Pathogenetic and clinical aspects of ANCA-associated vasculitis
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CHAPTER 5

Complement activation is involved in renal damage in human antineutrophil cytoplasmic autoantibody associated pauci-immune vasculitis

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Abstract

[Objective] This study was to investigate the evidence for complement activation in renal biopsy specimens of patients with MPO-ANCA-associated pauci-immune vasculitis.

[Methods] Renal biopsy specimens from seven patients with MPO-ANCA positive pauci-immune necrotizing crescentic glomerulonephritis (NCGN) were used to detect the staining of membrane attack complex (MAC), C3d, C4d, mannose-binding lectin (MBL), factor B and factor P using immunohistochemistry and immunofluorescence. Renal tissue from seven patients with minimal change disease (MCD) and two normal renal tissues were used as controls.

[Results] MAC, C3d, factor B and factor P could be detected in glomeruli and small blood vessels with active vasculitis of patients with pauci-immune AAV, but not or scarcely in patients with MCD and in normal renal tissues. C3d and factor B co-localized with MAC, factor P colocalized with C3d. MBL and C4d were not detected in patients with AAV.

[Conclusion] The alternative pathway of the complement system is involved in renal damage of human pauci-immune AAV.
Complement activation in renal damage of human AAV

Introduction

Antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is characterized by pauci-immune necrotizing crescentic glomerulonephritis (NCGN). "Pauci-immune" in renal histology indicates the relative lack of immunoglobulin and complement deposition within the kidney by routine immunofluorescence (IF) and no electron-dense deposits by electron microscopy (EM) [1]. However, substantial evidence suggests that ANCA play a crucial role in the pathogenesis of AAV. Following immunization of MPO-knockout (Mpo-/-) mice with mouse myeloperoxidase (MPO), the direct pathogenic effect of anti-MPO IgG in AAV has been convincingly demonstrated by adoptive transfer of anti-MPO positive splenocytes into Rag2-/- mice and passive transfer of anti-MPO IgG alone into wild type mice [2]. Recent studies in the same animal model provided strong evidence that the complement system is involved in the pathogenesis of AAV. Xiao et al demonstrated that induction of glomerulonephritis with anti-MPO IgG or anti-MPO splenocytes requires activation of the alternative pathway of complement but not the classic or the lectin pathway [3]. Huugen et al further showed that treatment with C5 inhibiting antibodies could markedly attenuate anti-MPO IgG/lipopolysaccaride-induced NCGN [4].

Although human AAV is characterized by paucity of immune deposits, complement activation is actively involved in the majority of human glomerulonephritides [5, 6]. Hinglais et al [7] already found over two decades ago when ANCA serology was not routinely used, deposition of MAC and C3d in renal sections of various glomerulonephritides including diffuse crescentic glomerulonephritis without anti-GBM antibodies. In 2004, Haas et al studied renal sections of 126 cases of ANCA-associated glomerulonephritis by immunofluorescence (IF) and electron microscopy (EM). They found some patients had deposits of immunoglobulins or complement, either by IF or EM [8]. Recent studies from Neumann et al [9] in patients with primary AAV and Yu et al [10] in patients with propylthiouracil-induced AAV also demonstrated deposits of immunoglobulins or complement in renal biopsy specimens, either by IF or EM. These data indicate that, in human pauci-immune AAV, complement activation might be involved in active vasculitis. However, in a considerable number of patients with AAV, deposits of immunoglobulins or complement
can not be found by routine IF or EM. Therefore, it remains inconclusive whether the complement system plays a role in the development of human AAV. In this study we investigated complement activation in renal biopsy specimens of patients with MPO-ANCA-associated crescentic glomerulonephritis without immune deposits by routine IF and EM.

Materials and methods

Patients

Consecutive renal biopsies (n=7) showing pauci-immune NCGN were selected from patients with AAV. “Pauci-immune” was defined as absence of deposition of IgG, IgA, IgM, C3c and C1q by routine direct immunofluorescence as assessed on a scale of 0 to 4+ [1], and absence of electron dense-deposits by electron microscopy. All the seven patients had a positive test for perinuclear ANCA (P-ANCA) by indirect immunofluorescence and MPO-ANCA by antigen-specific ELISA. Patients with secondary vasculitis or with any other coexisting renal disease were excluded. As complement activation has been considered not to be involved in minimal change disease (MCD) [5, 11], renal biopsy specimens from seven patients with MCD were used as disease controls. Patients with MCD was negative on routine direct immunofluorescence for IgG, IgA, IgM, C3c and C1q and no electron-dense deposits found by electron microscopy. Renal tissue, obtained from the normal part of a nephrectomized kidney due to renal carcinoma and a 13-year-old girl investigated for episodes of macroscopic hematuria, was used as normal control. Their renal tissue was considered normal by light microscopy, immunofluorescence and electron microscopy.

