Hypoalbuminaemia in antineutrophil cytoplasmic antibody-associated vasculitis: incidence and significance

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Abstract

Objective

Hypoalbuminaemia has been proved to be a biomarker of poor prognosis in many diseases. The objective of this study was to investigate the significance of hypoalbuminaemia in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).

Methods

Data of 117 AAV patients were analysed retrospectively. The relationship between hypoalbuminaemia and disease severity were studied. The influence of albumin on the pathogenetic role of ANCA was investigated in vitro.

Results

Among all patients, 52 had light hypoalbuminaemia (30g/L<=albumin<35g/L) and 40 had nephrotic hypoalbuminaemia (albumin <30g/L). Patients with hypoalbuminaemia had higher inflammation levels and more severe kidney injury than patients without hypoalbuminaemia, but no significant difference of the urinary protein levels were found between patients with nephrotic and light hypoalbuminaemia. Multivariate analysis showed serum albumin correlated with age (r=-0.566, p=0.018), C-reactive protein (r=-0.521, p=0.032) and haemoglobin (r=0.512, p=0.036). Patients with nephrotic hypoalbuminaemia had higher incidence of infection, end stage renal disease and all cause mortality during treatment than patients with light hypoalbuminaemia or normal serum albumin. In vitro study indicated albumin could inhibit the binding between ANCA and neutrophils in a concentration dependent manner. Albumin also inhibited the ANCA-induced respiratory burst and neutrophil extracellular traps formation.

Conclusion

Serum albumin have an inhibitory effect on the binding between ANCA and its antigen. The incidence of hypoalbuminaemia in AAV with kidney involvement is high but is not caused by heavy proteinuria. Hypoalbuminaemia is correlated with the high inflammation level and poor prognosis of AAV. Therapy targeting hypoalbuminaemia might benefit patients with AAV.

Key words

hypoalbuminaemia, antineutrophil cytoplasmic antibody, nephrotic syndrome, neutrophil extracellular traps

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Introduction

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterised by positive serum ANCA and is classified into four types: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), renal limited variant of MPA (RLV) and eosinophilic granulomatosis with polyangiitis (EPGA) (1, 2). AAV is a kind of inflammatory disease with high levels of inflammatory cytokines such as interleukin-6 and tumour necrosis factor-α in serum (3). Serum albumin is a kind of negative acute phase reaction protein. Hypoalbuminaemia, the happening of which is associated with the severity of many kinds of inflammatory diseases, is very common in active AAV. However, the significance of hypoalbuminaemia in AAV has so far not been given enough attention.

Hypoalbuminaemia has been proved to be a powerful predictor of poor prognosis in many kinds of diseases. The mortality of patients with hypoalbuminaemia was high in chronic renal failure (4, 5), heart disease, stroke and end stage liver disease (6-8). Hypoalbuminaemia can also predict the development of acute kidney injury in hospitalised patients (9). Since AAV is a kind of severe systemic disease with high probability of death or end stage renal disease (ESRD), the investigation of the influence of hypoalbuminaemia on the prognosis of AAV should be of great significance. It is noteworthy that the existence of albumin in serum can influence the immunological characteristics of immunoglobulin. Spontaneous aggregation of native immunoglobulins can occur in hypoalbuminaemic serum (10). On the other hand, previous studies indicate that the antigen recognition capability of purified ANCA-containing IgG is much stronger than that of ANCA-containing serum (11, 12). Therefore, we hypothesise that serum albumin might be a protective factor in AAV and hypoalbuminaemia can aggravate the disease. To validate this hypothesis, we analysed the clinical and laboratory data of 117 AAV patients retrospectively, and investigated the influence of albumin on the pathogeneic role of ANCA *in vitro*.

Methods

Participants

One hundred and seventeen patients with myeloperoxidase (MPO)-ANCA and diagnosed as AAV from 2012 to 2017 in the Tianjin Medical University General Hospital, were enrolled in this retrospective study. All the patients fulfilled the Chapel Hill Consensus Conference (CHCC) classification (13) and were routinely tested for ANCA and antiglomerular basement membrane antibody. Exclusion criteria were diabetes, hepatitis, cirrhosis, pregnancy, previous malignancy, infection at onset of the disease and positive antiglomerular basement membrane antibody. The research was in compliance of the declaration of Helsinki and the protocol was approved by the ethics committee of Tianjin Medical University General Hospital. Informed consent was obtained from each participant.

Measurement of ANCA

ANCA tests were performed by both indirect immunofluorescence (IIF) assay and antigen-specific enzyme-linked immunosorbent assay (ELISA) for all patients at the time of presentation before immunosuppressive treatment was instituted. IIF assays were performed according to the manufacturer's instructions (EUROIMMUN, Lübeck, Germany). Ethanol-fixed human polymorphonuclear leukocytes were used to detect ANCA, and monkey liver sections were used to exclude antinuclear antibodies. cytoplasmic ANCA and perinuclear ANCA were distinguished according to staining patterns by two experienced technicians. MPO-ANCA and proteinase 3 (PR3)-ANCA were tested using ELISA (EUROIMMUN, Lübeck, Germany).

