C-reactive protein: the underlying cause of microvascular dysfunction in rheumatoid arthritis

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Objective. RA is a chronic autoimmune inflammatory condition associated with increased cardiovascular morbidity and mortality. Endothelial dysfunction, a marker of early atherosclerotic disease, occurs in some inflammatory diseases but this relationship has not been previously explored within the microvasculature of patients with RA. We therefore assessed forearm microvascular endothelial function in patients with RA and determined its relationship to RA disease activity and inflammation.

Methods. A total of 128 RA patients with no previous history of cardiovascular disease were evaluated. Endothelium-dependent and -independent forearm skin microvascular function was measured using laser Doppler imaging after iontophoretic delivery of acetylcholine (ACH) and sodium nitroprusside (SNP), respectively. Parameters of RA disease activity and inflammation were also checked.

Results. There was a significant negative correlation between the level of inflammation measured by log10 CRP and maximum vasodilatation measured by peak ACh response ($r^2 = -0.209, P = 0.018$, Pearson correlation test). In a multiple regression model, age ($\beta = -0.449, P < 0.0001$) and log10 CRP ($\beta = -0.193, P = 0.026$) were independently negatively associated with ACh responses. When RA patients were sub-divided according to their systemic inflammatory status (CRP > 10 mg/l vs CRP ≤ 10 mg/l), the high CRP group showed lower vasodilator responses to ACh ($P = 0.018$, analysis of variance (ANOVA)) and SNP ($P = 0.05$, ANOVA) than the low CRP group.

Conclusions. In this large cross-sectional study, we found for the first time systemic inflammation (CRP) to be independently associated with microvascular dysfunction in patients with RA. This strong correlation was independent of other conventional vascular risk factors.

Key words: Endothelium, Inflammation, Microcirculation, Rheumatoid arthritis, C-reactive protein, Cardiovascular disease.

Introduction

Patients with RA have a reduced life expectancy which is predominantly due to cardiovascular disease (CVD) [1–3]. The reason for this excess risk is not clear. Evidence supporting an increased prevalence of hypertension [4] and dyslipidaemias [5] in RA is now available, but when adjustment is made for these risk factors, the risk ratio is only minimally attenuated [6], suggesting that mechanisms other than the conventional vascular risk factors may contribute to this excess CV risk.

Recently, similarities have been found between the inflammatory process seen in RA and atherosclerosis [7, 8]. These features include raised plasma levels of TNF-α, IL-6, concentrations of CRP and local expression of adhesion molecules. It is now recognized that the inflammatory process is a major contributor to the pathological processes seen in CVD, and may play an aetiological role. It seems likely therefore that the deleterious effect to the CV system in RA could be mediated by the inflammation associated with the disease itself, a process we already know is involved in atherogenesis.

The vascular endothelium plays an essential role in maintaining blood vessel health by releasing a variety of vasoactive substances and mediators of inflammation and coagulation. When the endothelial function is impaired, there is an imbalance in these substances resulting in a vasoconstrictor, pro-inflammatory and pro-coagulant endothelium that may lead to both thrombosis and atherosclerotic disease. Changes in endothelial function occur early in the development of CVD and are found in asymptomatic subjects with CV risk factors [9, 10].

In RA, impaired endothelial function has been observed in the macrocirculation [11–13], but less is known about microvascular function. The microvasculature is an important vascular bed to study as it is affected early in the development of endothelial dysfunction [10, 14] and abnormalities here have been shown to correlate with CV risk factors [14–16] and established coronary artery disease [17].

The objective of this study was to assess microvascular endothelial function in patients with RA and to determine its relationship with RA disease activity and inflammation.

Patients and methods

One hundred and twenty-eight consecutive patients meeting the 1987 ACR classification criteria for RA [18] and attending the rheumatology clinics in Ninewells Hospital and Medical School, Dundee and Perth Royal Infirmary, Perth, UK were studied. Exclusion criteria included previous history of CVD, diabetes mellitus, uncontrolled hypertension or hypercholesterolaemia and any other inflammatory conditions.

Clinical and laboratory measurements as well as assessment of microvascular function were performed in all subjects.

This study was approved by the Tayside Committee on Medical Research Ethics and written consent was obtained from all participants according to the Declaration of Helsinki.