Routine renal histopathology

Staining for IgG, IgA, IgM, C3c and C1q on fresh frozen renal tissue was routinely performed immediately after the renal biopsy was taken using corresponding FITC-conjugated antibodies (Dako A/S, Copenhagen, Denmark). For light microscopy, renal paraffin sections were routinely stained with hematoxylin & eosin, periodic acid-Schiff, Masson trichrome and periodic acid-silver methenamine. Sections were evaluated by experienced pathologists, not informed about the clinical data.
Detection of renal deposition of complement components by immunohistochemistry

To study complement activation, immunohistochemical staining was performed for C3d, MBL, C4d on 4μm deparaffinized sections of formaldehyde-fixed renal tissue using rabbit anti-human polyclonal antibody C3d (Dako A/S, Copenhagen, Denmark), mouse anti-human monoclonal antibody MBL (Abcam, Cambridge, UK) and rabbit anti-human polyclonal antibody C4d (Abcam, Cambridge, UK) as primary antibodies. Optimal antibody dilutions were pre-determined by means of titration on renal sections from patients with immune complex mediated crescentic glomerulonephritis or lupus nephritis. The optimal incubation time and temperature for detecting different complement components were also pre-determined. Antibodies against C3d, MBL, and C4d were used in dilutions of 1:2000, 1:500 and 1:40, respectively, in 0.01 mol/L phosphate buffered saline (PBS), pH 7.4. Sections were deparaffinized in xylene-ethanol at room temperature and rehydrated in PBS immediately before protease treatment. The sections were then treated with 0.4% pepsin (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 40min respectively. Digestion was terminated by repeated washings in PBS. Sections were immersed into freshly prepared 3% hydrogen peroxide in methanol solution for 10min at room temperature to quench endogenous peroxidase activity. To block non-specific staining, sections were incubated with 1% BSA in PBS at 37°C for 30min. The primary antibodies were added on each section directly. Antibodies against C3d, MBL, and C4d were incubated overnight at 4°C. The detection system used, Dako EnVision HRP (Dako A/S, Copenhagen, Denmark), was an avidin-free two-step indirect method with goat anti-rabbit and goat anti-mouse immunoglobulins conjugated with horse-radish peroxidase (HRP) as secondary antibodies. The secondary antibodies were incubated for 30min at 37°C. Next, sections were developed in fresh hydrogen peroxide plus 3-3-diaminobenzidine tetrahydrochloride solution for 2min respectively. Finally, in order to clearly visualize the location of deposited complement components, sections were counterstained with Periodic acid-Schiff (PAS) and Mayer’s hematoxylin. As negative controls, primary antibodies were replaced by normal rabbit IgG or normal mouse IgG.
The terminal product of complement activation, C5b-9, also designated as membrane attack complex (MAC), was detected on 5μm frozen renal tissue sections by immunohistochemistry. The primary antibody, mouse anti-human MAC (Dako A/S, Copenhagen, Denmark), was diluted 1:50 in PBS and incubated for 60min at room temperature. The detection system was identical to that described above. Sections were developed in fresh hydrogen peroxide plus 3-3-diaminobenzidine tetrahydrochloride solution for 2min. Sections were counterstained with hematoxylin. Renal sections from a patient with lupus nephritis were used as positive controls. Negative controls were performed by replacing the primary antibodies with normal mouse IgG.

