Concise Report

Circulating endothelial cells and rheumatoid arthritis: relationship with plasma markers of endothelial damage/dysfunction

Will Foster¹, Eduard Shantsila¹, David Carruthers², Gregory Y. H. Lip¹ and Andrew D. Blann¹

Objectives. RA is associated with endothelial cell dysfunction (ECD) and increased cardiovascular mortality and morbidity. Circulating endothelial cells (CECs) are a novel marker of severe endothelial damage. We hypothesized altered CECs in patients with RA compared with community controls (CCs) and hospital controls (HCs, with diabetes and hypertension) correlate with established plasma markers of inflammation and of ECD.

Methods. CECs (CD146-immunobeads), von Willebrand factor, soluble E-selectin, soluble intercellular adhesion molecule-1, soluble vascular endothelial adhesion molecule-1 (sVCAM, all ELISA) and C-reactive protein (CRP, immunonephelometry) were measured in 57 patients with RA, 45 CC and 23 HC patients.

Results. CECs in RA [median/interquartile range 8 (5–13.5) cells/ml] were elevated compared with either CC [4 (2–8.5) cells/ml] or HC [4 (1–8) cells/ml] (both P < 0.001). Levels of CECs did not correlate with other markers of ECD or of inflammation but did correlate inversely with sVCAM.

Conclusion. Evidence of endothelial damage in the form of mildly increased numbers of CECs is present in RA and is independent of plasma markers of inflammation and of ECD.

Key words: Rheumatoid arthritis, von Willebrand factor, C-reactive protein, Circulating endothelial cells, Soluble E selectin.

Introduction

RA is associated with increased cardiovascular mortality and morbidity, possibly related to thrombosis, endothelial damage and dysfunction, a pathology that predicts those individuals at increased risk of adverse cardiovascular events, and that may also be used as a surrogate marker for measuring beneficial effects of interventions [1, 2]. Indeed, the ‘vascular’ hypothesis of RA is long established and is supported by abnormal plasma markers of the endothelium including von Willebrand factor (vWF) and soluble E-selectin (sE-selectin) [3–5]. Recent interest has been directed towards the quantification of ex-luminal circulating endothelial cells (CECs). Their presence indicates shedding of the damaged intimal endothelial layer into the blood resulting in small numbers of these cells in health, with higher numbers in disease states that accelerate the vasculopathological process such as cardiovascular disease, where CEC numbers correlate with levels of vWF [6, 7]. Increased numbers of CECs are present in the other systemic inflammatory diseases of lupus and vasculitis [8, 9].

Whilst endothelial damage/dysfunction has been demonstrated in a wide range of patients with RA [10, 11], it is unclear if there are differences in levels of CECs. Moreover, it is not clear which mechanisms are involved in the generation of CECs, although the action of excess inflammatory cytokines (such as tumour necrosis factor) is possible [8, 9, 12]. Accordingly, we hypothesized that there would be raised numbers of CECs in the blood of patients with RA, and that such raised levels would correlate with levels of established plasma markers of inflammation and vascular damage [i.e. vWF, sE-selectin, soluble intercellular adhesion molecule-1 (sICAM) and soluble vascular endothelial adhesion molecule-1 (sVCAM) (the latter two both known to be abnormal in the connective tissue diseases)] [13, 14] and C-reactive protein (CRP). We tested our hypothesis in a simple cross-sectional study, comparing patients with RA with two control groups: hospital patients (HCs) attending outpatient clinics for the risk factors for atherosclerosis (i.e. diabetes and concurrent hypertension), and community controls (CCs), some of whom have mild disease not requiring hospital outpatient care.

Methods

Subjects

We recruited 57 patients with RA, 45 CCs and 23 diabetic hypertensives as an HC group. All RA patients had a diagnosis of RA made by a consultant rheumatologist according to the standard ARA criteria [15]. The disease activity score (DAS-28) was quantified according to established criteria [16]. HCs were hypertensive type 2 diabetic patients recruited from a general medical outpatient clinic. Hypertension was defined as systolic/diastolic blood pressures >160/100 pre-treatment or >140/90 on treatment. Diabetes was defined as patients with a fasting blood glucose exceeding 7 mmol/l or a random blood glucose exceeding 11.1 mmol/l supported by an oral glucose tolerance test. CCs were hospital staff and friends and relatives of patients, all of whom were free of diabetes and symptomatic cardiovascular, neoplastic or connective tissue disease by careful history and examination. This group was not attending hospital for treatment of symptomatic disease or its risk factors. Exclusion criteria for all subjects were atrial fibrillation, significant valvular heart disease, previous coronary artery bypass surgery, primary angioplasty for acute ST elevation myocardial infarction, infection or pyrexial illness, recent (<3 months) ischaemic stroke, chronic and systemic illnesses including renal failure (on supportive therapy), hepatic impairment and/or HRT.