Clinical and laboratory measurements

All clinical characteristics of RA were documented (these included: duration of disease, CV risk factors, evidence of erosions on hand and feet X-rays, RF positivity and current medication).

A clinical evaluation was performed measuring height and weight, blood pressure (mean of three measurements taken on the left arm with the patient in a seated position after 25 min rest), DAS28 [validated disease activity index that combines 28 tender joint count, 28 tender joint count, CRP and a 100-mm general health visual analogue scale (VAS)] [19], VAS of pain (0–100 mm), physician’s global assessment of disease activity (0–100 mm) and Stanford HAQ [20].

Blood samples were taken for full blood count, urea electrolytes and liver function tests, CRP and plasma viscosity (PV).

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Assessment of microvascular function

Measurements of microvascular function were conducted in a temperature-controlled laboratory (22 ± 1°C). Subjects were given a 30-min equilibration period and were lying in a supine position. We assessed forearm microvascular function, as described previously [21–23], by measuring skin vascular responses to iontophoresis of 1% acetylcholine (ACh; Sigma-Aldrich Co. Ltd, Poole, UK) and sodium nitroprusside (SNP; David Bull Laboratories, Warwick, UK). Iontophoresis is a non-invasive drug delivery method that stimulates the migration of charged ions across the skin without inducing systemic effects. Two measurement sites were prepared on the volar aspect of the forearm by gently removing surface keratinocytes with adhesive tape and cleaning the area with alcohol. The iontophoresis chamber (Moor Instruments Ltd, Axminster, UK) consisted of a Perspex ring of a 20 mm internal diameter with a wire electrode running round the inner surface. The two chambers were fixed to the skin using double-sided adhesive tape and filled with 2 ml solution. The leads from the electrodes were connected to the iontophoresis controller. When an electrical potential difference is established, ions of the drug migrate across the skin, and the dose delivered is defined by the duration of current x time. Skin perfusion was measured using a laser Doppler imager (moorLDI, Moor Instruments). A 2-mW helium–neon laser scans the surface of the skin, and light back scattered from moving erythrocytes is shifted in frequency by an amount proportional to their velocity. For each scan, the computer builds up a colour-coded image representing skin blood flow over the scan area, a relative measure called the laser Doppler flux. For each dose response, the mean of the two highest stable flux values was calculated. The imager was programmed to take 20 separate scans lasting 60 s each. Two baseline scans of skin perfusion were taken before the iontophoresis protocol was administered. After the baseline scans, four scans were performed at 10 μA, four at 15 μA, four at 20 μA, four at 50 μA and two at 100 μA.

Statistical analysis

SPSS 13.0 for Windows was used for all statistical analyses. Results are presented as mean ± s.d. Between-group differences in vascular responses to ACh and SNP were tested using analysis of variance (ANOVA) for repeated measures, followed by a modified post hoc Student’s t-test at each dose when a significant difference was found. Two-tailed independent Student’s t-test was used to compare group differences. Data that were not normally distributed were log-transformed to achieve normality. Pearson correlation test was used to assess the univariate relationship (P < 0.05) as shown in Fig. 1.

Results

Baseline demographic and clinical data are summarized in Table 1. One hundred and twenty-eight patients, aged 31–81 yrs, were studied. Of these, 91 (71%) were females and 37 (29%) were males. Eighty-nine (70%) of the patients were RF positive and 72 (56%) had erosive disease. One hundred and nineteen (92%) were on a DMARD, of which MTX was the commonest (78 [60%]). Thirty patients were on anti-TNF-α drugs (eight on etanercept, four on adalimumab and two on infliximab). Eighty-eight patients (68%) were taking regular NSAIDs and 30 (23%) were on prednisolone (mean ± s.d. dose of 4.6 ± 2 mg). Twenty-six patients had hypertension that was successfully controlled with angiotensin-converting enzyme (ACE)-inhibitors (19), calcium channel blockers (10), β-blockers (four), diuretics (one) and moxonidine (one). All six patients with hypercholesterolaemia were treated with statins.

Of the parameters tested on univariate analysis (age, gender, previous history of hypertension or hypercholesterolaemia, smoking habits, RF positivity, presence of erosions, BMI, systolic blood pressure, diastolic blood pressure, PV, log10CRP, DAS28, HAQ, MTX use and prednisolone use), only age and log10CRP showed significant correlations with peak ACh response (r² = −0.393, P < 0.0001; r² = −0.209, P = 0.018, respectively). There was a significant negative correlation between age and peak SNP response (r² = −0.338, P < 0.0001), but no correlation between log10CRP and peak SNP response (r² = −0.163, P = 0.067).