**Detection of renal deposition of complement components by immunofluorescence using laser scanning confocal microscopy**

Sections cut at a thickness of 5μm from frozen renal biopsy tissue were air-dried at room temperature for 30 min and fixed in iced acetone for 10min. Endogenous peroxidase activity was blocked by immersion of the sections into freshly prepared 3% hydrogen peroxidase-blocking solution for 10min at room temperature. Between stages, the sections were washed three times with PBS for 10min. To block non-specific staining, sections were incubated in 1% BSA in PBS at 37°C for 30min. Sheep anti-human factor B (Serotec, Oxford, UK dilution 1:25 in PBS), mouse anti-human factor P (Abcam, Cambridge, UK, dilution 1:25), rabbit anti-human C4d (dilution 1:20), and rabbit anti-human C3d (dilution 1:2000) were incubated at 4°C overnight as primary antibodies, respectively. FITC-labeled rabbit anti-sheep IgG (Southernbiotech, Birmingham, USA, dilution 1:200), FITC-labeled goat anti-mouse IgG and goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology, Beijing, China, both diluted 1:100), were used as secondary antibodies at 37°C for 30min respectively. Then, mouse anti-human MAC (Dako A/S, Copenhagen, Denmark, dilution 1:50) was added to the sections with factor B and C3d, or rabbit anti-human C3d was added to the sections with factor P for 60min at 37°C. TRITC-labeled goat anti-mouse IgG or TRITC-labeled goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology, Beijing, China, both diluted 1:100) was used as secondary antibody for 30min at 37°C. Sections were stored shortly at 4°C before being scored using a confocal microscope (Olympus viewer 1000,
Sections of renal tissue from patients with lupus nephritis and IgA nephropathy were used as positive controls. Negative controls were performed by omitting or replacing the primary antibodies.

**Criteria for semiquantitative scoring**

**Renal histopathology**

In a previously standardized protocol for scoring renal biopsies of patients with ANCA-associated vasculitis [12, 13], the number of glomeruli with lesions was expressed as a percentage of the total number of glomeruli in a biopsy.

All sections were evaluated by two pathologists. Both pathologists scored the biopsies separately, blinded to patients’ data and the scores of the other observer, according to the standardized protocol for scoring renal biopsies. Each glomerulus was scored separately for the presence of fibrinoid necrosis, crescents (cellular/fibrocellular/fibrous), glomerular sclerosis and granulomatous reactions. Differences in scoring between the two pathologists were resolved by re-reviewing the sections and coming to a consensus.

**Renal immunohistochemistry**

The extent of glomerular staining for C3d and MAC was evaluated at 400X magnification and scored semiquantitatively: 0, no staining; 1, weak and spotty intraglomerular staining; 2, moderate and segmental intraglomerular staining; and 3, strong and diffuse (involving more than 50% of the glomerular surface area) intra-glomerular staining.

**Renal immunofluorescence**

The intensity of immunofluorescence staining of complement components in glomeruli was graded on a scale of 0 to 4+ [1]: 0, no staining; 1, mild staining; 2, moderate staining; 3, moderate-high staining; 4, high staining on a high-power field.

Co-localization of different complement components was judged by merging of the green fluorescence of FITC and the red fluorescence of TRITC.

**Statistical analysis**

Differences in quantitative parameters between groups were assessed using Student t-test (for data normally distributed) or
nonparametric test (for data not normally distributed). Differences in semiquantitative data were tested using Kruskal Wallis H one-way analysis and Mann-Whitney U test. Spearman’s correlation was used to measure the relationship between two non-parametric variables or one non-parametric variable with one parametric variable. Differences in qualitative data were compared using the Chi square test. A \( p \)-value of less than 0.05 was considered statistically significant. Analysis was performed with SPSS statistical software package (version 11.0; Chicago, IL).

Results

Routine renal histopathology

Renal biopsy specimens from the seven patients with MPO-ANCA-associated vasculitis did not show any staining for IgA, IgG, IgM, C3c and C1q by routinely performed direct immunofluorescence. No electron dense deposit was observed by electron microscopy.

Clinical data of patients with AAV and MCD are listed in Table 1.

<table>
<thead>
<tr>
<th>Features</th>
<th>AAV (N=7)</th>
<th>MCD (N=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median and range)</td>
<td>67; 51-75</td>
<td>29;14-54</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>5/2</td>
<td>3/4</td>
</tr>
<tr>
<td>Urinary protein (g/24h; normal range:0-0.15)</td>
<td>1.46±1.33</td>
<td>4.48±3.89</td>
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<td>Serum C3 (g/L; normal range: 0.85-1.93)</td>
<td>0.89±0.27</td>
<td>1.04±0.14</td>
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<tr>
<td>Serum C4 (g/L; normal range: 0.12-0.36)</td>
<td>0.19±0.06</td>
<td>0.21±0.04</td>
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</table>

Deposition of complement components by immunohistochemistry and their association with renal histopathology

Renal histological parameters of the 7 patients with MPO-ANCA associated glomerulonephritis are listed in Table 2.

