After coverslips had adhered and dried, IF was detected (x20 objective) using an Zeiss Axiosplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany). Negative controls included primary anti-mouse IgG and secondary antibodies.

Flow cytometry

EPCs were quantified by FACS, as previously described [7]. Briefly, PBMCs were analysed for the expression of the following cell-surface antigens: allopseudocyanin-conjugated CD34 phycoerythrin-conjugated CD117 (both from BD Biosciences), CD31 (Santa Cruz Biotechnology, SantaCruz, CA, USA), CD133 (Miltenyi Biotech, Bergisch Gladbach, Germany) and unconjugated KDR (Sigma-Aldrich) followed by FITC-conjugated secondary antibody (BD Biosciences). Identical IgG isotypes served as negative controls. Each analysis consisted of between 100 000 and 200 000 events.

Statistical analysis

Data are presented as mean ± s.e.m. Statistical analysis was performed using MedCalc software (Mariakerke, Belgium). Comparisons between two groups were compared by the Mann–Whitney test or Student’s unpaired t-test, where appropriate. Univariate correlation was assessed by Pearson’s correlation coefficient (r). Multivariate analysis was applied to examine associations between clinical characteristics and EPCs. P-values are two-tailed; n-values refer to the number of patients. P ≤ 0.05 was considered statistically significant.

Results

CFU number, but not circulating EPCs are decreased in RA patients

FACS analysis was performed on cells expressing EPC markers within the lymphocytic population, as shown in supplementary figure 1A (available as supplementary data at Rheumatology Online), in agreement with previous reports [5, 6, 9]. Of the five EPC populations analysed by FACS analysis, only CD34/CD31 and CD34/CD133/KDR-positive cells, were decreased in RA patients compared with controls, although they did not attain statistical significance (CD34/CD31: 0.15±0.02% versus 0.22±0.04%, P = 0.087 and CD34/CD133/KDR: 0.048±0.02% versus 0.07±0.01%, P = 0.07; see supplementary figure 1B, available as supplementary data at Rheumatology Online).

Endothelial colony formation has previously been used as an alternative method to detect endothelial progenitors in PBMCs in normal and diseased individuals, including patients with RA [4–8]. CFUs were obtained by culturing PBMCs, as detailed in Patients and methods section. The endothelial phenotype of cells forming CFUs was confirmed by staining with endothelial and progenitor markers. IF staining revealed the expression of endothelial markers CD31, vWF and acLDL/lectin and the progenitor markers CD34 and CD117, but not of CD133 (see supplementary figure 1C and D, available as supplementary data at Rheumatology Online). Comparison of the average number of CFUs measured in RA patients with that found in controls, revealed a reduction in CFU number in patients (7.1±1.7 and 13.6±2.9, respectively, P = 0.042; see supplementary figure 1E, available as supplementary data at Rheumatology Online), which is in agreement with data obtained by Grisar et al. [5].

Levels of EPCs but not CFU number are negatively associated with ESR and RF in RA patients

Univariate regression was applied to determine whether an association existed between the disease activity index (DAS-28 score) and serological markers (ESR, CRP and RF) and levels of EPCs and CFU number. Of the four markers analysed, only RF and ESR revealed a significant correlation between the EPC populations. Actually, out of the five EPC populations analysed, four were negatively correlated with RF (Fig. 1A–D) while ESR negatively correlated only with CD34/CD133/KDR-positive cells, (Fig. 1E). No correlation was observed with CRP and DAS-28 score values.

CFU number is associated with CV risk factors, whereas levels of EPCs are associated with parameters of poor prognosis in RA patients

We next examined the association between CV risk factors, CVD and elevated levels of serological markers and pharmacological treatment upon the levels of EPC combinations and CFU number (Table 1). Multivariate analysis revealed a significant association between CFU number and presence of CV risk factors [analysis of variance (ANOVA), P = 0.018]. On the other hand, CV risk factors and pharmacological treatment were not associated with levels of any EPC population. In contrast, the presence of CVD and elevated levels of serological markers were significantly associated with the number of CD34/CD117 (ANOVA, P = 0.04) and CD34/CD133/KDR-positive cells (ANOVA, P = 0.022).