Clinical and laboratory findings

Clinical data including gender, age (years), time from onset (days) and the level of Birmingham Vasculitis Activity Score (BVAS) were collected. General symptoms and organ involvement at the moment of diagnosis were also assessed. Fever was defined as body temperature when more than 38.5°C. Weight loss was defined as loss of more than 2 kg over 1 months preceding di-

agnosis. Rash was defined as new skin lesions during the active period of AAV which could not be explained by any other cause. Arthritis was diagnosed when there was pain, stiffness, redness, warmth or swelling of joints. Mucous/eye involvement included oral/genital ulcer, conjunctivitis, uveitis and fundus hemorrhage. Ear involvement included hearing loss and otitis media. Nasal involvement included sinusitis, nasal polyposis and epistaxis. Lung involvement was diagnosed when there was haemoptysis or chest x-ray and/or computed tomography revealed nodule or infiltration. Asthma was diagnosed when symptoms included episodes of wheezing, coughing, chest tightness and shortness of breath. Hypereosinophilia was diagnosed when the eosinophil count in the peripheral blood exceeded 4.5×10^9/L. Haemorrhage of digestive tract was diagnosed based on the existence of gastrointestinal bleeding or the positive result of fecal occult blood. Peripheral neuropathy was diagnosed if new mononeuropathy or multiple mononeuropathies occurred. Sensory neuropathy is defined as an objective sensory deficit in a glove or stocking distribution. Motor neuropathy is defined as objective motor weakness. Neuropathy was diagnosed once other causes of neuropathy were excluded. Multiple mononeuropathies refers to mononeuropathy affecting two or more nerves in different areas.

Laboratory data included the following: haemoglobin (HB, 115–150 g/L), platelet (PLT, 125–350 10^9/L), white blood cell (WBC, 3.5–9.5 10^9/L), serum albumin (35–55 g/L), proteinuria (<0.15 g/24h), serum creatinine (Scr, 62–133 μmol/L), serum ferritin (SF, 4.6–204 ng/mL), erythrocyte sedimentation rate (ESR, 0–15 mm/h), C-reactive protein (CRP, <0.80 mg/dL), rheumatoid factor (RF, <20 IU/mL), Complement 3 (C3, 79-152 mg/dL) and 4 (C4, 16–38 mg/dL).

Proteinuria was defined as urine total protein more than upper normal limit (>0.15 g/24h). Nephrotic proteinuria was defined as urine total protein ≥3.5 g/24h (14). Hypoalbuminaemia was defined as albumin less than the lower normal limit (<35g/L). Hypoalbuminaemia reaching nephrotic level (nephrotic hypoalbuminaemia) was defined as albumin<30g/L. Light hypoalbuminaemia was defined as 30g/L ≤albumin<35g/L.

Isolation of neutrophils

Neutrophils were isolated from a normal donor by density centrifugation using a Histopaque gradient. Briefly, a double gradient was formed by layering an equal volume of 3 mL Histopaque-1077 (10771, Sigma-Aldrich) over 3 mL Histopaque-1119 (11191, Sigma-Aldrich). Ethylene diamine tetraacetic acid (EDTA) anti-coagulated whole blood (6 mL) was carefully layered onto the upper Histopaque-1077 medium. After centrifugation at 700 g for 30 min, neutrophils between two Histopaque medium were carefully isolated. Cells were washed by addition of 5 mL of isotonic phosphate buffered saline (PBS) (0.20 g/L of KCl, 0.20 g/L of KH_2PO_4, 8 g/L of NaCl, 1.15 g/L of Na_2HPO_4) to the tubes. Purity of neutrophils was >90% as assessed by flow cytometric analysis.

Purification of IgG

IgG fractions from 5 patients with positive ANCA and 5 healthy donors were purified by protein G affinity column (GE Healthcare Life Sciences) with 0.01 mol/L PBS as starting buffer and 0.1 mol/L glycine, pH 2.7 as eluting buffer, at a flow rate of 1 mL/min at room temperature. IgG was eluted and neutralised to pH 7.0 by 2 mol/L Tris-HCl, pH 9.0 immediately, and dialysed against PBS overnight.

Binding of ANCA-containing IgG and MPO detected by enzyme-linked immunosorbent assays (ELISA)

Half of the polystyrene microtitre plates (Nunc Immunoplate, Roskilde, Denmark) were coated with MPO (Lee BioSolutions) at 2.0 μg/mL in 0.05 mol/L bicarbonate buffer, pH 9.6, overnight at 4°C. The other half of the plates were used as antigen-free wells which were coated with bicarbonate buffer alone. All the wells were blocked with 1% bovin serum albumin in 0.05% PBS for 1 h at 37°C. ANCA-containing IgG (5 mg/mL) diluted 1:100 with PBS containing 0.1% Tween 20 (PBST) were added to the wells in duplication and incubated at room temperature for 1 h. IgG form normal people was used as control. In some wells, 125 μg/mL human ceruloplasmin (Sigma) and (or) human albumin (Rongsheng, Chengdu, China) with final concentrations of 25 g/L or 40 g/L were added. Incubation resumed for 1h with alkaline phosphatase–conjugated mouse anti-human IgG (Fc specific; Sigma) diluted 1:20,000 in PBST. Then P-nitrophenyl phosphate (Sigma) 1 mg/mL in substrate buffer (1 mol/L diethanolamine and 0.5 mmol/L $MgCl_2$, pH 9.8) was used as substrate, and colour development was measured spectrophotometrically at 405 nm (Bio-Rad, Tokyo, Japan).

