Angiopoietin-2 is highly correlated with inflammation and disease activity in recent-onset rheumatoid arthritis and could be predictive for cardiovascular disease

Johanna Westra¹, Lodewijk de Groot¹, Shannon L. Plaxton¹, Elisabeth Brouwer¹, Marcel D. Posthumus¹, Cornelis G. M. Kallenberg¹ and Marc Bijl¹

Abstract

Objective. To investigate whether serum levels of endothelial cell activation markers in early RA patients can serve as biomarkers for inflammation and disease activity, and are associated with radiological progression and development of cardiovascular disease (CVD).

Methods. Serum levels of VEGF, soluble vascular cell adhesion molecule (sVCAM)-1 and angiopoietin-2 (Angpt-2) were measured by ELISA in 176 patients with recent-onset RA, at the time of diagnosis and after 2 years. Markers of inflammation and disease activity were assessed, as well as radiological damage of hands and feet, at diagnosis and after 2 years. Prevalence of CVD of all patients after 12.5 years disease duration was retrieved from medical records.

Results. Patients with RA had higher levels of VEGF and Angpt-2 at disease onset compared with healthy controls, which correlated with markers of inflammation, but were not predictive of radiological progression after 2 years. Angpt-2 levels, moreover, significantly correlated with measures of disease activity. Nearly 18% of RA patients developed CVD after an average of 12.5 years of disease, and these patients had a significantly higher level of Angpt-2 at the onset of RA compared with patients who did not develop CVD.

Conclusions. In early RA, markers of endothelial activation are highly correlated with inflammation and disease activity, but not with radiological progression. Angpt-2 could be predictive for the development of CVD since Angpt-2 levels were significantly higher in CVD patients than in non-CVD patients.

Key words: Rheumatoid arthritis, Radiological damage, Cardiovascular disease, Angiopoietin-2, Endothelial cell.
levels and radiological progression after 1 year, but in other studies radiological damage has been related to levels of MMPs, which actually degrade cartilage and bone [5–7].

Another major factor in endothelial activation is angiopoietin-2 (Angpt-2), which causes vascular destabilization, thereby rendering the endothelium responsive to stimulation by inflammatory and angiogenic cytokines [8, 9]. A study investigating synovial tissue showed that Angpt-2, both at the mRNA and protein level, was highly expressed in the synovial membrane of early RA [10]. Angpt-2 is stored and rapidly released by endothelial Weibel–Palade bodies upon stimulation by pro-inflammatory stimuli, hypoxia, shear stress and VEGF. Recently, it was shown that Angpt-2, and not Angpt-1, induces oedema formation in the mouse paw in an animal model [11]. Angpt-2 is also elevated in hypertensive patients, in particular those with atherosclerosis, reflecting endothelial activation [12].

Vascular cell adhesion molecule (VCAM)-1 is expressed on the membrane of endothelial cells and shed after activation to form a soluble molecule [soluble VCAM-1 (sVCAM-1)]. sVCAM-1 levels are reported to be elevated in RA patients compared with controls and seem to respond to therapy [13, 14].

Recently, chronic systemic inflammation has been identified as a major factor in atherosclerosis development and it has been shown that atherosclerosis is induced by increased endothelial activation [15]. The aim of this study was to investigate in early RA patients whether the markers of endothelial cell activation (VEGF, Angpt-2, sVCAM-1) can serve as biomarkers for inflammation and disease activity, and whether they are related to radiological progression after 2 years of disease. In a subgroup of 40 patients with long-standing disease duration, we compared levels of endothelial cell activation markers with levels at the time of diagnosis. Finally, we retrospectively investigated whether markers of endothelial cell activation at the onset of disease are related to the development of cardiovascular disease (CVD) in RA patients.