A step-wise multivariate regression analysis was carried out to test the independent determinants of microvascular function. Peak ACh was set as the dependent variable and age, gender, smoking habits, systolic and diastolic blood pressure, previous history of hypercholesterolaemia and/or hypertension, DAS28, log10CRP and RF positivity were entered as independent variables. Age (β = −0.28, P < 0.0001) and log10CRP (β = −0.193, P = 0.026) were the only variables independently associated with peak ACh response. When MTX, anti-TNF-α and prednisolone use were entered in the model, age and log10CRP still remained the sole independent determinant of ACh response and their β- and significance levels did not change.

When peak SNP was set as the dependant variable, only age (β = −0.396, P < 0.0001) out of the previous parameters remained as an independent determinant.

To further study the relationship between inflammation and microvascular responses to ACh and SNP, patients were subdivided into two groups according to their systemic inflammatory status (high level of inflammation, CRP > 10 mg/l vs low level of inflammation, CRP ≤ 10 mg/l) as previously described [24]. Apart from the parameters of RA disease activity, there were no differences in the baseline demographics between the two groups (Table 1).

The dose-dependent vascular responses to ACh were significantly lower in the high CRP group than in the low CRP group (P = 0.018, ANOVA) as shown in Fig. 1.

The dose-dependent vascular responses to SNP were also significantly lower in the high CRP group than in the low CRP group (P = 0.05, ANOVA; Fig. 2).

In this study, we included patients with a previous history of hypertension and hypercholesterolaemia who were well controlled on medication. As both these risk factors and the medications used to treat them can affect vascular function we repeated the analysis (high CRP vs low CRP groups) excluding these patients. There was still a statistically significant difference in the dose-dependent ACh response (P = 0.048, ANOVA), but in the SNP response, although there was a trend towards better dose-dependent response in the low CRP group, this did not reach statistical significance (P = 0.110, ANOVA).

CRP is an important marker of inflammation but RA disease activity is primarily assessed by disease activity indexes such as the DAS28 that includes tender and swollen joint counts and a general healthVAS, besides the CRP. We evaluated the dose-dependent vascular responses of patients with active (DAS28 > 5.1, n = 41), moderate (DAS28 3.2 – 5.1, n = 41) and mild RA disease activity (DAS28 < 3.2, n = 46) and found no differences between the groups (ACh P = 0.493, SNP P = 0.908).

As further secondary analysis, we compared and found no differences in the demographics, disease activity scores (DAS28 P = 0.119, log10CRP P = 0.328) and dose-dependent vascular responses of RF-positive and RF-negative patients (ACh P = 0.710, ANOVA; SNP P = 0.362, ANOVA). No significant differences were observed either between the vascular responses of patients taking MTX for at least 6 months and those not on MTX.
(ACh \( P = 0.354; \) SNP \( P = 0.831 \)). There was, however, a trend towards better peak ACh response in the MTX group, but this did not reach statistical significance (457/C6 118 PU vs 429/C6 107 PU, \( P = 0.185 \)). MTX takers had lower log10CRP (0.93/C6 0.38 mg/l vs 1.19/C6 0.48 mg/l, \( P = 0.001 \)) and DAS28 values (3.68/C6 1.5 vs 4.92/C6 1.7, \( P < 0.0001 \)) but longer disease duration (14/C6 9 yrs vs 8/C6 8 yrs, \( P = 0.001 \)) than non-takers.

### Discussion

In this large cross-sectional study, we report the novel finding that systemic inflammation (CRP) is an independent determinant of microvascular dysfunction in patients with RA. Indeed, we have shown that the association between inflammation and microvascular function is primarily located at the level of the endothelium, as shown by a stronger correlation between the ACh response and CRP than between SNP response and CRP.