Assessment of binding of ANCA on neutrophils

Neutrophils (2.5×10^6/ml HBSS) were incubated with goat-derived heat-aggregated IgG (0.5 mg/mL) for 15 min at room temperature to saturate the Fcγ-receptors. To explore the binding between ANCA and neutrophils in whole blood, cells were subsequently washed with PBS and incubated with undiluted serum from a patient with serum albumin concentration of 23 g/L. As controls, the albumin concentrations were adjusted to be 30 g/L and 40 g/L by adding albumin (Rongsheng, Chengdu, China) into serum. To explore the binding between purified ANCA-containing IgG and neutrophils, cells were subsequently washed with PBS and incubated with ANCA-containing IgG (5 mg/mL) for 30 min at room temperature. In some tubes, human albumin (Rongsheng, Chengdu, China) with final concentrations of 25 g/L or 40 g/L were added. After incubation, cells were washed twice with PBS, and incubated for 30 min at room temperature with mouse anti-human IgG-PE (Southern-Biotech, Birmingham, USA) Subsequently, cells were washed with PBS, fixed in 1% paraformaldehyde containing PBS on ice and assessed by flow cytometry analysis (BD FACS-Calibur).

Measurement of respiratory burst of neutrophils

Measurement of the respiratory burst of neutrophils using dihydrorhodamine

(DHR) is based on the fact that reactive oxygen radicals cause oxidation of the nonfluorescent DHR to the green fluorescent rhodamine. In brief, neutrophils (2.5×10^6/mL in HBSS) were incubated with cytochalasin B (5 μg/mL, Sigma) for 5 min at 37 ºC to enhance the oxygen radical production. Then, neutrophils were loaded with 0.05 mM DHR (Sigma) and 2 mM sodium azide (NaN_3) at 37 ºC. Next, neutrophils were primed with TNF-α (2 ng/mL) for 15 min at 37 ºC. ANCA-containing IgG (5 mg/mL) were added. In some tubes, human albumin (Rongsheng, Chengdu, China) with final concentrations of 25 g/L or 40 g/L were added. We analysed samples using Calibur flow cytometer (BD FACS-Calibur). Data were collected from 10,000 cells per sample. The median fluorescence intensity (MFI) representing the amount of generated oxygen radicals was reported.

Induction of neutrophil extracellular traps (NETs) by ANCA

Neutrophils (1×10^6/mL) were coated on the Poly-L-Lysine-coated slides in PBS at 37 °C for 1 h and then were treated with 5 ng/mL TNF-α (H8916, sigma-Aldrich) and ANCA-containing IgG (5 mg/mL) at 37 °C for 4 h. In some wells, human albumin (Rongsheng, Chengdu, China) with final concentrations of 25 g/L or 40 g/L were added. Netting neutrophils were stained using 4',6-diamidino-2-phenylindole (DAPI) (C1006, Beyotime). The results were visualised with fluorescence microscopy (Leica DMI4000 B) and evaluated with the Image Pro Plus analysis software 6.0. The supernatant was collected to measure the free DNA level using Quant-iT PicoGreen DNA quantification kit (Invitrogen) in accordance with the manufacturer's instructions. Briefly, to generate a standard curve, the 2 μg/mL DNA stock solution were diluted and incubated with the aqueous working solution at room temperature for 5 min. Fluorescence signals were measured using a fluorescence microplate reader with excitation 480 nm and emission 520 nm. The supernatant of each well was also diluted and incubated with the aqueous working solution. Then the fluorescence values were measured. The DNA concentration of the sample from the standard curve was determined in DNA Standard Curve.

Statistical analyses

Differences of quantitative parameters between groups were assessed using the *t*-test (for data that were normally distributed) or non-parametric test (for data that were not normally distributed). Categorical variables are presented as frequencies. The mortality rate was calculated with the Kaplan-Meier method and the curves were compared using the Log-Rank test. *P*-values lower than 0.05 were considered significant. The software SPSS, v. 19.0 for Windows (IBM, Chicago, IL, USA), was used for statistical analysis.

Table I. Comparison of baseline clinical characteristics of patients with different serum albumin levels.

	No HA (Alb ≥35g/L)	Light HA (30g/L ≤Alb<35g/L)	Nephrotic HA (Alb <30g/L)
Male/female	13/12	28/24	23/17
Age (years)	61.52 ± 10.48	66.21 ± 12.04*	68.53 ± 13.65*
Time from onset (days)	66.36 ± 22.48	43.25 ± 21.00*	45.97 ± 21.04*
Fever (Y/N)	4/21	40/12*	31/9*
Weight loss (Y/N)	16/9	29/23	25/15
Rash (Y/N)	2/23	10/42	5/35
Arthritis (Y/N)	13/12	25/27	23/17
Mucous/Eye involvement (Y/N)	5/20	8/44	11/29
Ear involvement (Y/N)	8/17	22/30	18/22
Nasal involvement (Y/N)	0/25	0/52	0/40
Lung involvement (Y/N)	1/24	12/40*	9/31*
Asthma (Y/N)	0/25	0/52	0/40
Hypereosinophilia (Y/N)	0/25	0/52	0/40
Haemorrhage of digestive tract (Y/N)	4/21	10/42	9/31
Peripheral neuropathy (Y/N)	3/22	10/42	7/33
Haemodialysis at admission (Y/N)	1/24	9/43	7/33
BVAS	10.56 ± 6.85	18.70 ± 7.24*	16.82 ± 7.01*

BVAS: Birmingham vasculitis activity score; HA: hypoalbuminaemia.
*$p<0.05$, compared with data of group no HA.

