The association between anti-plasminogen antibodies and disease activity in ANCA-associated vasculitis

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Abstract

Objective. Previous studies have shown that in patients with ANCA-associated vasculitis (AAV), anti-plasminogen antibodies were associated with reduced renal function and the presence of fibrinoid necrosis and cellular crescents in renal histology. The purpose of the current study was to investigate whether anti-plasminogen antibodies are associated with the systemic disease activity of AAV.

Methods. One hundred and four Chinese patients with AAV were recruited. Anti-plasminogen antibodies were detected in sequential serum samples at initial onset and remission of the disease. Associations of anti-plasminogen antibodies with clinicopathological parameters were analysed.

Results. The prevalence of anti-plasminogen antibodies was significantly higher in AAV patients than in healthy controls (19/104 vs 0/50, \( \chi^2 = 8.8, P = 0.003 \)). The prevalence of anti-plasminogen antibodies was significantly higher in the active stage of AAV than in remission (19/104 vs 1/48, \( \chi^2 = 7.5, P = 0.013 \)). The level of anti-plasminogen antibodies (expressed as a percentage of the positive controls) correlated with the ESR (\( r = 0.207, P = 0.042 \)), serum creatinine (\( r = 0.302, P = 0.002 \)), d-dimer (\( r = 0.273, P = 0.009 \)) and the percentage of glomeruli with crescents in renal specimens (\( r = 0.393, P = 0.004 \)). The level of Birmingham vasculitis activity scores and the prevalence of arthralgia and gastrointestinal involvement in patients with anti-plasminogen antibodies were significantly higher than in patients without anti-plasminogen antibodies [22.5 (s.d. 5.63) vs. 19.4 (s.d. 4.66), \( P = 0.015 \); 63.2% vs. 25.8%, \( P = 0.002 \); 57.9% vs. 21.1%, \( P = 0.001 \), respectively].

Conclusion. Circulating anti-plasminogen antibodies were associated with systemic disease activity and renal disease activity of AAV.

Key words: plasminogen, antibodies, ANCA, vasculitis.
Methods

Patients
Blood samples of 104 consecutive patients with active AAV at initial onset, diagnosed at Peking University First Hospital from 2008 to 2011, were collected before commencing immunosuppressive treatment. All the patients met the Chapel Hill Consensus Conference (CHCC) definition of AAV [9]. Patients with secondary vasculitis or with co-morbid renal diseases, such as anti-GBM nephritis, IgA nephropathy, diabetic nephropathy, lupus nephritis or membranous glomerulonephropathy, were excluded. Blood samples of 48 patients with AAV who achieved remission after immunosuppressive therapy were also collected at their regular ambulatory visits. The time of sampling was 15.5 (s.d. 3.0) months after remission was achieved. All 48 patients were among the 104 patients described above. Fifty-three of the 104 received renal biopsy at diagnosis and before commencing immunosuppressive therapy. Fifty age- and gender-matched healthy blood donors were enrolled as normal controls. Twenty patients with anti-GMB disease were enrolled as disease controls. Sera from all subjects were kept at −70°C until use. The disease activity of AAV was assessed according to the BVAS [10]. Remission was defined as the ‘absence of disease activity attributable to active disease qualified by the need for ongoing stable maintenance immunosuppressive therapy’ (complete remission), or a ‘50% reduction of the disease activity score and absence of new manifestations’ (partial remission), as described previously [11]. Estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease equations [12]. This research was in compliance with the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Peking University First Hospital. Written informed consent was obtained from each participant.

Detection of serum ANCA
ANCA tests were performed by both IIF assays and antigen-specific ELISAs. Standard IIF assays were performed according to the manufacturer’s instructions (Euroimmun, Lübeck, Germany). Two highly purified known ANCA antigens, PR3 and MPO, purified as previously reported [13], were used as solid-phase ligands in ELISA. Serum levels of PR3- and MPO-ANCA were measured according to the manufacturer’s instructions using a commercial ELISA kit (Euroimmun, Lübeck, Germany). The results of ELISA-ANCA were expressed as relative units per millilitre.

