Circulating angiopoietin-2 is a marker and potential mediator of endothelial cell detachment in ANCA-associated vasculitis with renal involvement

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Abstract

Background. The endothelial-specific angiopoietin (Ang)-Tie ligand–receptor system has been identified as a non-redundant regulator of endothelial cell detachment in vitro. Binding of circulating angiopoietin-2 (Ang-2) to the Tie2 receptor antagonizes Tie2 signalling and leads to disassembly of cell–cell junctions. Here, we ask whether circulating Ang-2 correlates with the severity of endothelial damage in ANCA-associated vasculitis (AAV) with renal involvement.

Methods. Ang-2 was measured in sera obtained from 45 patients with AAV and 20 healthy controls by in-house ELISA. The disease activity was monitored by BVAS and the enumeration of circulating endothelial cells (CECs).

Results. Ang-2 was significantly elevated in active AAV with renal involvement compared to controls and patients in remission. In contrast, Ang-2 was normal in patients with active granulomatous disease limited to the respiratory tract. Linear regression analysis demonstrated a strong association of Ang-2 with BVAS (r² = 0.49, P < 0.0001) and the number of CECs (r² = 0.48, P < 0.001). An Ang-2 cut-off value >4.15 ng/ml for a positive result yielded 100% specificity and 65% sensitivity for active systemic vasculitis. The positive predictive value was 99% and the negative predictive value 84%.

Conclusions. Circulating Ang-2 is elevated and closely correlates with BVAS and CEC numbers in AAV with renal involvement. These data indicate that Ang-2 might be a potential mediator of endothelial cell detachment in AAV.

Keywords: ANCA-associated vasculitis; angiopoietin-2; circulating endothelial cells; disease activity; necrotizing glomerulonephritis

Introduction

Interaction of primed neutrophils with endothelial cells, possibly mediated by anti-neutrophilic cytoplasmatic anti-bodies (ANCA), is considered a crucial event in the pathogenesis of severe endothelial lesions in ANCA-associated vasculitis (AAV) [1,2]. In this regard, the interaction between neutrophils and ANCA has been the subject of much study whereas mechanisms for endothelial damage and detachment remain less well defined.

The presumable sequence of adherence, penetration and destruction of the vessel wall by ANCA-activated neutrophils was already proposed in the 1980s [3,4]. It had been proposed that once injured endothelial cells lose their anchorage to neighbouring cells and/or extracellular matrix, they detach from the basement membrane and are released into peripheral blood [5,6]. In an attempt to provide a useful biomarker of vascular damage, we have earlier given evidence for this process by visualizing and enumerating these so-called circulating endothelial cells (CECs) in AAV [7–10]. CECs were found to correlate with disease activity and help to distinguish limited granulomatous disease from active systemic vasculitis [8–10]. However, molecular mechanisms for endothelial detachment in AAV are poorly understood [11].

In 1996/1997, the Ang-Tie ligand–receptor system was identified as the second class of vascular-specific receptor tyrosin kinases (RTKs) (the first being the VEGF/VEGF–receptor system) [12,13,14]. Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are antagonistic ligands that bind to the extracellular domain of the Tie2 receptor, which is almost exclusively expressed by endothelial cells [15,16]. Binding of the agonist Ang-1 to the Tie2 receptor induces rapid auto-phosphorylation that promotes vessel integrity, inhibits vascular leakage and suppresses inflammatory gene expression [17–19].

In contrast, binding of the antagonistic ligand Ang-2 abrogates protective Tie2 signalling [13,20,21]. Ang-2 constitutes an endothelial Weibel-Palade body (WPB) stored molecule that is rapidly secreted and induced upon stimulation whereas Ang-1 is constitutively expressed by pericytes and vascular smooth muscle cells providing a stabilization signal [14,22–25]. Once released from WPB in
response to several stimuli, Ang-2 facilitates auto/paracrine endothelial cell activation and disassembly of cell–cell contacts in a non-redundant fashion, thus leading to increased vascular inflammation and endothelial cell detachment in vitro [20,23,26]. In AAV, endothelial activation is probably caused by serine proteases that are released from ANCA IgG-stimulated neutrophils [27] and potentiated by cytokines such as TNF-α [28].