Results

Comparison of the baseline clinical characteristics among patients with different levels of serum albumin

Among the 117 patients, 52 had light hypoalbuminaemia (30g/L ≤albumin <35g/L) and 40 had nephrotic hypoalbuminaemia (albumin <30g/L). The clinical characteristics of 3 groups were shown in Table I. There was no significant difference of the gender distribution among 3 groups. Patients with hypoalbuminaemia were older and had a shorter time from onset to diagnosis than patients without hypoalbuminaemia. Patients with hypoalbuminaemia had higher incidence of fever and lung involvement than patients without hypoalbuminaemia. There were no significant difference of the incidence of weight loss, rash, arthritis, mucous/eye involvement, ear involvement, nasal involvement, asthma, hypereosinophilia and peripheral neuropathy among 3 groups. There was a tendency of higher proportion of haemodialysis at admission in patients with both light hypoalbuminaemia and nephrotic hypoalbuminaemia than patients without hypoalbuminaemia, but the difference did not reach statistical significance (p=0.153 and p=0.139, respectively). Patients with hypoalbuminaemia had higher BVAS than patients without hypoalbuminaemia. No significant difference of any baseline clinical characteristic was found between patients with light and nephrotic hypoalbuminaemia.

Comparison of the laboratory characteristics among patients with different levels of serum albumin

As shown in Table II, patients with hypoalbuminaemia had higher CRP, WBC, and proteinuria than patients without hypoalbuminaemia. Among above 3 characteristics, the levels of CRP and WBC were much higher in patients with nephrotic hypoalbuminaemia than in patients with light hy-

Table II. Comparison of laboratory characteristics of patients with different serum albumin levels.

	No HA (Alb ≥35g/L)	Light HA (30g/L ≤Alb<35g/L)	Nephrotic HA (Alb <30g/L)
ANCA levels (IU/mL)	126.28 ± 60.88	131.49 ± 68.90	143.68 ± 63.06
CRP (mg/dL)	0.53 (0.12, 15.10)	2.52 (0.10, 11.40)	9.59 (0.21, 23.90)*#
C3 (mg/dL)	103.69 ± 24.22	90.41 ± 28.69	91.17 ± 29.41
C4 (mg/dL)	22.34 ± 5.80	23.80 ± 7.25	23.96 ± 12.22
RF (IU/mL)	20 (20, 443)	20 (20, 484)	24 (20, 780)
HB (g/L)	112.36 ± 24.31	87.65 ± 18.18*	90.13 ± 22.82*
WBC (10^9/L)	6.92 ± 2.19	9.66 ± 4.85*	11.37 ± 4.71*#
PLT (10^9/L)	248.72 ± 83.31	272.12 ± 130.14	328.18 ± 235.23*
ESR (mm/h)	40.00 ± 16.44	86.57 ± 37.72*	80.24 ± 35.64*
SF (μg/L)	141.49 (11.23, 566.28)	151.05 (10.91, 1278.37)	467.85 (116.58, 1314.10)*
Proteinuria (g/24h)	0.218 (0.013, 2.320)	1.300 (0.052, 5.570)*	0.765 (0.015, 6.600)*
eGFR (mL/min/1.73m^2)	80.59 ± 45.68	33.12 ± 31.01*	40.59 ± 32.26*

ANCA: antineutrophil cytoplasmic antibody; CRP: C reactive protein; C3: complement 3; C4: complement 4; eGFR: estimated glomerular filtration rate; ESR: erythrocyte sedimentation rate; HB: haemoglobin; HA: hypoalbuminaemia; PLT: platelet; RF: rheumatoid factor; SF: serum ferritin; Upro: urinary protein; WBC: white blood cell.
*p<0.05, compared with data of group with no HA; # p<0.05, compared with data of group with light HA.

Table III. Univariate and multivariate analysis of the relationship between serum albumin and other parameters of patients.