Venous blood was obtained first into vacutainers with no anticoagulant to provide serum (for CRP, lipids, urea and electrolytes, soluble adhesion molecules, etc.), then into EDTA vacutainers for haematology and for CECs. Plasma and serum were obtained by centrifugation at 3000 r.p.m. (1000 g) for 20 min at 4°C. All aliquots were stored at −70°C to allow batch analysis of vWF, sE-selectin, sVCAM and sICAM. CECs were measured in whole
blood. The local research ethics committee approved the study which was conducted according to the Declaration of Helsinki. All subjects gave written informed consent before taking part.

**Laboratory**

CECs were measured by the immunobead–CD146 method fully described elsewhere [6, 8, 9]. Briefly, immunomagnetic beads (Dynabeads; Biocytex, Marseille, France) were coated with an antibody (sENDO) directed against human CD146. Whole blood and (4.5 ml) was collected into a vacutainer and mixed with an equal volume of sterile filtered phosphate-buffered saline (PBS), and 100 µl of a prepared immunobead suspension was added. After 45 min of rotary mixing to allow binding of beads to CECs, the beads and any bound cells were separated from the rest of the blood constituents by the use of a magnet and removal of the supernatant. The beads and cells were washed by repeated re-suspensions in PBS and removal of the supernatant after magnet application. Finally, the suspension was reduced to a small volume (e.g. 50 µl), spread onto a microscope slide and air dried and the CECs were counted using fluorescence light microscopy. CECs were defined as cellular structures that bound at least four beads and were auto-fluorescent. We have previously shown that these cells stain positively for expressed nitric oxide synthase (eNOS) which confirms their endothelial phenotype [6]. CEC slides were prepared by one of us (W.F.) but counting was performed by an independent operator (E.S.), blinded to the origin of the sample.

Plasma vWF, sE-selectin, sVCAM and cICAM were measured by ELISA using commercial reagents and standards (Dako-Patts, Ely, UK and R&D Systems, Abingdon, UK). CRP (immuno nephelometry) and erythrocyte sedimentation rate (ESR) were measured by the routine service of the hospital pathology laboratory.

**Data analysis**

Continuous data were subjected to the Shapiro–Wilks test to determine distribution. If normally distributed, they are presented as median (interquartile range) and are analysed by the Kruskal–Wallis test and, if normally distributed, were log-transformed for sub-group analysis. Categorical data were compared using the chi-squared test. Correlations were sought using Spearman’s ranks test method. A P-value of <0.05 was considered as statistically significant. All analyses were performed on Minitab release 14.

**Results**

Clinical, demographic and laboratory data are presented in Table 1. HCs were significantly older than the RA groups and the CCs. There were more men in the HC groups but the sex ratio difference between the RA and CC groups was not different. The HCs had higher systolic blood pressure than the two other groups (all the HCs were diabetics and most had treated hypertension and were taking a statin). There were more smokers in the RA group than in the other two groups. The median disease duration in the RA group was 10.7 yrs. CECs were significantly elevated in RA compared with the CC or HC groups with no difference between the two control groups (Fig. 1). As expected [13, 14, 17], there were significant differences in levels of vWF and the soluble adhesion molecules.

Correlations between the CEC counts and the plasma makers were as follows. In the 43 CCs the correlation and probability with vWF was \( r = 0.146 \), \( P = 0.345 \); CRP \( r = -0.085 \), \( P = 0.594 \); sE-selectin \( r = 0.181 \), \( P = 0.241 \); sICAM \( r = 0.036 \), \( P = 0.813 \); sVCAM \( r = 0.161 \), \( P = 0.296 \); ESR \( r = -0.08 \), \( P = 0.648 \). In the 57 RA patients, the data were vWF \( r = -0.063 \), \( P = 0.643 \); CRP \( r = 0.022 \), \( P = 0.87 \); sE-selectin \( r = -0.105 \), \( P = 0.439 \); sICAM \( r = -0.256 \), \( P = 0.055 \); sVCAM \( r = -0.275 \), \( P = 0.039 \); ESR \( r = -0.04 \), \( P = 0.778 \); disease duration \( r = 0.111 \), \( P = 0.409 \); DAS \( r = -0.053 \), \( P = 0.813 \). A sample size of 25 for the HCs does not allow meaningful correlation data.

**Discussion**

Our primary finding is that CECs are mildly elevated in patients with RA, supporting the presence of endothelial damage in this condition. Levels of CECs were unrelated to any laboratory and inflammatory markers. The only notable correlation was inversely between the adhesion molecule sVCAM-1 and CECs.