We hypothesized that Ang-2 is elevated in active systemic AAV, and if so, should correlate with the extent of vascular inflammation and endothelial cell detachment. To prove this theory, we measured serum concentrations of circulating Ang-2 (i) during the course of AAV with renal involvement and (ii) among different patient groups (active AAV, AAV in remission, active AAV limited to the respiratory tract) in a cross-sectional fashion. In addition to the Birmingham Vasculitis Activity Score (BVAS), endothelial cell detachment was quantified by enumeration of CECs.

Methods

Patients and study design

All patients were recruited from the Department of Nephrology at Hannover Medical School. The study was done in accordance with the Declaration of Helsinki and approved by the institutional review board (No. 4373). Informed consent was obtained. A total of 45 patients with AAV were enrolled in the present cross-sectional study (Table 1).

- Of those, 15 patients had active AAV with biopsy-proven pauci-immune glomerulonephritis (BVAS 18.2 ± 6.8) and were analysed at initial presentation before initiation of immunosuppressive therapy. Eight of those patients had a new onset AAV. Seven patients had a relapse of AAV while they were on immunosuppression or received maintenance oral prednisolone ≤7.5 mg/day. A follow-up was performed at 1 and 6 months.

- In addition, 20 AAV patients with biopsy-proven pauci-immune glomerulonephritis vasculitis in remission were analysed (length of remission 35.3 ± 45.4 months, BVAS = 0). All of those patients were on immunosuppression or received maintenance oral prednisolone ≤7.5 mg/day.

- We also studied 10 patients with active ‘limited’ isolated granulomatous disease of the respiratory tract (LGD). LGD was defined as an isolated disease of the respiratory tract with granulomatous inflammation, no active vasculitis on biopsy and no constitutional symptoms. None of these patients had renal involvement. All of those patients were off immunosuppression.

- Twenty healthy BMI- and age-matched volunteers of the Hannover Medical School staff served as controls.

Detailed clinical characteristics of patient groups and healthy controls are provided in Table 1. The diagnosis of AAV was established in accordance with the Chapel Hill classification and the criteria of the American College of Rheumatology [29]. The diagnostic criteria for microscopic polyangiitis (MPA) were necrotizing pauci-immune vasculitis and glomerulonephritis of the small vessels typical presentation with involvement of the upper respiratory tract in the presence of granulomatous inflammation on histology. The diagnostic criteria for microscopic polyangiitis (MPA) were necrotizing pauci-immune vasculitis and glomerulonephritis of the small vessels without granuloma. ‘Limited’ granulomatous disease (LGD) was defined as an isolated disease of the respiratory tract with granulomatous inflammation, no active vasculitis on biopsy and no constitutional symptoms [10]. Disease activity was assessed by the Birmingham Vasculitis Activity Score (BVAS) [30].

Treatment protocol

All patients with active AAV were treated with 1 mg/kg BW per day prednisolone orally. By Day 15 tapering of the steroid regimen was initiated, with a reduction of 10 mg/week. In all patients with renal involvement, oral prednisolone was started on Day 4 after 500 mg prednisolone was given on Days 1–3 intravenously. Cyclophosphamide was administered orally or intravenously for 3–6 months followed by azathioprine or mycophenolate mofetil [31].

Laboratory testing and enumeration of CECs

 Peripheral blood samples at initial presentation were obtained before or within 24 h of the initiation of treatment. All samples were centrifuged and stored at −80°C. C-reactive protein and serum creatinine were measured by routine techniques. Creatinine clearance was estimated using the Cockcroft–Gault formula. Proteinuria was quantified by 12-h urine collections. Erythrocyturia was quantified by direct enumeration of red blood cells in a high-power field in full urine.

CECs were enumerated as a laboratory marker of microvascular injury according to a European consensus as described elsewhere [7,8]. In brief, detection of CECs was performed by anti-CD146-driven immunomagnetic isolation. CECs were subsequently stained using endothelial-specific Ulex Europaeus lectin-1 (UEA-1), and finally enumerated (cells/ml) by fluorescence microscopy [7,8].

Ang-2 enzyme-linked immunosorbent assay (ELISA)

Serum Ang-2 concentrations were measured by an in-house sandwich ELISA assay as previously described in detail elsewhere [32,33]. Monoclonal Ang-2 and anti-Ang-2 antibodies were purchased from R&D Systems (Minneapolis, MN, USA). In our hands, the intra-assay coefficient of variation for Ang-2 was 3.8%, and inter-assay coefficient of variation was 4.2%. The sensitivity threshold was 0.2 ng/ml.