	Univariate analysis			Multivariate analysis		
	Correlation coefficient	p-value	95% confidence interval	β-coefficient	p-value	95% confidence interval
Age	-0.235	0.011	-0.419, -0.036	-0.566	0.018	-0.887, 0.021
BVAS	-0.267	0.004	-0.423, -0.097	-0.038	0.886	-0.793, -0.654
CRP	-0.522	0.000	-0.654, -0.366	-0.521	0.032	-0.918, 0.147
RF	-0.321	0.001	-0.499, -0.148	-0.330	0.196	-0.845, 0.439
HB	0.332	0.000	0.137, 0.491	0.512	0.036	-0.377, 0.937
WBC	-0.374	0.000	-0.523, -0.214	-0.064	0.808	-0.785, 0.591
PLT	-0.188	0.042	-0.311, -0.090	0.130	0.620	-0.414, 0.735
ESR	-0.391	0.000	-0.562, -0.194	0.184	0.479	-0.599, 0.833
SF	-0.407	0.010	-0.610, -0.118	-0.104	0.691	-0.727, 0.703
eGFR	0.345	0.000	0.161, 0.491	0.026	0.920	-0.704, 0.722

BVAS: Birmingham vasculitis activity score; CRP: C reactive protein; eGFR: estimated glomerular filtration rate; ESR: erythrocyte sedimentation rate; HB: haemoglobin; PLT: platelet; RF: rheumatoid factor; SF: serum ferritin; WBC: white blood cell.

poalbuminaemia. Patients with hypoalbuminaemia had lower HB and eGFR than patients without hypoalbuminaemia. No significant differences of HB and eGFR were found between patients with nephrotic and light hypoalbuminaemia. Patients with nephrotic hypoalbuminaemia had higher PLT and SF than patients without hypoalbuminaemia. No significant differences of PLT and SF were found between patients with nephrotic and light hypoalbuminaemia or between patients with light hypoalbuminaemia and patients without hypoalbuminaemia, although there was a tendency of higher SF in patients with nephrotic hypoalbuminaemia than patients with light hypoalbuminaemia (p=0.123). No significant difference of the levels of ANCA, C3, C4, and RF were found among 3 groups.

Univariate and multivariate analysis of the relationship between serum albumin and other parameters of patients

Univariate correlation analysis showed that albumin correlated with age, BVAS, CRP, RF, HB, WBC, PLT, ESR, SF and eGFR. Multivariate correlation analysis showed that albumin still correlated with age, CRP and HB (Table III).

Influence of hypoalbuminaemia on the prognosis of patients

All patients received corticosteroid treatment. The majority of patients (92 of 117) received weekly intravenous cyclophosphamide. Treatment protocols were comparable among 3 groups. Patients were followed up with the longest time of 60 months. Thirty-three patients (one hypoalbuminaemia, 18 light hypoalbuminaemia and 14 with nephrotic hypoalbuminaemia) progressed to ESRD. Both patients with light hypoalbuminaemia and patients with nephrotic hypoalbuminaemia had worse renal survivals than patients without hypoalbuminaemia (p=0.013 and p=0.004, respectively). No different renal survival between patients with light and nephrotic hypoalbuminaemia was found (p=0.536) (Fig. 1A).

After immunosuppressive treatment, 6 of 25 patients without hypoalbuminaemia, 15 of 52 patients with light hypoalbuminaemia and 29 of 40 patients with nephrotic hypoalbuminaemia had infection at least once. The incidence of infection of patients with nephrotic hypoalbuminaemia was higher than the other 2 groups (Fig. 1B).

Twenty-five patients (one without hypoalbuminaemia, 12 with light hypoalbuminaemia and 12 with nephrotic hypoalbuminaemia) died during the period of follow-up. The average serum albumin of deceased patients was significantly lower than that of survived patients (p=0.002) (Fig. 1C). Patients with nephrotic hypoalbuminaemia had a higher mortality than patients without hypoalbuminaemia (p=0.008). Neither different mortality between patients without hypoalbuminaemia and patients with light hypoalbuminaemia (p=0.062) nor different mortality between patients with nephrotic hypoalbuminaemia and patients with light hypoalbuminaemia (p=0.246) was found (Fig. 1D).

Inhibition of albumin on the binding between ANCA and neutrophils

To verify the hypothesis that hypoproteinaemia might enhance the pathogenicity of ANCA, we firstly investigated the influence of albumin on the binding between ANCA and its antigen using ELISA. As shown in Figure 2A, IgG from normal people did not recognise coated MPO, while MPO-ANCA-containing IgG purified from patients could bind MPO. The existence of al-

