Supplementary material

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MATERIALS AND METHODS

Detection of deposition of complement activation products in renal specimens by immunohistochemistry

Immunohistochemical staining for Bb, C3d and C5b-9 was performed on 4 μm deparaffinized sections of formaldehyde-fixed renal tissue using mouse anti-human Bb monoclonal antibodies (Quidel Corporation, San Diego, CA), rabbit anti-human C3d polyclonal antibodies (Dako A/S, Copenhagen, Denmark) and mouse anti-human C5b-9 monoclonal antibodies (Abcam, Cambridge, UK) as primary antibodies. Antibodies against Bb, C3d and C5b-9 were diluted in 0.01 mol/L phosphate buffered

saline (PBS, pH 7.4) in 1:50, 1:500, and 1:50, respectively. After deparaffinized in xylene-ethanol at room temperature and rehydrated in PBS, sections were immersed into freshly prepared 3% hydrogen peroxide for 10 min at room temperature to quench endogenous peroxidase activity. The sections for C3d staining were treated with 0.4% pepsin (Zhongshan Golden Bridge Biotechnology, Beijing, China) 45 min for antigen retrieval. The sections for staining Bb and C5b-9 were treated with 0.5mg/ml proteinase K. Digestion was terminated by repeated washings in PBS. To block non-specific staining, sections were incubated with 3% bovine serum albumin (BSA) in PBS at room temperature for 30 min. The primary antibodies were added on each section after the removal of blocking BSA without washing. Primary antibodies were incubated overnight at 4°C. The secondary antibodies from the detection system, Dako EnVision HRP (Dako A/S, Copenhagen, Denmark), were incubated for 30 min

at 37°C. Next, sections were developed in fresh hydrogen peroxide plus 3-3-diaminobenzidine tetrahydrochloride solution for 1 min respectively. Finally, the sections were incubated with hematoxylin, dehydrated through alcohols and xylene. The sections were examined by light microscopy. Sections of renal tissue from patients with lupus nephritis were used as positive controls. Negative controls were performed by omitting or replacing the primary antibodies. Renal tissues from the normal part of two nephrectomized kidneys due to renal carcinoma were used as normal controls. The renal tissues were considered normal by light microscopy, immunofluorescence and electron microscopy.

Detection of MPO-ANCA IgG subclasses

MPO-ANCA IgG subclasses (IgG1, IgG2, IgG3 and IgG4) were measured using ELISA. In brief, highly purified human native MPO were coated to plates at 2.0 μg/ml in coating buffer (0.05 mol/L bicarbonate buffer, pH9.6). The volume in each well was 100μL in this step and subsequent steps and every sample was added in duplication, all incubations were carried out at 37°C for 1h, and the plates were washed three times with PBS containing 0.1% Tween-20 (PBST) (Chemical Reagents, Beijing, China) between stages. Sera from patients were diluted at 1:100 with PBST. Mouse anti-human IgG1, IgG2, IgG3 or IgG4 (SouthernBiotech, Birmingham, USA) were diluted at 1:500 with PBST and the binding was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Fc specific; Sigma, St Louis, MO, USA) at a dilution of 1:5000, followed by addition of P-nitrophenyl phosphate (pNPP, 1mg/ml; Sigma) diluted in substrate buffer [1M diethanolamine and 0.5mM $MgCl_2$

(pH 9.8)]. The absorbance was recorded at 405nm. Every plate contained a positive control, a negative control and blank controls. Samples were considered positive if the absorbance exceeded mean + 3SD from 30 normal blood donors.

Statistical Analysis

For normally distributed data, differences of quantitative parameters between groups were assessed using the *t*-test, and descriptive statistics for these data were presented as mean±standard deviation. For non-normally distributed data, differences of quantitative parameters between groups were assessed using non-parametric test, and descriptive statistics for these data were presented as median and interquartile range (IQR). The associations between two continuous variables were analyzed using Pearson's correlation (for two parametric variables) or Spearman's rank correlation (for two non-parametric variables or one non-parametric variable with one parametric variable). In order to adjust for the influence of urinary protein excretion on urinary complements concentration, an analysis of covariance model was further used for adjustment: the model regarded the urinary level of complement components as a dependent variable, group as a factor and urinary protein excretion (urinary protein-creatinine ratio) as a covariate. Since most of the urinary levels of complement components were not normally distributed, by making logarithmic transformation, we converted the original data to its normal distribution representation for such adjustment. A P value of 0.05 or less was considered statistically significant. Analysis was performed with SPSS statistical software package (version 13.0, Chicago, IL, USA).

RESULTS

Urinary levels of C1q and MBL

The urinary C1q levels were significantly higher in patients with AAV in active stage compared with AAV patients in remission and normal controls (3.69 (0.91-4.76) ng/mg Cr vs. 0.17 (0.04-0.39) ng/mg Cr, P<0.001; 3.69 (0.91-4.76) ng/mg Cr vs. 0.02 (0.01-0.10) ng/mg Cr, P<0.001, respectively). There was no significant difference in urinary C1q levels between patients with active AAV and patients with lupus nephritis. However, the levels of urinary C1q in patients with AAV in remission were still significantly higher than those of normal controls (0.17 (0.04-0.39) ng/mg Cr vs. 0.02 (0.01-0.10) ng/mg Cr, P=0.001).

The urinary MBL levels were significantly higher in patients with AAV in active stage compared with AAV patients in remission and normal controls (6.36 (2.15-43.57) ng/mg Cr vs. 0.30 (0.16-0.87) ng/mg Cr, P<0.001; 6.36 (2.15-43.57) ng/mg Cr vs. 0.03 (0.01-0.07) ng/mg Cr, P<0.001, respectively). There was no significant difference in urinary MBL levels between patients with active AAV and patients with lupus nephritis. However, the levels of urinary MBL in patients with AAV in remission were still significantly higher than those of normal controls (0.30 (0.16-0.87) ng/mg Cr vs. 0.03 (0.01-0.07) ng/mg Cr, P<0.001).

Association between MPO-ANCA IgG subclasses and urinary levels and renal deposition of complement activation products

All four MPO-ANCA IgG subclasses could be detected in the 29 sera from

patients with active AAV: IgG1 in 27 patients (27/29, 93.1%), IgG2 in 23 patients (23/29, 79.3%), IgG3 in 26 patients (26/29, 89.7%) and IgG4 in 26 patients (26/29, 89.7%). Urinary C5a levels in patients with positive MPO-ANCA IgG1 subclass were significantly higher than those with negative MPO-ANCA IgG1 subclass (2.94 (0.74-10.38) ng/mg Cr vs. 0.09 (0-0.14) ng/mg Cr, P=0.02). Urinary C5a levels in patients with positive MPO-ANCA IgG3 subclass were significantly higher than those with negative MPO-ANCA IgG3 subclass (3.01 (0.87-11.07) ng/mg Cr vs. 0.19 (0-0.67) ng/mg Cr, P=0.02). There was no significant correlation between MPO-ANCA IgG subclasses and the renal deposition of complement activation products.