MAC

MAC represents the terminal product of complement activation. The number of glomeruli scored per biopsy of frozen sections for MAC in patients with AAV was 3.43±1.72 (range 2-7). MAC was detected clearly along the glomerular capillary wall and mesangial area of crescentic glomeruli of patients with AAV in a granular pattern (Figure 1A), but was scarcely found in renal biopsy specimens from patients with MCD or in normal control renal tissue. In patients with AAV, 24/24 (100%) glomeruli in
Complement activation in renal damage of human AAV

Frozen sections were positive for deposition of MAC, the intensity of which was scored as 1 (5/24 glomeruli), 2 (4/24 glomeruli), and 3 (15/24 glomeruli) (Table 2). In patients with MCD, only 2/28 (7.14%) glomeruli were positive and only scored as 1 (1/28 glomeruli) and 3 (1/28 glomeruli). The extent of MAC staining in glomeruli was negatively correlated with the percentage of glomeruli with normal histology and mild mesangial proliferation in patients with AAV ($r=-0.767$, $P<0.05$). Compared with the deposition of MAC in glomeruli in patients with MCD, the deposition of MAC in patients with AAV was significantly stronger ($\chi^2=44.57$, $P<0.01$; by Mann-Whitney U test, mean rank 39.71 vs. 15.18, $P<0.01$).

In addition, patients with AAV had granular staining of MAC in small arteries (Figure 1B).

No deposit of MAC was observed in frozen sections from the 2 normal control renal tissues (total 8 glomeruli).

### Table 2. Renal parameters and histology in 7 patients with MPO-ANCA associated glomerulonephritis

<table>
<thead>
<tr>
<th>patient</th>
<th>Scr at the time of renal biopsy (µmol/L)</th>
<th>M/N (%)</th>
<th>CC (%)</th>
<th>C3d in M/N on IHC</th>
<th>C3d in CC on IHC</th>
<th>MAC in glomeruli of frozen sections on IHC</th>
<th>Factor B in glomeruli on IF</th>
<th>Factor P in glomeruli on IF</th>
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<tbody>
<tr>
<td>1</td>
<td>801</td>
<td>0</td>
<td>13.3</td>
<td>2(1)</td>
<td>3(2)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>0(1)</td>
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<td></td>
<td></td>
<td>3(1)</td>
<td></td>
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<tr>
<td>2</td>
<td>762</td>
<td>48.8</td>
<td>26.8</td>
<td>0(16)</td>
<td>0(1)</td>
<td>3(3)</td>
<td>0(2)</td>
<td>1(1)</td>
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<tr>
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<td>1(4)</td>
<td>1(10)</td>
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<tr>
<td>3</td>
<td>392</td>
<td>57.1</td>
<td>4.76</td>
<td>0(5)</td>
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<td>1(1)</td>
<td>0(1)</td>
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<td>4</td>
<td>306</td>
<td>50.0</td>
<td>5.56</td>
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<td>1(1)</td>
<td>0(2)</td>
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<tr>
<td>5</td>
<td>217</td>
<td>0</td>
<td>15.4</td>
<td>3(2)</td>
<td>3(7)</td>
<td>0(1)</td>
<td>1(1)</td>
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<tr>
<td>6</td>
<td>169</td>
<td>25.0</td>
<td>25.0</td>
<td>3(2)</td>
<td>2(1)</td>
<td>2(1)</td>
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<tr>
<td>7</td>
<td>97</td>
<td>67.6</td>
<td>18.9</td>
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<td>1(5)</td>
<td>1(3)</td>
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M/N: mild mesangial proliferation and/or normal glomeruli; CC: cellular crescent; IHC: immunohistochemistry; IF: immunofluorescence. The number out of the parenthesis is the score at the scale of 0-3. The number in the parenthesis is the number of glomeruli at the corresponding score.
**Figure 1** MAC and C3d staining. A) and B) Frozen sections for MAC by immunohistochemistry; C)-F) Paraffin sections for C3d by immunohistochemistry, from the same patient with AAV; G)-I) Frozen sections for co-localization of C3d and MAC by immunofluorescence. A) Positive staining of MAC is showed in the whole glomerulus with cellular crescent of a patient with AAV, and a capillary tuft near the crescent is clearly demonstrated positive (indicated by arrow). B) Positive staining of MAC in the arteriole wall of a patient with AAV. C) No C3d staining in a normal glomerulus of a patient with AAV. D) Positive staining of C3d at the site of fibrinoid necrosis (indicated by arrow). E) Positive staining of C3d along the wall of a small artery with fibrinoid necrosis. F) Granular positive staining of C3d along the glomerular capillary wall near cellular crescent (indicated by arrows). G) Granular positive staining of C3d by immunofluorescence along the glomerular capillary wall and mesangial area in a patient with AAV. H) Granular positive staining of MAC by immunofluorescence along the glomerular capillary wall and mesangial area in the same section with Figure 1G. I) C3d and MAC co-localized completely along the glomerular capillary wall and mesangial area, merged in yellow. (A and E: Original magnification×200; B-D and F: Original magnification×400; G-I: Original magnification×600)