Discussion

It has previously been reported that EPCs are decreased in active RA but not inactive RA [5]. EPCs have also been shown to be negatively correlated with disease activity in RA and in type 2 diabetes [5, 7, 9]. Here we show by multivariate analysis that factors indicative of poor outcome (i.e. levels of serological markers ESR and RF) are negatively associated with levels of circulating EPCs in RA patients, corroborating similar observations made in diabetic patients [7].

In recent years, the characterization of endothelial progenitor cells has remained controversial (see ref. [8] for detailed discussion). We therefore decided to analyse EPCs by FACS analysis, in addition to the CFU culture method [8]. Our findings revealed that the number of CFUs was reduced ~2-fold in RA patients compared with controls, confirming previous reports [5, 6]. In contrast, FACS analysis of absolute levels of five
different EPC populations did not reveal any significant difference between the two groups, in agreement with findings by Herbrig et al. [6] but at variance with results by Grisar et al. [5]. Therefore, it would appear that levels of circulating EPCs may not always easily discriminate between RA patients and controls. Here we report that multivariate analysis may, however, detect a correlation between serological markers, which are indicative of poor prognosis and levels of EPCs, which would suggest that EPC levels may be used to further define the progression of the disease. The impaired status of endothelial progenitor cells in RA patients is also supported by the observation that the number of CFUs was reduced in RA patients and negatively associated with CV risk factors in these patients.

Why the results obtained following analysis of EPCs by FACS or by the CFU methodology differ is not clear. For example, the CFU culture method is composed of a heterogeneous population of cells that can express endothelial and progenitor markers [15]. In contrast, EPCs quantified by FACS analysis are mainly derived from a homogeneous population of PBMCs, using a range of endothelial lineage and progenitor-specific surface markers. Both methods are distinctly different, measuring different cellular characteristics involving different cell types that can sometimes reveal similarities that may or may not reflect information specific to EPC biology [8, 15]. Therefore, comparison of data obtained following FACS analysis of EPCs and the CFU culture method requires careful interpretation, due to inherent differences involved.
In addition to comparing absolute levels of EPCs in RA patients and controls, examining the relationship or association between their levels and clinical characteristics in RA and other diseases can provide more specific information [5, 7, 9]. In fact, Grisar et al. [5] were the first group to show that EPCs defined by the expression of CD34/CD133/KDR-positive cells negatively correlated with DAS-28 score in patients with RA. We also looked for a similar association using a range of EPC marker combinations. However, in the present study, since all patients had already been pre-selected to have a high DAS-28 score, values were not sufficiently spread to permit an association by univariate regression. Unlike DAS-28 score however, RF and ESR values were wide-ranging throughout the cohort, therefore allowing analysis against levels of EPCs to be performed. Interestingly, our analysis revealed that ESR, but not RF, was negatively associated with CD34/CD133/KDR-positive cells; however, RF was shown to be negatively correlated with four other EPC populations examined.

RF is generally recognized as a marker of poor prognosis in RA patients. Moreover, it is also increasingly identified as an independent predictor of both endothelial dysfunction and CV disease [16, 17]. Likewise, EPCs are negatively associated with endothelial dysfunction and their levels are decreased in diseases with CV complications [5–7, 9, 18]. The inverse correlation observed between levels of circulating EPCs and RF, that we identified in RA patients in this study, is a novel association that further underlines the link between these two biomarkers and poor outcome status.

Taken together, these findings further underline the association between select combinations of EPCs and disease status, previously reported in diabetes, [7, 9] and now identified in RA. Although a first-phase examination of EPC levels by flow cytometry did not point towards a deficit in RA patients, further analysis by multivariate regression did reveal a significant association between factors indicative of poor prognosis and levels of EPCs. The finding that RF was negatively associated with four of the five EPCs examined prompts further investigation and would be best assessed in a longitudinal prospective study. Lastly, in this study, we utilized both FACS analysis and the CFU methods. Since neither method has yet been successfully proven to identify the ‘true’ EPC, interpretation of these data remains complex. Studies testing the angiogenic capacity of candidate or ‘putative’ EPCs in *in vivo* inflammatory models should help to improve our understanding of the role and identity of this elusive cell population in the context of RA.

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### Supplementary data

Supplementary data are available at Rheumatology Online.

### References