Detection of anti-plasminogen antibodies
Anti-plasminogen antibodies were detected by following the method established previously [8, 14]. In brief, purified normal human plasminogen (Abcam, Cambridge, UK) diluted at 5 µg/ml in 0.05 M bicarbonate buffer (pH 9.6) was coated onto the wells of one-half of a polystyrene microtire plate (Costar, Mankato, MN, USA). The wells in the other half were coated with the same bicarbonate buffer alone to act as antigen-free wells to exclude non-specific binding. The volume of each well for this step and for subsequent steps was 100 µl. All incubations were carried out at 4°C overnight and the plates were washed three times with 0.01 M PBS containing 0.1% Tween 20 (PBST). Then the plates were blocked for 1 h with PBST containing 1% BSA at 37°C. The sera diluted at 1:100 with PBST were added in duplicate to both antigen-coated and antigen-free wells. Each plate contained a blank control, negative control and a known positive control. After incubation and washing, the wells were then incubated with 1:5000 diluted ALP-conjugated goat anti-human IgG antibody. The results were recorded as the net optical absorbance (average value of antigen wells minus average value of antigen-free wells) at 405 nm in an ELISA reader (Bio-Rad 550; Bio-Rad Laboratories, Tokyo, Japan). Positivity was defined as the mean +2 s.d. above the mean of 50 healthy control subjects. The quantitative level of anti-plasminogen antibody was expressed as the percentage of the positive control.

Renal histopathology
Fifty-three renal biopsy specimens were evaluated using direct immunofluorescence and light and electron microscopy. For light microscopy, paraffin sections stained with silver, periodic acid-Schiff, haematoxylin and eosin, and trichrome were forwarded to two pathologists. Two pathologists who were blinded to the patients’ data evaluated the slides separately, according to a previously standardized protocol for scoring renal biopsies of patients with ANCA-AAV [15–17]. Differences in scoring between the two pathologists were resolved by re-reviewing the biopsies and coming to a consensus. Local segmental sclerosis, fibrinoid necrosis and crescents were calculated as percentages of the total number of glomeruli. Interstitial and tubular lesions were scored semi-quantitatively on the basis of the percentage of the tubulointerstitial compartment that was affected: interstitial infiltrate (– for 0%, + for 0–20%, ++ for 20–50% and +++ for >50%), interstitial fibrosis (– for 0%, + for 0–50% and ++ for >50%) and tubular atrophy (– for 0%, + for 0–50% and ++ for >50%).

Statistical analysis
Quantitative data are expressed as mean (s.d.), median (range) or median [interquartile range (IQR)] or number (%) as appropriate. Differences in continuous variables between groups were assessed using the t-test (for data that were normally distributed) or non-parametric t-test (for data that were not normally distributed) as appropriate. Differences of semi-quantitative results were tested using the Mann–Whitney U test. Differences in categorical variables were compared using the χ² test. The Pearson test was used for correlation analysis. The probability of progressing to end-stage renal disease (ESRD) and relapse was derived from Kaplan–Meier analysis. Differences were considered significant if the P-value was <0.05. The statistical analysis was performed using the SPSS statistical software package (version 16.0; SPSS, Chicago, IL, USA).
Results

General patient data

Among the 104 patients with AAV, 47 (45.2%) were male and 57 (54.8%) were female, with an age of 59.3 (S.D. 15.1) years at diagnosis. All 104 AAV patients in our study presented renal involvement of vasculitis. Seven patients were cytoplasmic ANCA (cANCA) positive and all these sera recognized PR3; 97 patients were pANCA positive and all these sera recognized MPO. Among the 104 patients with AAV, 16 were classified as GPA, 84 were classified as MPA, 2 were classified as EGPA and 2 were classified as renal-limited vasculitis. The level of initial serum creatinine was 306.1 (S.D. 217.5) (range 40.0–1046.0) μmol/l. The level of BVAS in the 104 patients in the active stage was 20.0 (S.D. 5.0) and in the 48 patients in remission it was 0. The general data for these patients are listed in Table 1.

Prevalence of anti-plasminogen antibodies

Among the 104 patients with AAV, 19 were positive for anti-plasminogen antibodies. The prevalence of anti-plasminogen antibodies in active AAV patients was significantly higher than in normal controls (19/104 vs 0/50, χ² = 8.8, P = 0.003). In the 20 patients with anti-GBM disease, who only expressed antibodies reactive with GBM, not ANCA, only one had anti-plasminogen antibodies.

In the 7 PR3-ANCA-positive patients and the 97 MPO-ANCA-positive patients, the prevalence of anti-plasminogen antibodies was 42.8% (3/7) and 16.4% (16/97), respectively (3/7 vs 16/97, χ² = 1.53, P = 0.216). Of the 16 GPA patients, 3 (18.8%) were positive for anti-plasminogen antibodies, as compared with 15 of 84 (17.9%) MPA patients. No significant differences in gender or age were found between anti-plasminogen-positive patients and anti-plasminogen-negative patients.