Materials and methods

Patients

One hundred and seventy-six consecutive patients with early RA who participated in a prospective follow-up study between January 1993 and December 2001 at the Department of Rheumatology of the University Medical Center Groningen were included in this study. All patients gave informed consent. We obtained approval from the Medical Ethical Review Committee (University Medical Center Groningen) for both the prospective study and the study investigating cardiovascular risk factors. Patients met the 1987 ACR criteria of RA [16], had joint symptoms existing for <1 year at presentation and had previously not taken DMARDs. All patients started on SSZ 2000–3000 mg/day, and, in the case of insufficient response, MTX, in increasing dosages up to 25 mg/week, was added. If there was still an insufficient response, SSZ was replaced by another DMARD.

Peripheral joints were examined for tenderness and soft-tissue swelling. The following articular indices were determined: Ritchie articular index (RAI), tender joint count (TJC), swollen joint count (SJC) and 28-joint DAS (DAS-28). Radiological damage in hands and feet was assessed by Sharp’s method with some modifications as described by van der Heijde et al. [17]. Medical records of all patients were screened after 12.5 years disease duration regarding presence of CVD, defined as a history of ischaemic heart disease, cerebrovascular accidents or peripheral vascular disease.

Forty patients of the original cohort participated in a separate study investigating cardiovascular risk factors in RA after a median disease duration of 12.3 years (range 9.5–14.9 years), and serum samples of these patients were obtained. The aim of our study was to investigate levels of endothelial cell activation markers after long-standing disease and compare these with levels at the onset of disease. At the time of diagnosis, this group of 40 patients did not differ from the other RA patients, except for age, which was 45.4 years compared with 52.8 years in the other 136 patients (data not shown). Healthy control (HC) groups were included in the study, which were age and sex matched to the original RA cohort and also to the smaller cohort of 40 patients. Characteristics of all groups are presented in Table 1.

Laboratory measurements

Serum samples were collected at the time of diagnosis and after 2 years. ESR, CRP, immunoglobulin M (IgM)-RF and ACPAs were measured by routine techniques. VEGF, Angpt-2 and sVCAM-1 levels were determined by commercially available ELISA (DuoSet; R&D Systems, Abingdon, UK) according to the manufacturer’s instructions.

Statistical analysis

Comparisons between patients and controls were made by Mann–Whitney tests for continuous variables. Chi-square test was used to test difference in gender distribution in groups. Comparisons between paired samples were made using paired t-tests or Wilcoxon signed rank test where appropriate. Univariate correlations between categorical variables were assessed by Pearson correlation coefficient, when variables were normally distributed; otherwise Spearman correlation coefficient was used. Multivariate logistic regression analysis was used to assess the influence of parameters that tested significant in univariate analysis on CVD. Analyses were performed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA).

Results

Markers of endothelial activation in patients and controls

In RA patients, significantly elevated serum levels were found for VEGF [median (range) 330.5 (33–1979); HC: 113.5 (16–443)] and Angpt-2 [median (range) 569 (141 to >10000); HC: 510 (158–1821)] at the time of diagnosis,
whereas sVCAM-1 levels [median (range) 319 (156–914); HC: 327 (191–543)] were not increased. After 2 years, VEGF levels [median (range) 263 (31–1022)] were significantly reduced ($P < 0.001$), but were still elevated compared with HC (Fig. 1A). DAS-28 levels were significantly decreased after 2 years following treatment, but were still high and only five patients were in remission (DAS-28 < 2.6) (Fig. 1B and Table 1). VEGF, sVCAM-1 and Angpt-2 levels seemed relatively constant in each patient, and levels between time of diagnosis and after 2 years were highly correlated (VEGF: $r = 0.773$, $P < 0.0001$; sVCAM-1: $r = 0.622$, $P = 0.0001$; Angpt-2: $r = 0.583$, $P < 0.0001$).

Correlations with inflammatory and disease markers

Both VEGF and Angpt-2 showed a highly significant positive correlation with inflammatory markers (CRP, ESR) at time point 0, and Angpt-2 also correlated with CRP and ESR after 2 years. Moreover, Angpt-2 correlated with clinical parameters of inflammation (DAS-28). Angpt-2 levels were significantly correlated with sVCAM-1 and IgM-RF levels (Table 2).