**C3d**

The number of glomeruli scored for C3d per biopsy of paraffinized sections in patients with AAV was 21.9±12.4 (range 8-41).

No positive staining of C3d was found in normal glomeruli from renal specimen of patients with AAV (Figure 1C), while only scantly positive
staining was found in glomeruli with mild mesangial proliferation. Positive staining of C3d was detected at the area of glomerular fibrinoid necrosis (Figure 1D) and was also found in small arteries and arterioles with fibrinoid necrotic lesions (Figure 1E). Granular positive staining of C3d was mainly along the glomerular capillary wall near cellular crescents (Figure 1F) and mesangial area in cellular crescents from patients with AAV.

C3d staining can be found in glomeruli with cellular, fibrocellular, fibrous crescents and glomerular sclerosis. Because it has less specificity for C3d staining in scarred area, we focused on C3d staining at active lesions such as cellular crescents (Table 2). The extent of positive staining of C3d in cellular crescents was much stronger than those with mild mesangial proliferation and/or normal glomeruli (M/N) in AAV and those with MCD. Among the 3 groups, by Kruskal Wallis test, $\chi^2=62.8$, $P<0.001$; by Mann-Whitney U test, the extent of positive staining of C3d in glomeruli with cellular crescents versus that in M/N, $P<0.001$; versus that in MCD, $P<0.001$, respectively (Figure 2).

The extent of C3d staining in glomeruli was negatively correlated with the percentage of glomeruli with normal histology and/or mild mesangial proliferation in patients with AAV ($r=-0.883$, $P<0.01$).

In patients with MCD, C3d staining was scarcely observed in glomeruli. No staining of C3d (total 27 glomeruli in paraffin section) was observed in the 2 normal renal tissues.

**MBL**

The number of glomeruli scored for MBL per biopsy of paraffinized sections in patients with AAV was 18.3±14.1 (range 8-41).

Deposition of MBL was not observed in glomeruli of normal controls, patients with MCD and patients with AAV (Figure 3A). Positive staining could be detected in a renal specimen from a patient with IgA nephropathy with mesangial proliferation and sclerosis in the absence of C1q staining, which was used as a positive control (Figure 3B).

**C4d**

The number of glomeruli scored for C4d per biopsy of paraffinized sections in patients with AAV was 20.3±13.5 (range 8-46).

Deposition of C4d was scanty in glomeruli of normal controls, patients with MCD and patients with AAV (Figure 3C). However, positive staining could be detected strongly in a renal biopsy specimen from a
patient with immune complex mediated crescentic glomerulonephritis, which was used as a positive control (Figure 3D).

Figure 2
Distribution of C3d staining in glomeruli with MCD, M/N and cellular crescents of patients with AAV as assessed on a score of 0 to 3. MCD: minimal change disease. M/N: glomeruli with mesangial proliferation and/or normal glomeruli. Numbers marked in each bar indicate the number of glomeruli at its corresponding score of C3d deposition. For C3d staining, among the 3 groups, by Kruskal Wallis test, $\chi^2=62.8$, $P<0.001$; by Mann-Whitney U test, the extent of C3d staining in glomeruli with cellular crescent versus that in MCD, $P<0.001$; versus that in M/N, $P<0.001$, respectively.