The presence of increased numbers of CECs in the blood of RA patients broadly supports other data in the connective tissue diseases and may be important given the association between CEC and increased morbidity and mortality in a variety...
of conditions [6–9]. However, this increase was small (only 2-fold) when compared to other studies. Clancy et al. [8] reported mean CEC numbers of 32 cells/ml compared to 5 cells/ml in healthy controls, whereas Woywodt et al. [9, 18] reported much higher levels (88–136 CECs/ml) in active vasculitis. We attribute lower numbers of CECs in our patients with RA to the relatively low level of disease activity, especially when compared with SLE and vasculitis. Against expectation, CECs failed to correlate with any of the other markers of endothelial damage/dysfunction, disease activity or duration. This may perhaps be because vascular biology is a dynamic phenomenon and that endothelial desquamation is the end product of significant damage (often preceded by dysfunction) that may not fluctuate to the same extent [2]. Thus, at any one time endothelial (dys)function represents the endothelial biology at that instant, whereas, number of CECs is the result of preceding insults to the endothelium, cumulatively resulting in damage that causes shedding of cells into the bloodstream [7–9].

An alternative explanation for lack of correlation is the effects of different pharmacotherapies, the complex nature of which is impossible to address at this level. Many patients were taking immunosuppressive agents (methotrexate, steroids, Table 1) which may have different effects on CECs than on the plasma markers.

It is somewhat surprising that the HC group did not show elevated CECs [19]. However, whilst these patients were a high cardiovascular risk group (type 2 diabetics with documented hypertension), they were also aggressively managed with medical therapy in a dedicated outpatient clinic. The small sample size of hypertension), they were also aggressively managed with medical therapy in a dedicated outpatient clinic. The small sample size of this group does not allow sub-analyses, e.g. for HbA1c. Hyper-tension per se does not raise CEC levels, although it does influence vWF levels [20] as is illustrated in our cohort (Table 1). The majority of HCs (74%) were treated with statins, and almost all (96%) were treated with anti-hypertensives and anti-platelet agents. Thus whilst (untreated) patients at high risk would be expected to have elevated markers of endothelial dysfunction, including elevated CECs, it is perhaps therefore less surprising that aggressive management of their risk factor profile with statins and anti-hypertensive treatment results in less endothelial insult. As endothelial damage may be thought of as a continuum from endothelial activation through dysfunction to damage/apoptosis [3], the shedding of cells into the blood may represent the extreme of endothelial damage and it may well be that treatments that reduce endothelial injury will have the greatest impact on measures at this end of the spectrum.

The particular factors involved in CEC shedding are not clear. From our data it appears that active inflammation is not involved in the process of vascular cell injury in RA as no correlation was found between CRP, ESR and numbers of CECs. However, we cannot exclude a longer term involvement of inflammation. As discussed, shedding of endothelial cells is the result of long-term damage, and it is plausible that inflammation is involved in this—perhaps a cumulative effect of chronically elevated inflammatory mediators. Other investigators have found that patients with severe sepsis have elevated levels of CECs, particularly in the case of septic shock [21], although inflammation per se (as opposed to direct endotoxin effect, for example) has not been causally linked. In addition, levels of inflammatory mediators in sepsis are typically much higher but over a shorter time-course than those generally seen in RA.

The inverse association of sVCAM-1 and CECs in RA patients warrants further investigation. Perhaps sVCAM-1 (or its release from the cell membrane) is protective towards the endothelium, or factors involved in protecting the endothelium against damage resulting are also implicated in sVCAM-1 expression/shedding. Alternatively, sVCAM-1 may scavenge CECs, so preventing them from being sampled during phlebotomy, or perhaps sVCAM-1 interferes with the CEC assay preventing detection of CECs that are present in the blood sample. We are not aware of any such interference having been previously recognized by other authors investigating CECs.

The exact nature of CECs remains elusive and controversial [22, 23]. Although we defined our CECs in the same way as numerous other workers [6–9, 18–21], it remains possible that in all these studies, and ours, the anti-CD146 antibody also captures other cells such as CD34+ endothelial progenitor cells [24]. Consequently, some caution in interpretation (which is therefore needed for every study using immunobead technology) is required and this by itself could explain non-correlation with vascular and inflammatory plasma markers.

In conclusion, we have demonstrated increased CECs in RA patients with no evidence of a direct link with inflammation or endothelial dysfunction. The finding that sVCAM-1 is negatively correlated with numbers of CECs deserves further investigation. However, we feel a major outcome is that raised CECs in RA fail to correlate with the gold standard endothelial plasma marker (vWF). This is in stark contrast to cardiovascular disease where CECs do indeed correlate with vWF (and sometimes with sE-selectin) [5, 25] again emphasizing the differences between these two major diseases.

**Rheumatology key messages**

- There are mildly increased numbers of CECs in the blood of patients with RA.
- These fail to correlate with markers of inflammation and endothelial injury.

**Acknowledgements**

The authors thank the R&D Committee of the Sandwell and West Birmingham NHS Trust for its support.

**Disclosure statement:** The authors have declared no conflicts of interest.

**References**