Angpt-2 as well as sVCAM-1, but not VEGF, correlated positively with age, both in patients and controls (Fig. 2). Correlations for age with Angpt-2 were 0.408 ($P < 0.0001$) in HC and 0.188 ($P = 0.0124$) in RA, and for sVCAM-1 0.443 ($P < 0.0001$) in HC and 0.231 ($P = 0.002$) in RA. Interestingly, for Angpt-2 and age, the slope of the regression lines is similar in HC and RA, showing that RA patients from disease onset have higher Angpt-2 levels compared with HCs of the same age.

At the time of diagnosis, 49% of the patients had no radiological damage and 37% had a Sharp-van der Heijde score of 1–5, whereas only 1.7% had a score >15. After 2 years, 21% still had no damage, whereas 18% had a Sharp-van der Heijde score of 1–5 and 29.5% had scores >15. There was a significant correlation between cumulative CRP values (calculated using monthly CRP values) and increase in radiological damage ($X_{tt}$) ($r = 0.263$, $P = 0.0005$) over 2 years (Fig. 3A) as our group has reported before [18]. Calculating cumulative CRP values using values from time points 0, 1 and 2 years also gave a positive correlation with increase in radiological damage ($P < 0.05$). There was, however, no correlation between cumulative values of VEGF, Angpt-2 and sVCAM-1 and radiological damage after 2 years. No correlation was found between baseline levels of VEGF, Angpt-2 and sVCAM-1 levels and disease damage after 2 years. Moreover, there was no difference in levels of these markers between patients who developed erosions (progressive) and those who did not (non-progressive) (Fig. 3B). ACPA status had no effect on VEGF, Angpt-2 and sVCAM-1 levels (data not shown).

Markers of endothelial activation in patients after long-standing RA

VEGF levels [median (range) 240 (11–1074); HC: 117.5 (16–443)], sVCAM-1 [median (range) 491 (274–909); HC: 348 (224–691)] and Angpt-2 [median (range) 1198 (287 to 1198)]
Fig. 1 Serum levels of VEGF, Angpt-2 and sVCAM-1 and DAS-28 scores in 176 early RA patients at the time of diagnosis and after 2 years. RA patients (n = 176) with joint symptoms for <1 year were included in a prospective study. (A) VEGF, Angpt-2 and sVCAM-1 levels were measured by ELISA in serum samples at the time of diagnosis and after 2 years, and compared with age- and sex-matched HCs. Comparisons between RA patients and HCs were made by Mann-Whitney tests, and between RA patients at different time points by paired t-tests. Horizontal bars represent median values. (B) Box and whisker plots of DAS-28 levels at time points 0 and 2 years, showing median, 25% and 75% percentile and range; a significant decrease was found after 2 years of therapy (paired t-test, P < 0.0001).

Table 2 Correlations between VEGF and Angpt-2 and markers of inflammation and disease activity at 0 and 2 years

<table>
<thead>
<tr>
<th>CRP, T = 0</th>
<th>ESR, T = 0</th>
<th>sVCAM-1, T = 0</th>
<th>DAS-28, T = 0</th>
<th>SJC, T = 0</th>
<th>RF, T = 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF, T = 0</td>
<td>0.414</td>
<td>0.398</td>
<td>NS</td>
<td>0.364</td>
<td>0.235</td>
</tr>
<tr>
<td>Angpt-2, T = 0</td>
<td>0.277</td>
<td>0.257</td>
<td>0.267</td>
<td>0.160</td>
<td>NS</td>
</tr>
<tr>
<td>CRP, T = 2</td>
<td>ESR, T = 2</td>
<td>sVCAM-1, T = 2</td>
<td>DAS-28, T = 2</td>
<td>SJC, T = 2</td>
<td>RF, T = 2</td>
</tr>
<tr>
<td>VEGF, T = 2</td>
<td>NS</td>
<td>0.183</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Angpt-2, T = 2</td>
<td>0.219</td>
<td>0.213</td>
<td>0.340</td>
<td>0.248</td>
<td>0.232</td>
</tr>
</tbody>
</table>