Table 1. Characteristics of patient groups and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Active AAV</th>
<th>AAV in remission</th>
<th>Active LGD</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>15</td>
<td>20</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>WG/MPA</td>
<td>9/6</td>
<td>9/11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>New onset/relapse</td>
<td>8/7</td>
<td>–</td>
<td>1/9</td>
<td>–</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>10/5</td>
<td>12/8</td>
<td>6/4</td>
<td>12/8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.3 ± 15.7</td>
<td>56.5 ± 14.9</td>
<td>43.2 ± 15.08</td>
<td>57.6 ± 12.5</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>26.2 ± 4.3</td>
<td>27.4 ± 2.7</td>
<td>25.7 ± 3.0</td>
<td>25.2 ± 3.5</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>204.5 ± 107.7</td>
<td>137.6 ± 78.4</td>
<td>82.6 ± 16.0</td>
<td>79.4 ± 10.8</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>42.5 ± 28.2</td>
<td>58.0 ± 18.2</td>
<td>94.4 ± 23.4</td>
<td>82.6 ± 16.1</td>
</tr>
<tr>
<td>CEC (cells/ml)</td>
<td>139.4 ± 187.5</td>
<td>22.0 ± 11.5</td>
<td>6.3 ± 3.4</td>
<td>–</td>
</tr>
<tr>
<td>BVAS</td>
<td>18.2 ± 6.8</td>
<td>0</td>
<td>8.4 ± 1.3</td>
<td>–</td>
</tr>
</tbody>
</table>

AAV, active ANCA-associated vasculitis; WG, Wegener’s granulomatosis; MPA, microscopic polyangiitis; eGFR, estimated glomerular filtration rate (Cockroft–Gault formula); CEC, circulating endothelial cells; BVAS, Birmingham Vasculitis Activity Score.
Serum Ang-2 concentrations were elevated 6-fold in patients with active AAV (6.1 ± 3.5 ng/ml SD) compared to healthy controls (1.0 ± 0.7 ng/ml, \( P < 0.0001 \)) (Figure 1). In contrast, Ang-2 concentrations were normal in stable long-term remission of AAV (1.7 ± 0.7 ng/ml). Similarly, patients with active ‘limited’ granulomatous disease of the respiratory tract (LGD) had normal Ang-2 concentrations (1.7 ± 1.1 ng/ml) that were not different from those in healthy controls (1.0 ± 0.7 ng/ml, \( P = 0.134 \)) (Figure 1).

In patients with active AAV, Ang-2 concentrations were not different between new onset (\( n = 8 \)) and relapse of AAV (\( n = 7 \)) (\( P = 0.65 \)). Likewise, Ang-2 concentrations were not different between MPA (\( n = 6 \)) and WG (\( n = 9 \)) (\( P = 0.524 \)).

Circulating Ang-2 concentrations decline during therapy

Ang-2 concentrations at initial presentation and 6 months after the start of immunosuppressive treatment were different by Friedman’s test (\( P < 0.0001 \)) (Figure 2). After 1 month of immunosuppressive treatment, the Ang-2 concentrations had decreased substantially (2.5 ± 1.3 ng/ml) (Wilcoxon signed-rank test; \( P = 0.002; n = 15 \)). The ang-2 concentrations remained within the normal range at 6 months (1.4 ± 0.86 ng/ml) (initial presentation versus 6 months: \( P = 0.008; 1 \) month versus 6 months \( P = 0.11; n = 8 \)) (Figure 2). The consistent decline in Ang-2 was observed in all but one female patient, in which Ang-2 had increased from 3.5 to 4.8 ng/ml after 1 month of immunosuppressive therapy. Consistently, this was paralleled by an increase in BVAS from 18 to 21 points. Of note, eGFR was 42.5 ± 28.2 ml/min at initial presentation and did not change significantly during the follow-up at 1 month (45.5 ± 30.0) and 6 (44.0 ± 21.0) months, respectively (\( P = 0.79 \)).

Circulating Ang-2 correlates with disease activity and the extent of endothelial cell detachment

Next we analysed the correlation of Ang-2 with disease activity in all (\( n = 35 \)) AAV patients (active and remission). Circulating Ang-2 showed a strong positive correlation with BVAS that was confirmed by linear regression analysis (\( r^2 = 0.51, P < 0.0001, n = 35 \)) (Figure 3A).