Anti-plasminogen antibodies in AAV patients in the active stage, remission and relapse

Follow-up data were available for all 104 patients. Eleven of 104 patients died in the acute phase of AAV before remission was achieved. The other 93 patients achieved remission (complete or partial). As described above, blood samples from the remission stage of AAV were available in 48 of the 93 patients. The prevalence of anti-plasminogen antibodies was significantly higher in AAV patients with active disease than those in remission (19/104 vs 1/48, χ² = 7.53, P = 0.013). The levels of anti-plasminogen antibodies (expressed as a percentage of the positive controls) were significantly higher in AAV patients with active disease than those in remission or normal controls [median 15.6 (IQR 9.1–29.4) vs 7.6 (3.1–12.4), P < 0.001; median 15.6 (IQR 9.1–29.4) vs 1.0 (0–6.71), P < 0.001, respectively].

For patients with sequential blood samples (n = 48), 7 patients were positive for anti-plasminogen antibodies in the active stage and only 1 of these patients remained positive in remission. For these 48 patients, the level of anti-plasminogen antibodies (expressed as a percentage of the positive controls) was significantly higher in the active stage than in remission [median 16.2 (IQR 10.9–29.4) vs 7.6 (3.1–12.4), P < 0.001] (Fig. 1).

Among the 48 patients in remission, 3 were PR3-ANCA positive and the other 45 were MPO-ANCA positive. In the three patients with PR3-ANCA, ANCA remained positive despite remission. In the 45 MPO-ANCA patients, ANCA became negative in remission in three patients. However, there was no significant difference in ANCA levels (in ELISA) between the active stage and in remission [median 138.0 (IQR 90.0–200.0) vs 134.0 (113.3–200.0)]

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of clinical and pathological data of AAV patients with and without anti-plasminogen antibodies</th>
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<tbody>
<tr>
<td>Feature</td>
<td>Anti-plasminogen antibodies positive (n = 19)</td>
</tr>
<tr>
<td>Male/female</td>
<td>8/11</td>
</tr>
<tr>
<td>Age, mean (s.d.), years</td>
<td>64.1 (9.51)</td>
</tr>
<tr>
<td>PR3-ANCA/MPO-ANCA</td>
<td>3/16</td>
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<tr>
<td>Renal involvement of vasculitis, n (%)</td>
<td>19 (100)</td>
</tr>
<tr>
<td>Dialysis-dependent at diagnosis, n (%)</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td>Arthralgia, n (%)</td>
<td>12 (63.2)</td>
</tr>
<tr>
<td>Alveolar interstitial infiltration, n (%)</td>
<td>12 (63.2)</td>
</tr>
<tr>
<td>ENT, n (%)</td>
<td>10 (52.6)</td>
</tr>
<tr>
<td>Gastrointestinal tract, n (%)</td>
<td>11 (57.9)</td>
</tr>
<tr>
<td>Nervous system, %</td>
<td>7 (36.8)</td>
</tr>
<tr>
<td>ANCA level, mean (s.d.), RU/ml</td>
<td>124 (56.8)</td>
</tr>
<tr>
<td>CRP, median (IQR), g/l</td>
<td>19.2 (4.68–33.0)</td>
</tr>
<tr>
<td>ESR, median (IQR), mm/1h</td>
<td>84.0 (55.0–122.0)</td>
</tr>
<tr>
<td>Initial serum creatinine, median (IQR), μmol/l</td>
<td>428.0 (258.0–645.0)</td>
</tr>
<tr>
<td>Initial eGFR, median (IQR), ml/min/1.73m²</td>
<td>10.0 (6.5–21.8)</td>
</tr>
<tr>
<td>BVAS, mean (s.d.)</td>
<td>22.5 (5.63)</td>
</tr>
<tr>
<td>D-dimer, median (IQR), mg/l</td>
<td>1.3 (0.74–2.71)</td>
</tr>
<tr>
<td>Glomeruli with crescents, % (range)</td>
<td>54.7 (0–86.96) (n = 15)</td>
</tr>
</tbody>
</table>

RU: relative units.
Levels of anti-plasminogen antibodies in AAV patients in the active stage and remission.