Endothelial dysfunction plays a key role, and occurs early, in the pathogenesis of atherosclerotic disease [9, 10]. Measuring endothelial function may help in the early detection of treatable cardiovascular abnormalities [25]. Endothelial function can be evaluated by assessing the ability of an artery to dilate in response to chemical and physical stimuli (through imaging) or by measuring biomarkers of endothelial activation, dysfunction and damage. Several imaging methods have been reported, both invasively at coronary artery level and non-invasively at the peripheral macro- and microcirculation. Endothelial responses can be measured with coronary angiography by assessing the vasodilator response to infused ACh [26, 27]. This technique, however, is invasive and not ethical unless coronary angiogram is required. Endothelial function can also be evaluated in the periphery, by brachial artery flow-mediated dilatation (FMD) and laser Doppler flowmetry after iontophoresis of vasoactive substances. FMD consists of high-resolution ultrasonographic determination of brachial artery diameter changes after post-occlusive reactive hyperaemia [9] and it has shown a close relationship with coronary artery vasomotor response to ACh [28]. Laser Doppler imaging after iontophoretic delivery of ACh (endothelium dependent) and SNP (endothelium independent) has been successfully used to study the vascular microcirculation [10, 12, 23], has good reproducibility [22] and it correlates with FMD [29]. In this study, we have confirmed that it is a reliable non-invasive method that can be readily used in RA patients to assess microvascular function. Measuring the response of the microcirculation to different stimuli may add value to studying the macrocirculation, as there is increasing evidence linking microvascular dysfunction to cardiovascular outcomes [30]. Moreover, although conduit function measured by FMD may be a stronger determinant of future cardiovascular outcomes, there is a stronger correlation between microvascular function and the presence of cardiovascular disease.

#### Table 1. Demographic, clinical and laboratory parameters of all patients and in patients sub-divided according to inflammatory status

<table>
<thead>
<tr>
<th></th>
<th>All RA patients</th>
<th>CRP &gt; 10 group</th>
<th>CRP ≤ 10 group</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>128</td>
<td>66</td>
<td>62</td>
<td>0.214</td>
</tr>
<tr>
<td>Female: male</td>
<td>91:37</td>
<td>43:22</td>
<td>44:15</td>
<td>0.161</td>
</tr>
<tr>
<td>Age, yrs</td>
<td>58 ± 12</td>
<td>59 ± 11</td>
<td>57 ± 9</td>
<td>0.496</td>
</tr>
<tr>
<td>RF, Pos/Neg/NotK</td>
<td>89/19/20</td>
<td>48/7</td>
<td>41/12</td>
<td>0.179</td>
</tr>
<tr>
<td>Erosions, Yes/No/NotK</td>
<td>72/20/36</td>
<td>44/7</td>
<td>32/13</td>
<td>0.069</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26 ± 5</td>
<td>28 ± 5</td>
<td>27 ± 5</td>
<td>0.229</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>137 ± 16</td>
<td>137 ± 18</td>
<td>137 ± 14</td>
<td>0.994</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>84 ± 8</td>
<td>83 ± 8</td>
<td>84 ± 8</td>
<td>0.907</td>
</tr>
<tr>
<td>Smoking, C/Ex/Ne</td>
<td>24/53/51</td>
<td>11/31/23</td>
<td>13/22/28</td>
<td>0.684</td>
</tr>
<tr>
<td>Treated hypertension</td>
<td>26</td>
<td>15/50</td>
<td>11/52</td>
<td>0.434</td>
</tr>
<tr>
<td>Treated hyperChol</td>
<td>6</td>
<td>1/64</td>
<td>5/58</td>
<td>0.088</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.17 ± 1.7</td>
<td>4.98 ± 1.5</td>
<td>3.32 ± 1.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Log₁₀CRP, mg/l</td>
<td>1.03 ± 0.44</td>
<td>1.39 ± 0.29</td>
<td>0.66 ± 0.21</td>
<td>0.0001</td>
</tr>
<tr>
<td>PV, mPa/s</td>
<td>1.75 ± 0.15</td>
<td>1.81 ± 0.16</td>
<td>1.69 ± 0.10</td>
<td>0.0001</td>
</tr>
<tr>
<td>HAQ</td>
<td>1.36 ± 0.7</td>
<td>1.53 ± 0.7</td>
<td>1.20 ± 0.7</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. Pos: positive; Neg: negative; NotK: not known; SBP: systolic blood pressure; DBP: diastolic blood pressure; Smoking C: current; Ex: ex smoker; Ne: never; hyperChol: hypercholesterolaemia.
Little is known, however, about microvascular function in CVD [32]. Atherosclerosis, whereas FMD may better represent established microvascular bed may be more sensitive in the early stages of risk factors [31]. It has been suggested, therefore, that assessing the microvascular responses. To ensure that including patients--independent impairment was observed in patients with high RA disease activity [11, 13].