Since all AAV patients (active and remission) had biopsy-proven glomerulonephritis, we quantified proteinuria, erythrocyturia and creatinine clearance to address vascular inflammation within the renal vasculature. Ang-2
Angiopoietin-2 correlates with disease activity and the extent of endothelial cell detachment. Scatter plots of (A) Birmingham Disease Activity Score (BVAS) and (B) circulating endothelial cell (CEC) numbers in 35 AAV patients (active AAV n = 15; AAV in remission n = 20).

was associated with proteinuria ($r^2 = 0.47; P = 0.0002$) and erythrocyturia ($r^2 = 0.24; P = 0.005$). Interestingly, there was no association between Ang-2 and the estimated creatinine clearance (Cockcroft–Gault formula) ($r^2 = 0.002, P = 0.83$). Ang-2 was not detectable in urine samples from six healthy controls (data not shown).

Finally, Ang-2 concentrations were correlated with individual CEC numbers to identify an association between Ang-2 and endothelial cell detachment in vivo. Indeed, a highly significant positive correlation was detected between Ang-2 and CEC number ($r^2 = 0.56, P < 0.0001; n = 35$) (Figure 3B). No correlation was detected between Ang-2 levels and ANCA titres ($r^2 = 0.03, P = 0.37$).

Application of different Ang-2 cut-off concentrations identifies active AAV

Although this study was not designed to analyse the value of Ang-2 as a disease marker, we calculated ROC curves to address the diagnostic value of Ang-2 as a novel endothelial marker for active systemic AAV with renal involvement ($n = 15$) compared to active LGD ($n = 10$). An Ang-2 value of $>4.15$ ng/ml resulted in 100% specificity and a sensitivity of 68.8% in diagnosing active AAV. The AUC was $0.94 \pm 0.05$ (95% CI $0.84-1.03, P < 0.0004$).

When active AAV ($n = 15$) was compared to AAV in remission ($n = 20$), an Ang-2 value of $>2.9$ ng/ml resulted in 87.5% specificity and a sensitivity of 100% in diagnosing active AAV (AUC $0.97 \pm 0.039$ (95% CI $0.81-1.02, P < 0.0001$)).

Comparable results were obtained when CEC numbers were used to detect active vasculitis (active AAV versus active LGD: AUC $0.93 \pm 0.053$ (95% CI $0.83-1.04, P < 0.001$); active AAV versus AAV in remission: AUC $0.91 \pm 0.048$ (95% CI $0.82-1.01, P < 0.0001$)).

Last, we calculated sensitivity, specificity and predictive values by $2 \times 2$ tables including all patients ($n = 45$) enrolled in this pilot study (active AAV, AAV in remission, and active LGD). An Ang-2 cut-off value $>4.15$ ng/ml identified active AAV with 100% specificity and 65% sensitivity. The positive predictive value was 99% and the negative predictive value 84%. Changing the cut-off value to $>2.9$ ng/ml decreased specificity to 88% and increased sensitivity to 87%, respectively. The positive predictive value was 62% and the negative predictive value 92%.

Discussion

In the present study, we investigated circulating Ang-2 in patients with AAV. The results are as follows: (1) median Ang-2 concentrations were 6-fold higher in active AAV with renal involvement compared to healthy controls; (2) Ang-2 was not elevated in patients with active granulomatous disease limited to the respiratory tract (LGD) or systemic AAV in remission; (3) Ang-2 declined after initiation of successful immunosuppressive therapy in AAV with renal involvement and (4) clinical disease activity (BVAS) and the extent of endothelial cell detachment (CECs) were closely associated with individual Ang-2 concentrations.