RU/ml, \( P = 1.0 \) and 116.0 (76.0, 200.0) vs 99.0 (22.0, 200.0) RU/ml, \( P = 0.197 \) for PR3-ANCA and MPO-ANCA, respectively]. This indicated that the association between the levels of anti-plasminogen antibodies and disease activity was closer than the association between ANCA levels and disease activity.

Among the 48 AAV patients with sequential serum samples, 10 patients experienced relapses during a follow-up of 23.5 (IQR 3.25–50.75) months. We further measured anti-plasminogen antibodies in sequential serum samples of these 10 patients. Only 1 of 10 patients was positive for anti-plasminogen antibodies at the relapse stage. Anti-plasminogen antibodies were positive at initial presentation for that patient, which became negative in remission and positive again at subsequent relapse. For the other 38 patients who did not experience relapse, we further measured anti-plasminogen antibodies in several serum samples per patient at different time points during remission. Anti-plasminogen antibodies in these sera were all negative.

Association between anti-plasminogen antibodies and clinicopathological parameters in AAV patients

Fibrin degradation products (FDPs), d-dimer, ANCA level, CRP, ESR and serum creatinine measurements were done at the same time as serum samples were collected for this study. Correlation analysis showed that the level of anti-plasminogen antibodies (expressed as a percentage of the positive controls) correlated with the level of ESR (\( r = 0.207, \ P = 0.042 \)), serum creatinine (\( r = 0.302, \ P = 0.002 \)) and d-dimer (\( r = 0.273, \ P = 0.009 \)). The level of anti-plasminogen antibodies was negatively correlated with eGFR (\( r = -0.306, \ P = 0.002 \)). Correlation analysis also showed that the level of anti-plasminogen antibodies correlated with the percentage of glomeruli with crescents in the renal specimens (\( r = 0.393, \ P = 0.004 \)) (Fig. 2). The patients with anti-plasminogen antibodies had a significantly higher level of BVAS than patients without anti-plasminogen antibodies [22.5 (s.d. 5.6) vs 19.4 (s.d. 4.7), \( P = 0.015 \)]. The prevalence of arthralgia, pulmonary infiltration, gastrointestinal tract involvement and nervous system involvement in patients with anti-plasminogen antibodies was significantly higher than in patients without anti-plasminogen antibodies (63.2% vs 25.8%, \( P = 0.002 \); 63.2% vs 35.2%, \( P = 0.025 \); 57.9% vs 21.1%, \( P = 0.001 \); 36.8% vs 12.9%, \( P = 0.013 \), respectively). No significant differences in muscle pain, skin rash, ENT involvement, FDPs and dialysis-dependence at diagnosis were found in patients with or without anti-plasminogen antibodies.

The renal histopathological parameters of 53 AAV patients were further analysed (Table 1). The number of glomeruli per biopsy was 23 (range 5–53). Fifteen of these 53 patients were positive for anti-plasminogen antibodies. The percentage of glomeruli with crescents in patients positive for anti-plasminogen antibodies was significantly higher than in patients negative for anti-plasminogen antibodies [54.7 (range 0–86.9) vs 21.4 (0–84.0), \( P = 0.002 \)]. In the Kaplan–Meier analysis, the probability of progressing to ESRD (Fig. 3A) and relapse (Fig. 3B) was not significantly different between patients with and without anti-plasminogen antibodies (\( P = 0.46 \) and \( P = 0.34 \), respectively).

**Discussion**

The concept of complementary protein–protein interactions led to the discovery of a previously unidentified autoantigen [18, 19]. Potential cPR3m (a protein complementary to the middle part of PR3)-homologous structures were found in various pathogens, and anti-cPR3 reactivity was increased in PR3-ANCA-positive patients compared with MPO-ANCA patients [20]. These findings were disputed by Tadema et al. [21] in a separate study that failed to detect increased anti-cPR3 reactivity in PR3-ANCA-AAV patients. The anti-plasminogen antibodies reacted with a highly restricted motif on the plasminogen, also found in cPR3105 [14]. Meanwhile, Berden et al. [8] reported the presence and functionality of anti-plasminogen antibodies in both PR3- and MPO-ANCA patients, suggesting a discordance between anti-plasminogen and anti-cPR3 antibodies in MPO-ANCA patients. More importantly, it was found that anti-plasminogen antibodies correlate with reduced renal function and the percentages of glomeruli with fibrinoid necrosis and cellular crescents in AAV patients [8]. Thus it was reasonable to speculate that the level of serum anti-plasminogen antibodies might be associated with the systemic disease activity of AAV.