Elevated serum CRP level has been extensively reported as an independent predictor of CVD [37, 38] and is associated with poor prognosis in unstable angina [39]. In RA, a raised baseline CRP level is associated with death from CVD [40] and correlates well with surrogate markers of CVD, such as arterial stiffness [24], carotid intima–media thickness of the common carotid artery [41] and macrovascular endothelial function measured by FMD of the brachial artery [13]. With the present study we have observed for the first time that CRP also correlates with microvascular function. These findings are of great interest as changes in this vascular bed may constitute the initial stage in the development of atherosclerosis [42].

Interestingly, we did not find any relationship between endothelial dysfunction and either DAS28, number of swollen and tender joints or PV. There may be different explanations for these findings. On the one hand, it is probably no coincidence that it is CRP and not other parameters of disease activity that are closely linked to microvascular dysfunction. CRP is a very sensitive marker that reflects ongoing inflammation more accurately than most other inflammatory parameters and even minimally raised levels positively correlate with atherothrombotic events [43]. Indeed, there is now increasing evidence to suggest that CRP may not only be a marker of increased vascular risk but may have a role as a direct contributor to the atherosclerotic process [44]. Another explanation for this lack of correlation between disease activity and DAS28 may be the fact that the components of DAS28 do not represent solely disease activity but may represent joint damage or patient’s perceptions of their disease [45].

Several other factors such as the presence of cardiovascular risk factors, RF positivity or use of MTX are known to have either harmful or protective effects in the vascular bed. As pre-planned secondary analysis we studied the influence of these parameters on the microvascular responses. To ensure that including patients with a previous history of hypertension and hypercholesterolaemia, albeit well controlled on medication, did not influence the results of this study, we looked for any correlation between a previous history of these diseases and ACh and SNP responses, and none was found. Moreover, when we excluded all 32 patients with a previous history of high blood pressure or elevated cholesterol levels, there was still a statistically significantly better endothelium-dependent response in the low than in the high CRP group.

Patients with seropositive (RF positive) RA have increased levels of all-cause and CV mortality [46]. Even in the early years of inflammatory polyarthritis, the presence of raised levels of RF increases the risk of premature death from CVD [47]. However, we did not find any correlation between RF positivity and endothelial function or any differences between the endothelium-dependent or -independent responses of seropositive and seronegative patients, and suggest that this previous association could also have been due to levels of inflammation.

Despite previous reports suggesting reduced all-cause and CV mortality with MTX use in RA [48], we did not find any differences in the vascular responses of patients taking MTX. It is not clear what mechanism is involved in the CV protection conferred by MTX, but suppression of systemic and vascular inflammation was postulated to be a key factor by Choi et al. [48].

Subgroup analysis in a recent study suggested that MTX treatment preserved vascular function in patients with RA by reducing arterial stiffness and improving macrovascular function measured by FMD [12]. Although in the present study we found the peak ACh response to be slightly better in the MTX patients, this did not reach statistical significance. Moreover, the lower levels of disease activity and inflammation observed in the MTX group could have accounted for those greater vascular responses.

These findings could suggest that the vasoprotective effects of MTX are at the level of the macrocirculation rather than microcirculation. Further prospective studies are required to understand the effects of MTX in the vascular bed.

A potential limitation of this study, particularly with regards to subgroup analysis, was the differences observed between the MTX and RF groups. MTX group comparisons could have been affected by the greater number of MTX patients (61%) and the significant differences observed in disease activity, inflammation and disease duration between MTX takers and non-takers. The greater proportion of RF-positive patients (70%) in this cohort could have contributed to the lack of difference observed in the vascular responses of RF-positive and -negative patients.

In conclusion, this is a large cross-sectional study evaluating microvascular function in patients with RA, for the first time showing a direct correlation with a key marker of inflammation: CRP. We have demonstrated that of all parameters of disease activity, CRP is the only independent determinant of microvascular endothelial dysfunction in patients with RA. This finding provides the evidence to underpin therapeutic studies of drugs, through anti-inflammatory mechanisms, to improve endothelial function in RA.
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