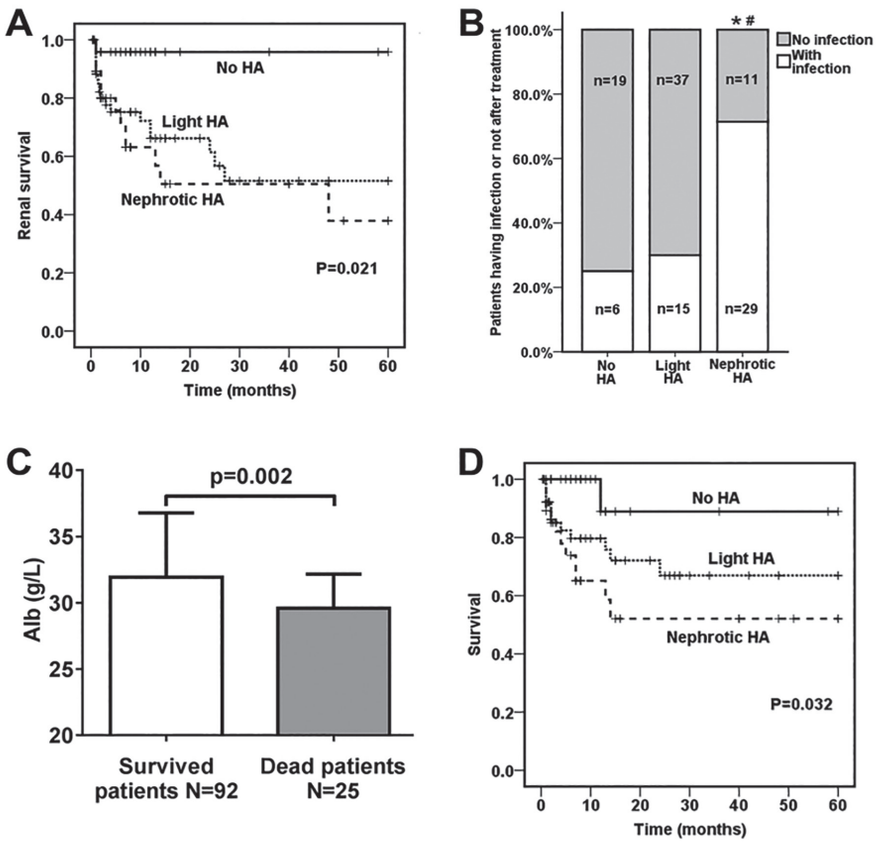

Fig. 1. Influence of hypoalbuminaemia on the prognosis of AAV. **A**: Comparison of the renal survival among patients with different levels of serum albumin. **B**: Comparison of the incidence of infection after immunosuppressive therapy among patients with different levels of serum albumin. **p*<0.001, compared with patients without hypoalbuminaemia; #*p*<0.001, compared with patients with light hypoalbuminaemia. **C**: Comparison of the serum abumin levels between survived patients and dead patients. **D**: Comparison of the mortality among patients with different levels of serum albumin.
Alb: albumin; HA: hypoalbuminaemia.

bumin in reaction system inhibited the binding between ANCA and MPO with the increase of albumin concentration (expressed as *A* values at 405 nm, albumin 0 g/L: 1.349±0.291, albumin 25 g/L: 0.836±0.334, albumin 40 g/L: 0.577±0.231). Ceruloplasmin, the native inhibitor of MPO, could also inhibit the binding between ANCA and MPO. Combination of albumin (40 g/L) and ceruloplasmin (125 μg/mL) inhibited the binding between ANCA and MPO further.

Then we investigated the influence of albumin on the binding between MPO-ANCA-containing IgG and neutrophils which expressed MPO on surface using FACS. As shown in Figure 2B and C, IgG from normal people did not bind neutrophils, while MPO-ANCA-containing IgG purified from patients cound bind neutrophils. The existence of albumin in reaction system inhibited the binding between MPO-ANCA-containing IgG and neutrophils with the increase of albumin concentration [expressed as mean fluorescence intensity (MFI), albumin 0 g/L: 255.8±70.0, albumin 25 g/L: 155.2±38.6, albumin 40 g/L: 111.0±13.3].

To further validate this result, serum was drawn from a representative patient. This patient had a serum albumin concentration of 23 g/L and a MPO-ANCA level of 164.3 RU/mL. By adding human albumin into the serum, the albumin concentration was adjusted to be 30 g/L and 40 g/L resceptively. As shown in Figure 2D, the binding between MPO-ANCA-containing serum and neurtophils isolated form a nomal donor decreased with the increase of albumin concentration of serum (expressed as MFI, albumin 0 g/L: 34.0±9.4, albumin 30 g/L: 29.2±6.8, albumin 40 g/L: 21.6±6.2).

Inhibition of albumin on the ANCA-induced activation of neutrophils

Since albumin could inhibit the binding between ANCA and neutrophils, we investigated whether albumin could inhibit ANCA-induced activation of neutrophils so as to inhibit the pathogenetic function of ANCA. As shown in Figure 3A-B, MPO-ANCA-containing IgG could induce respiratory burst of neutrophils. The existence of albumin inhibited the respiratory burst level with the increase of albumin concentration (expressed as MFI, albumin 0 g/L: 201.8±49.3, albumin 25 g/L: 131.2±46.6, albumin 40 g/L: 75.0±45.9).

Since respiratory burst of neutrophils is accompanied by NETs production and DNA release, we then investigated whether albumin could also inhibit the NETs production of neutrophils. Firstly, we observed the NETs formation using immunofluorescence. Along with the albumin concentration increased, the ANCA-induced NETs formation of neutrophils decreased gradually (Fig. 3C-F). Then we measured the level of free DNA in supernatant using ELISA. As shown in Figure 3D, the existence of albumin inhibited the DNA release from neutrophils in a concentration dependent manner (ng/mL, albumin 0 g/L: 2373.2±705.1, albumin 25 g/L: 1365.2±378.1, albumin 40 g/L: 757.6±309.6).