We identified the anti-plasminogen antibodies in ~18% of AAV patients. This prevalence was similar to two other studies [8, 14]. There was no significant difference in the prevalence of anti-plasminogen antibodies between MPO-ANCA- and PR3-ANCA-positive patients.

In AAV renal involvement, patients with anti-plasminogen antibodies had a significantly higher level of serum creatinine and higher percentages of glomeruli with cellular crescents. These results confirmed and further extended previous observations [8]. Plasminogen plays an important role in protecting the glomerulus from acute inflammatory injury [6]. Fibrin exudate is important...
**Fig. 2** Correlation between levels of anti-plasminogen antibodies and cliniopathological parameters in patients with AAV.

- **A** The correlation of anti-plasminogen antibodies and ESR.
- **B** The correlation of anti-plasminogen antibodies and eGFR.
- **C** The correlation of anti-plasminogen antibodies and D-dimer.
- **D** The correlation of anti-plasminogen antibodies and the percentage of glomeruli with crescents in renal specimens.

**Fig. 3** Kaplan-Meier analysis of the probability of (A) progressing ESRD and (B) relapse between patients with and without anti-plasminogen antibodies.

The solid line represented patients with anti-plasminogen antibodies. The dashed line represents patients without anti-plasminogen antibodies. **(A)** Kaplan-Meier analysis of the probability of progressing ESRD between patients with and without anti-plasminogen antibodies. **(B)** Kaplan-Meier analysis of the relapse between patients with and without anti-plasminogen antibodies.
in stimulating the crescent formation [22]. Anti-plasminogen antibodies might influence glomerular pathology through a disturbance of fibrinolysis in the microvasculature [8].

The most important finding in the current study was that anti-plasminogen antibodies are associated with the systemic disease activity of AAV. Patients with anti-plasminogen antibodies had a significantly higher level of BVAS than those without. The serum levels of anti-plasminogen antibodies were associated with ESR. Patients with anti-plasminogen antibodies had more common or more severe organ involvement, including lungs, gastrointestinal tract, nervous system and kidneys, than patients without anti-plasminogen antibodies. More importantly, it was found that the level of anti-plasminogen antibodies in remission is lower than in the active phase for every patient with sequential serum samples. Vasculitis is an inflammatory process of the blood vessels and is characterized by an influx of inflammatory cells with fibrinoid necrosis in the small vessel walls [14]. Plasminogen plays a major role in inflammatory cell recruitment [23]. Therefore we speculated that the complex of plasminogen and anti-plasminogen antibodies may participate in the inflammatory reaction. Anti-plasminogen antibodies might promote the development of inflammation and disease activity in AAV. Moreover, fibrinoid necrosis is a ubiquitous feature in the histology of ANCA-AAV [24]. This disturbance might be initiated or accelerated by anti-plasminogen antibodies through a disturbance of fibrinolysis in the microvasculature [10].

Previous studies found that anti-plasminogen antibodies can delay fibrinolysis [8] and correlated with venous thromboembolic event episodes in AAV patients [14]. Although we failed to find any correlation between anti-plasminogen antibodies and venous thromboembolic events since it was retrospective study, it was found in the current study that patients with anti-plasminogen antibodies have higher levels of d-dimer than those without. This supported, at least to some extent, the relationship between anti-plasminogen antibodies and thrombotic events [14].

One limitation of the current study was the failure to demonstrate that the levels of anti-plasminogen antibodies could predict relapse. There are several possible reasons. First, relapse events were uncommon in our cohort. That was probably because most of our patients were MPO-ANCA positive, and thus less likely to relapse compared with PR3-ANCA-positive patients. Moreover, the prevalence of anti-plasminogen antibodies was not high, even at the initial onset of AAV (19/104), let alone in relapse. Therefore it is not easy to get positive results of anti-plasminogen antibodies in a cohort without enough endpoint events (relapse). To achieve more convincing results of anti-plasminogen antibody in predicting relapse, a larger sample size, in particular, with more relapse events, is needed for further investigation. In conclusion, circulating anti-plasminogen antibodies were associated with systemic disease activity as well as renal disease activity in ANCA-AAV.

**Rheumatology key messages**

- Circulating anti-plasminogen antibodies were associated with the systemic disease activity of ANCA-AAV.
- Circulating anti-plasminogen antibodies were associated with the renal disease activity of ANCA-AAV.

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