Figure 3
MBL, C4d, factor B (fB) and factor P(fP) staining. A)-D) Paraffin sections for MBL and C4d by immunohistochemistry; E)-G) Frozen sections for co-localization of fB and MAC by immunofluorescence. H)-I) Frozen sections for co-localization of fP and C3d by immunofluorescence. A) No staining of MBL in a glomerulus with cellular crescent of a patient with AAV. B) As positive control, MBL is strongly positive in the mesangial area of a glomerulus of patient with IgA nephropathy. C) No staining of C4d in a glomerulus with cellular crescent of a patient with AAV. D) As positive control, C4d is strongly granular positive along the capillary wall and mesangial area in a glomerulus of a patient with immune complex mediated crescentic glomerulonephritis. E) Granular positive staining of fB by immunofluorescence along the glomerular capillary wall and mesangial area in a patient with AAV. F) Granular positive staining of MAC by immunofluorescence along the glomerular capillary wall and mesangial area in the same section with Figure 3E. G) Factor B and MAC co-localized almost completely along the glomerular capillary wall and mesangial area, merged in yellow. H) Granular positive staining of factor P by immunofluorescence along the glomerular capillary wall and mesangial area in a patient with AAV. I) Granular positive staining of C3d by immunofluorescence along the glomerular capillary wall and mesangial area in the same section with Figure 3H. J) Factor P and C3d co-localized along the glomerular capillary wall and mesangial area, merged in yellow. (A-C: Original magnification ×200; D: Original magnification ×400; E-J: Original magnification×600)
Complement activation in renal damage of human AAV
Co-localization of complement components by immunofluorescence

By immunofluorescence and laser scanning confocal microscopy (LSCM), positive staining of MAC, C3d, factor B and factor P in glomeruli of patients with AAV were further confirmed, but C4d was not detected. Furthermore, both C3d and factor B co-localized with MAC, and factor P co-localized with C3d along the capillary wall and mesangial area in glomeruli of patients with AAV (Figures 1, G-I; Figure 3, E-G). The detailed staining scales of factor B and factor P in glomeruli were shown in Table 2. In patients with MCD and the 2 normal controls, factor B and factor P staining was not detected.

Discussion

Although a few reports have described glomerular complement deposition in crescentic glomerulonephritis [7] and in patients with ANCA-associated vasculitis [8-10], solid evidence for involvement of complement activation in human AAV is lacking.

To demonstrate involvement of complement activation in the pathophysiology of human AAV, it is crucial to find evidence for complement activation in patients with AAV showing strict pauci-immune nature of their lesions. Therefore, in the current study, the seven patients with AAV were selected based on the absence of any detectable IgA, IgG, IgM, C3c and C1q by routine direct immunofluorescence and no electron-dense deposit by electron microscopy.

Activation of the classic, lectin and alternative complement pathways results in the conversion of C3 to C3a and C3b. The three pathways converge at the activation of C5 to form a potent chemo-attractant C5a and the membrane attack complex C5b-9 (MAC). C3b, which binds to an acceptor molecule on membrane, is inactivated to iC3b by factor I and a cofactor. iC3b is further degraded by factor I and CR1 to C3c and C3dg. C3dg is trimmed by plasma proteases to C3d, which binds covalently via thiolester bond to its acceptor molecule at the activated site [14]. It has been suggested that C3d could be detected not only at active lesions at the time of ongoing complement activation, but also at lesions after complement activation. C3c is in the free state and only indicates ongoing complement activation [15]. In renal tissue of patients with AAV, the lack of C3c
Complement activation in renal damage of human AAV deposition is quite common just as this group of patients showed. Whether on earth the complement system had been activated or not during renal damage process of AAV was not determined.