Injury and detachment of microvascular endothelial cells is a feature of AAV [2,6,34]. Conceptually, endothelial cells need to acquire a pro-inflammatory phenotype (i.e. endothelial activation) that includes surface presentation of surface adhesion molecules as well as loosening of cell–cell and cell–matrix contacts. Assuming that endothelial activation in active AAV represents an Ang-2-dependent process, the amount of Ang-2 within the circulation reflects the extent of activated endothelial surface [21,33]. We detected a strong correlation between serum Ang-2 concentrations and BVAS in the present study. In addition, surrogate markers for the extent of glomerular vascular lesions in AAV, such as erythrocyturia and proteinuria, correlated well with Ang-2 levels. Since circulating Ang-2 is available in several microvascular beds, it cannot be considered an organ-specific molecule. However, different Ang-2 cut-off values detected AAV with renal involvement with very good specificity and sensitivity. We could recently show that a high Ang-2 concentration predicted disease activity and was independently associated with renal involvement in a cohort of SLE patients [35]. Well in line with these results, Ang-2 levels in active AAV were not different between MP A and WG, indicating that the renal vasculature is an important source of circulating Ang-2 in AAV. The fact that Ang-2 does not correlate with renal function, but does correlate with erythrocyturia/proteinuria is intriguing, and might be explained by the fact that the latter are markers of active disease in...
the kidney, while renal dysfunction is caused mainly by irreversible scarring and not active inflammation.

Endothelial activation is characterized by increased vascular permeability, leukocyte adhesion and transmigration, but also provides an environment for and endothelial cell detachment [21]. Although the effect of Ang-2 on vascular permeability and leukocyte recruitment are well defined in pre-clinical models, the impact of Ang-2 on endothelial cell detachment has not been addressed in vivo. Strong evidence for the pathogenetic involvement of Ang-2 in endothelial cell detachment in vitro came from Scharpfenecker and colleagues [26]. They demonstrated that either exogenous recombinant Ang-2 stimulated release of endogenous Ang-2 or overexpression of Ang-2 in endothelial cells heavily disrupted the integrity and cell–matrix adhesion of the endothelial layer, which could be rescued by exogenous Ang-1 [26]. Parikh et al. showed that Ang-2 simultaneously mediates disassembly of cell–cell (e.g. VE-cadherin) and cell–matrix contacts (e.g. integrins), and causes active endothelial cell contraction in a Rho kinase-dependent fashion [36]. Ang-2-deficient mice do not exhibit any vascular inflammatory responses in experimental sepsis [20], and vessels in Ang-1-overexpressing mice are resistant to leakage caused by various inflammatory stimuli [37].

Based on these data, we hypothesized that excess circulating Ang-2 would impair endothelial cell anchorage, leading to detachment of endothelial cells into the circulation. In the present study, a strong correlation between the number of CECs and Ang-2 concentrations in patients with AAV was observed. These results should be interpreted with caution, because the fact that CECs correlated with Ang-2 release does not prove causality. Thus, Ang-2 concentrations might simply reflect the amount of CECs. However, this certainly is an oversimplified view since several investigators clearly demonstrated that Ang-2-driven shutdown of Tie2 signalling is an upstream event of loss of endothelial barrier function and endothelial cell detachment. This issue has to be addressed in preclinical models [38]. However, to our knowledge, an association between circulating Ang-2 and the extent of endothelial detachment has not been reported in vivo so far. Consistently, circulating Ang-2 had a prognostic value similar to that of CECs and is beyond that a readily available and reliable methodology to assess endothelial activation [33,35].

Our study has limitations. The sample size of the present study was small. Thus, the significance of ROC procedures and 2 × 2 tables is limited. However, post hoc standard error calculation (0.032 and 0.046) for ROC procedures confirmed an adequate sample size. Using a cross-sectional study design we cannot rule out that excess circulating Ang-2 is a consequence rather than a cause of impaired renal function in patients with active glomerulonephritis. However, because of the fact that Ang-2 (i) was absent in the urine of healthy controls (data not shown), (ii) did not correlate with estimated creatinine clearance (r² = 0.002, P = 0.83) and (iii) declined during the follow-up in the presence of stationary renal function, we favour the latter explanation.

Finally, elevated circulating Ang-2 has been shown to reflect vascular damage in several diseases, and elevated Ang-2 levels are not an exclusive feature of AAV. In future work with a large national database, we will investigate Ang-2 levels according to relapse, treatment regimens, additional patterns of organ involvement and renal survival.

So far, the therapeutic efficacy of selective Ang-2 blockade has already been demonstrated in different murine models [20,39,40]. Data in support of the clinical utility of Ang-2 inhibition by a recombinant Fc protein (NCT00102830) are currently being collected in four phase II trials in patients with solid tumours. The results of the present study constitute the intriguing concept that Ang-2 (as a biomarker) can not only identify AAV patients with renal involvement, but might also be a rational drug target (as a mediator) for additional endothelial-targeted therapy in selected patients.

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