≥ 10,000); HC: 800 (256–3930)] in the RA group after 12.3 years of disease were significantly higher compared with age- and sex-matched HCs (Fig. 4). At the time of diagnosis, sVCAM-1 levels were not higher compared with controls, but in long-standing RA sVCAM-1 levels were also significantly increased. Within the HCs, sVCAM-1 levels were not significantly elevated and Angpt-2 levels were significantly (P < 0.0001) elevated in the older compared with the younger HC group.

Again after 12.3 years of disease there was a high correlation between VEGF, sVCAM-1 and Angpt-2 levels compared with levels at the time of diagnosis (VEGF: r = 0.684, P < 0.0001; sVCAM-1: r = 0.372, P = 0.05; Angpt-2: r = 0.412, P = 0.010). Furthermore, in the RA group with long-standing disease, significant correlation between VEGF and DAS-28 (r = 0.3794, P = 0.017), and also between Angpt-2 and sVCAM-1 levels (r = 0.375, P = 0.032) was found.

Relation of endothelial activation markers and CVD

We retrospectively evaluated occurrence of cardiovascular events in RA patients. In our RA cohort, 31 patients had suffered a cardiovascular event, of which 3 were before diagnosis of RA. If we excluded these from our cohort, the median time between diagnosis RA and cardiovascular event during follow-up was 6 years (range 0.5–13 years).

In Table 3, characteristics of patients with and without CVD are given. At the time of diagnosis for RA, there
was a significant difference between patients who developed CVD and those who did not regarding Angpt-2 level ($P = 0.006$) (Fig. 5), male sex ($P = 0.007$) and age ($P < 0.001$), while there was a trend for a difference in CRP level ($P = 0.08$) and IgM-RF level ($P = 0.07$). To investigate which factors contributed to the development of CVD, we performed a multivariate logistic regression analysis with development of CVD as a dependent variable (Table 4). First, a univariate analysis was performed with different parameters and CVD. Next, variables with $P < 0.3$ were selected for multivariate logistic regression analysis with the development of CVD as a dependent variable. Testing these variables we found that age, male gender and RF level were the main contributors to the development of CVD.

Discussion

In this study, we show that the endothelial cell activation markers VEGF and Angpt-2 are elevated in recent-onset RA patients at the time of diagnosis, but also after 2 years. Moreover we show that these markers are highly correlated with markers of inflammation and disease activity, but not with radiological progression. Angpt-2 levels at the time of diagnosis, however, seem to be related to the development of CVD.

Angiogenesis, involved in up-regulation of many soluble and cell surface-bound mediators, has been associated with RA for a long time. These mediators include growth factors like fibroblast growth factor, epidermal growth factor, PDGF and VEGF, but also hypoxia-inducible factor-1 (HIF-1), as well as cytokines, chemokines and others. There has even been a debate as to what develops first in RA: angiogenesis or chronic inflammation [1, 3], but it is clear that both processes are closely interrelated. Moreover, inhibition of angiogenesis has been discussed and investigated as therapeutic intervention in RA. Targeting of specific molecules such as VEGF and HIF-1...
is under investigation in in vitro studies and in animal models [19].