In the present study, the presence of MAC, the final product of complement activation in glomeruli, arterioles and small arteries provided solid evidence for the overall complement activation. MAC may directly damage resident cellular components such as endothelium of glomerular capillaries and arterioles and glomerular mesangial cells, and through its direct effects on membranes resulting in cell lyses. A recent study revealed that MAC can up-regulate endothelial expression of leukocyte adhesion molecules which might be even more important in the generation of a neutrophilic inflammatory response than C5a [16]. Since the pathogenesis of AAV is thought to depend on activation of neutrophils [3, 17], MAC mediated neutrophil accumulation may also be an important pathogenic event in AAV as well. As a relative durable biomarker of local complement activation, positive staining for C3d in renal specimens of AAV provided further evidence for complement activation. More importantly, C3d could be detected at active lesions showing focal segmental fibrinoid necrosis and cellular crescents of glomeruli, but no or scanty staining was observed in normal glomeruli and in the glomeruli of mild mesangial proliferation. Furthermore, by laser scanning confocal microscopy, co-localization of MAC with C3d was observed in glomeruli of patients with AAV. These data demonstrate that complement activation directly involves in the pathogenic process of renal vasculitis.

As the percentage of normal glomeruli in patients with AAV has been demonstrated to be the best predictor for renal outcome [12], the negative correlation between the percentage of normal glomeruli and those with mild mesangial proliferation with the extent of C3d and MAC staining in glomeruli of patients with AAV might indirectly imply the involvement of complement activation in the process of renal damage.

Both classic and lectin pathways require activation of C4. C4d is a fragment of C4, produced during activation and binds covalently via internal thiolester bond to tissue elements at the site of activation. Therefore, it is a durable biomarker of the classic pathway activation [18]. The detection of C4d deposition in the peritubular capillaries of allografts is currently the best single biomarker of complement-fixing circulating antibodies to the
endothelium [19]. Glomerular C4d staining is commonly present in glomerular immune complex diseases [19]. Mannose-binding lectin (MBL) is a key molecule in the activation of the lectin pathway of complement by binding to carbohydrate ligands and then the activation of MBL-associated serine proteases (MASP) [20]. Deposition of MBL in association with IgA in the glomeruli, as a marker for lectin pathway activation, has been reported in a subpopulation of patients with IgAN [21, 22]. It cannot be excluded that MBL binds to a glycosylated molecule that is associated with polymeric IgA and present in the mesangial deposits of patients with IgAN [22]. Factor B and factor P are unique factors needed in the alternative complement pathway.

By immunohistochemistry techniques, we did not observe deposits of C4d and MBL in this subgroup of renal specimens of patients with AAV. Combined with the lack of staining of C1q, our study did not support the involvement of both classical pathway and lectin pathway activation of complement. On the contrary, granular factor B and factor P deposits could be detected along the glomerular capillary wall and mesangial area. Furthermore, factor B and MAC, factor P and C3d co-localized well along the glomerular capillary wall and mesangial area in the glomeruli of patients with AAV. This demonstrates the activation of the alternative pathway.

Intravital microscopy studies have confirmed that MPO-ANCA is capable of inducing glomerular accumulation of neutrophils and MPO deposition in vivo [23]. Importantly, activation of human neutrophils by human MPO-ANCA IgG induces the release of factors that activate complement resulting in the generation of C3a [3]. Increasing evidence indicates that neutrophils are a source of various complement factors including C3a and components that are unique to the alternative pathway, such as properdin [24]. It has also suggested that local neutrophil activation may result in disturbance of regulatory proteins of the complement system in necrotic cells or in injured regions of glomeruli [25-27], which play an important role in the inhibition of the complement activation. Therefore, in patients with renal damage with AAV, the vicious cycle of complement activation via the alternative pathway could be promoted and continued. Antibodies or immune complexes usually activate the classical pathway of complement. However, the possibility of initial seeding of C3b in glomeruli from the classical pathway activation in serum followed by activation of the
amplification loop of the alternative pathway could not be completely ruled out.

In AAV, renal specimens with active lesions will have neutrophils in capillary lumens adjacent to the foci of segmental necrosis in the initial stage of renal damage [28]. A similar clustering of neutrophils at sites of glomerular necrosis has been observed in a mouse model of pauci-immune crescentic glomerulonephritis caused by anti-myeloperoxidase ANCA [17]. The disappearance of C3c, which exists in a free stage after the complement activation, might be degraded by the proteinases released by the infiltrated neutrophils or phagocyted by neutrophils rapidly after the alternative pathway activation.

In conclusion, the present study demonstrates primarily that activation of the complement system via the alternative pathway has been involved in the development of human MPO-ANCA-associated vasculitis.

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