Discussion

Few studies have focused on the significance of hypoalbuminaemia in AAV until now. Yorioka *et al*. reported that after follow-up for a period of time, AAV patients who died had a significantly lower albumin and creatinine clearance than those who survived, but the authors analysed only 17 patients (15). Itabashi *et al*. surveyed 99 AAV patients and found significant decreases of albumin at 1 and 3 months after onset in the deceased patients. However, the authors did not find significant differences of the baseline albumin levels between the deceased patients and the survived patients. It is noteworthy that the study did not exclude patients with negative ANCA (16). To avoid the heterogeneity, all 117 patients enrolled in

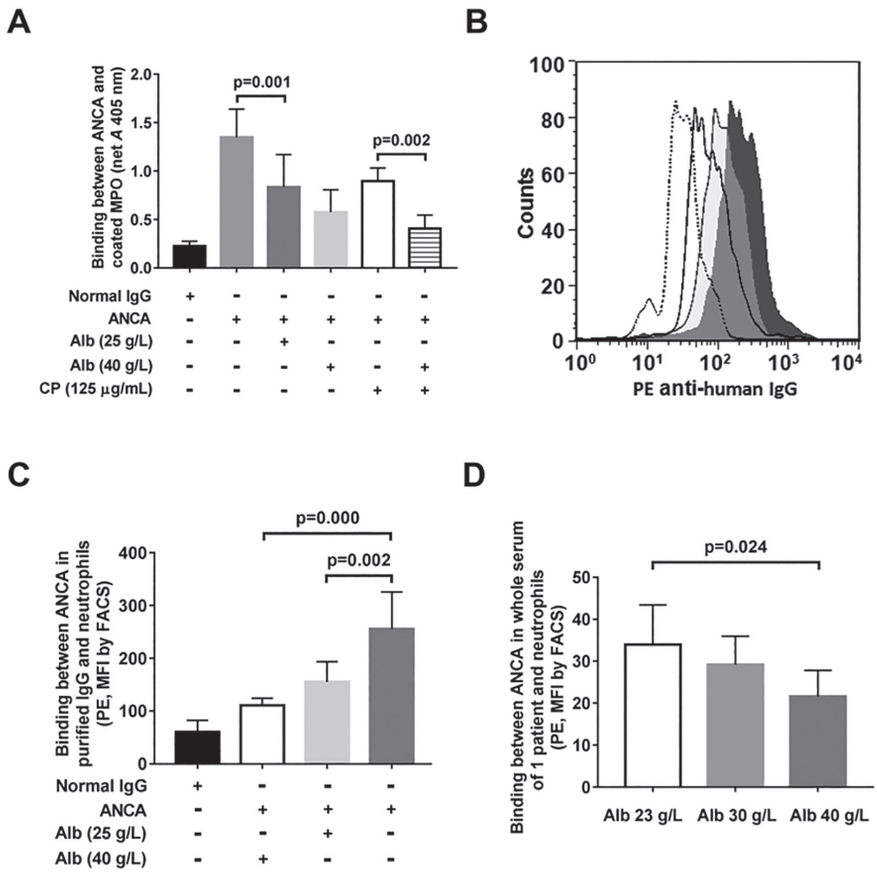

Fig. 2. Influence of albumin on the binding between ANCA and MPO and the binding between ANCA and neutrophils. **A**: Influence of albumin and ceruloplasmin on the binding between ANCA and coated MPO in ELISA. **B**: Representative histogram of the binding between ANCA and neutrophils. Neutrophils were obtained from a normal control. Histogram with dotted line represented IgG from a normal control. The others represented MPO-ANCA containing IgG from one patient. Hollow histogram with solid border represented the binding between neutrophils and 5 mg/mL IgG combined with 40 g/L albumin. Light grey histogram represented the binding between neutrophils and 5 mg/mL IgG combined with 25 g/L albumin. Dark grey grey histogram represented the binding between neutrophils and 5 mg/mL IgG without albumin. **C**: Statistical result of the binding between ANCA and neutrophils. Neutrophils were obtained from a normal control. The concentrations of both normal IgG (obtained from 5 normal controls) and MPO-ANCA containing IgG (obtained from 5 patients) were 5 mg/mL. **D**: Comparison of the binding between neutrophils and MPO-ANCA containing serum with different concentrations of albumin in serum. The serum was drawn from a patient with the original concentration of albumin 23 g/L. By adding human albumin into the serum, concentrations of 30 g/L and 40 g/L were obtained. The results were repeated 5 times and expressed by mean±standard error.
Alb: albumin; ANCA: antineutrophil cytoplasmic antibody; CP: ceruloplasmin.

the current study were MPO-ANCA positive. We found that patients with hypoproteinaemia had higher levels of inflammation, higher incidence of treatment-related infection, higher proportion of ESRD and higher mortality than patients without hypoproteinaemia. We also found the average baseline albumin level of deceased patients was significantly higher than that of survived patients.

The cause of hypoalbuminaemia in AAV needs further investigation. In the current study, we did not find different levels of proteinuria between patients with light and nephrotic hypoproteinaemia. This indicates that the hypoproteinaemia in AAV is not determined by proteinuria. It is noteworthy that patients with hypoalbuminaemia have lower eGFR than patients without hypoalbuminaemia, and correspondingly, the renal survival of patients with hypoalbuminaemia is worse. Actually, predicting the renal prognosis in AAV is not easy and even the newly proposed histological classification cannot draw a consistent conclusion in different validation studies (17, 18). Age should be an important factor influencing the renal prognosis (18) and the occurrence of hypoproteinaemia in AAV because we found patients with hypoproteinaemia were older than patients without hypoproteinaemia. So the relatively insufficient synthetic ability of liver in older people might be the most important reason why hypoalbuminaemia prefers to occur in the elderly in AAV.