In our study, we found that VEGF levels were significantly increased in a large group of patients with recent-onset arthritis. Also Angpt-2 levels were increased, whereas sVCAM-1 levels were comparable to those of sex- and age-matched HCs. RA patients were treated in the first 2 years according to a standard protocol, starting with SSZ, and in the case of insufficient response MTX was added. If there was still insufficient response, SSZ was replaced by another DMARD. This therapeutic regimen resulted in a decrease in DAS-28, although most patients did not reach remission, and VEGF and Angpt-2 levels were also still increased. These endothelial cell activation markers were significantly correlated with CRP, ESR, DAS-28 and SJC, indicating a close relationship with inflammation at the time of diagnosis. For Angpt-2, this was also true after 2 years of disease duration. The
Angiopoietin-2 in early RA

Fig. 5. Angpt-2 levels in RA patients who developed CVD compared with RA patients who did not and HCs. Occurrence of CVD was recorded in 176 RA patients after 12.5 years of disease. Angpt-2 levels measured at the time of diagnosis were compared in CVD patients and non-CVD patients. A significant difference was found in Angpt-2 levels (Mann–Whitney, P = 0.0061). Horizontal bars represent median values.

Table 4. Results of univariate and multivariate logistic regression analysis for CVD as dependent variable

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th></th>
<th>Multivariate analysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B (95% CI)</td>
<td>P-value</td>
<td>B (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>Age, years</td>
<td>1.086 (1.045, 1.128)</td>
<td>&lt;0.001</td>
<td>1.092 (1.047, 1.139)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>0.346 (0.156, 0.767)</td>
<td>0.009</td>
<td>0.369 (0.153, 0.893)</td>
<td>0.027</td>
</tr>
<tr>
<td>CRP</td>
<td>1.007 (0.997, 1.047)</td>
<td>0.183</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>1.005 (0.993, 1.018)</td>
<td>0.405</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>1.000 (0.999, 1.001)</td>
<td>0.654</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>1.004 (1.000, 1.007)</td>
<td>0.035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angpt-2</td>
<td>1.000 (1.000, 1.000)</td>
<td>0.379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM-RF</td>
<td>1.001 (1.000, 1.002)</td>
<td>0.021</td>
<td>1.001 (1.000, 1.002)</td>
<td>0.014</td>
</tr>
<tr>
<td>DAS-28</td>
<td>1.078 (0.809, 1.436)</td>
<td>0.606</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in bold are significant at P < 0.05. *The variable was not selected during multivariate regression analysis; **the value was not tested in multivariate regression analysis because of P > 0.3 in univariate analysis.

A link between angiopoietins and inflammation has been reviewed by Fiedler and Augustin [9]. Angpt-1 is required to maintain the endothelium in a quiescent state, whereas Angpt-2 is a destabilizing factor considered to be pro-inflammatory. Recently, Angpt-2 has been reported to be increased in SLE patients and in ANCA-associated vasculitis patients in relation to disease activity [20, 21]. We also found Angpt-2 to be a marker for disease activity with a high correlation to inflammation. Recently, Kennedy et al. [22] showed that the combination of Angpt-2 with TNF-α significantly increased cytokine release from synovial fibroblasts compared with TNF-α alone. This suggests that Angpt-2 sensitizes synovial cells to activation by TNF-α, thereby having a direct effect on inflammation.

In contrast, sVCAM-1 levels in RA were not increased compared with those in HCs, nor did they correlate with inflammation. Elevated levels of sVCAM-1 in RA have been reported, for instance, by Klimiuk et al. [13], who described higher levels in early RA patients compared with OA patients. In another study, increased sVCAM-1 levels were found in RA patients with longer disease duration, which decreased following therapy [14, 23]. We were able to obtain serum samples of 40 patients of our original cohort of 176 patients with RA after 12 years of disease. Indeed, in these samples next to increased levels of VEGF and Angpt-2, elevated sVCAM-1 levels were also found compared with an age- and sex-matched HC group. These findings suggest that prolonged rather than acute inflammation and endothelial cell activation are related. In concordance, a significant correlation was obtained between Angpt-2 and sVCAM-1, indicating increased endothelial cell activation in long-standing RA.

Whether these angiogenic mediators lead to joint destruction, is still a matter of debate. In 2001, Latour et al. [24] found that synovial tissue VEGF might be a marker for joint destruction in RA. Subsequently, it was reported that blockade of VEGF receptors in an animal arthritis model prevented bone destruction [25]. Recently, VEGF levels were mentioned by Kurosaka et al. [26] as a prognostic factor regarding joint destruction, but this conclusion was based primarily on the study by Clavel et al. [4], who described a significant relationship between serum VEGF levels and radiological progression after 1 year. Kurosaka et al. [26] furthermore found a correlation between VEGF and blood flow signals, measured by power Doppler ultrasonography, but this reflects angiogenesis rather than joint destruction. In our cohort, 49% of the patients had no radiological damage at the time of diagnosis, and this number had decreased to 21% after 2 years. There was an average increase of 9 points (range 0–70 points) in 2 years. We calculated cumulative values of CRP using monthly CRP values as described previously [18], or using yearly CRP values, and found a fair correlation between cumulative CRP and increase in radiological progression. This could not be found for endothelial cell activation markers. Categorizing patients as progressive (progression >1) or non-progressive patients did not show differences in VEGF, Angpt-2 or sVCAM-1 levels at the time of diagnosis or after 2 years. So, we could not find a direct relationship between these markers and joint destruction. The correlations between endothelial cell activation markers and markers of inflammation and disease activity therefore seem to be point associations, studied at the same time. Inflammation leads to joint destruction, but endothelial cell activation does not.

We investigated the development of CVD in our original group after 12.5 years of disease and found a prevalence...
of almost 18%. In a recent review, it was mentioned that in echocardiographic studies in established cohorts of RA patients prevalence rates of pericardial disease were found of between 1 and 30% [27]. Overall, the consensus is that CVD mortality is increased in RA, with standardized mortality rates of 1.13–5.15 [28]. Traditional risk factors like smoking, body mass index, male gender, age, dyslipidaemia and hypertension are of course the main risk factors for development of CVD, but in autoimmune rheumatic disease continuous inflammation and endothelial cell activation play a major role as well [29, 30]. In our study, we included a limited number of possible risk factors and found that age, male gender and RF level were the main contributors to the development of CVD. Concerning the role of IgM-RF, it has been described previously that seropositivity for RF is associated with increased cardiovascular mortality [31] in early RA patients, while also in a general population cohort RF was associated with increased cardiovascular mortality [32]. Our data showed a significant difference in the Angpt-2 levels between patients who developed CVD and those who did not. Moreover, in the long-standing RA group, Angpt-2 levels correlated significantly with sVCAM-1 levels, which is considered a marker of atherosclerosis [30]. In atherosclerotic plaques with high microvesSEL density, the balance between Angpt-1 and Angpt-2 is in favour of Angpt-2 [33], while circulating Angpt-2 was found to be a mediator for accelerated atherosclerosis in dialysis patients [34]. Recently, Lieb et al. [35] found that Angpt-2 and sTIE-2 are heritable traits associated with CVD risk factors in the third-generation cohort participants of the Framingham Heart Study. Moreover, they showed that Angpt-2 was positively related to age, smoking, systolic blood pressure, hypertension treatment and diabetes. Earlier we already mentioned that Angpt-2 is also elevated in hypertensive patients, in particular those with atherosclerosis [12]. So, Angpt-2 is already an independent predictor for CVD in patients with hypertension and other non-inflammatory disorders. In our study, we found that Angpt-2 is already increased in early RA patients and closely related to inflammation, disease activity, age and sVCAM-1, and therefore might be a valuable predictor of CVD in RA.

**Rheumatology key messages**

- Markers of endothelial cell activation, in particular VEGF and Angpt-2, are elevated in early RA.
- VEGF and Angpt-2 levels correlate with inflammation, but do not predict joint destruction.
- Angpt-2 levels correlate with age and are possibly predictors of CVD in early RA.

**Acknowledgements**

We want to thank Berber Doornbos-van der Meer for excellent technical assistance.

**Funding:** This work has been supported by infrastructural support of the Dutch Arthritis Association.

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

**References**