Patients with nephrotic hypoalbuminaemia had the highest level of CRP among 3 groups. Multivariate correlation analysis also showed that albumin negatively correlated with CRP. This phenomenon showed a higher inflammation level in patients with hypoalbuminaemia than in patients without hypoalbuminaemia. Previous studies have demonstrated the association between hypoalbuminaemia and susceptibility to infection (19, 20). In the current study, we found that, after immunosuppressive therapy, the incidence of infection in patients with nephrotic hypoalbuminaemia was higher than that in the other 2 groups. Some trials have suggested that resuscitation with albumin-containing solutions is associated with low mortality in patients with sepsis (21). We believe the therapy of rectifying hypoalbuminaemia in AAV is worthy of further study.

In the current study, we tried to explain the correlation between hypoalbuminaemia and the severe manifestation and poor prognosis of AAV in the aspect of the influence of albumin on the pathogenicity of ANCA. Although albumin is a kind of inert protein, its low molecular weight and high serum concentration still endow it the ability to interfere the antigen-antibody reaction *in vivo*. The isoelectric point of albumin is 4.7–4.9, so albumin carries negative charge in serum (PH7.35–7.45). The isoelectric point of MPO is about 11, so MPO carries a lot of positive charge in serum. Theoretically two kinds of protein carrying different charges will attract each other. Although this effect is not as strong as the antigen-antibody binding, it still can interfere the binding between MPO and ANCA. In the current study, we found albumin could both inhibit the ANCA-MPO binding alone and enhance the inhibition

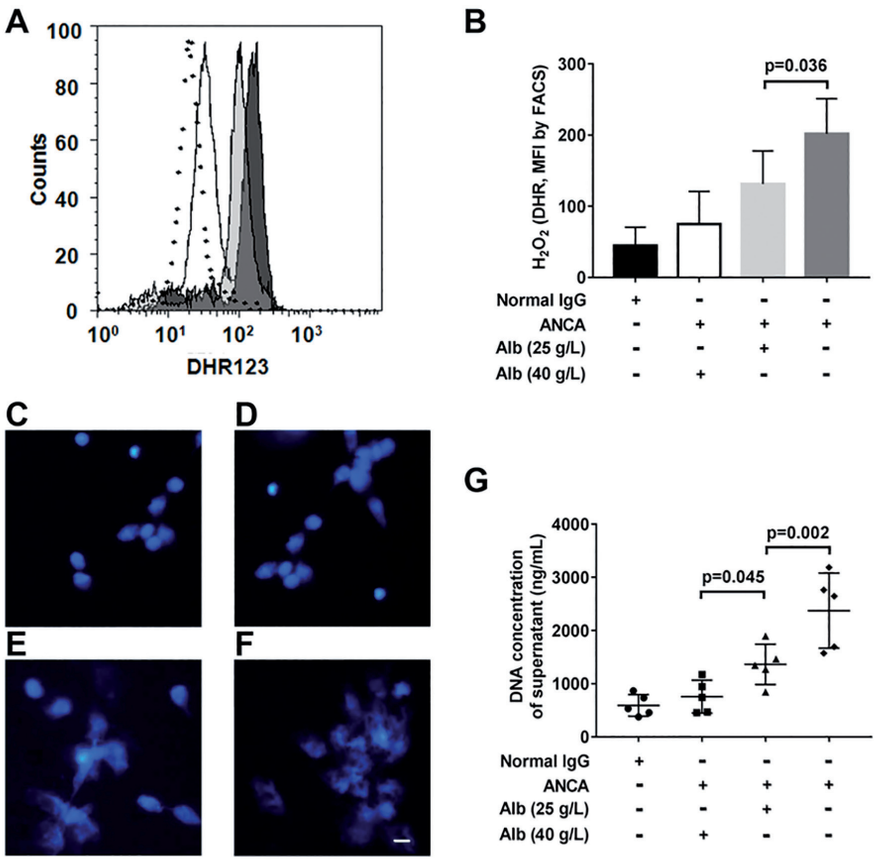

Fig. 3. Influence of albumin on the ANCA-induced activation of neutrophils.
A: A representative histogram of the ANCA-induced neutrophils respiratory burst. Neutrophils were obtained from a normal control. MPO-ANCA containing IgG was obtained from a patient. The results were expressed by the MFI of the oxidation of DHR. Histogram with dotted line represented IgG from a normal control. The others represented MPO-ANCA containing IgG from one patient. Hollow histogram with solid border represented the respiratory burst of neutrophils stimulated with 5 mg/mL IgG combined with 40 g/L albumin. Light grey histogram represented the respiratory burst of neutrophils stimulated with 5 mg/mL IgG combined with 25 g/L albumin. Dark grey histogram represented the respiratory burst of neutrophils stimulated with 5 mg/mL IgG without albumin.
B: Statistical result of the ANCA-induced neutrophils respiratory burst. Neutrophils were obtained from a normal control. The concentrations of both normal IgG (obtained from 5 normal controls) and MPO-ANCA containing IgG (obtained from 5 patients) were 5 mg/mL.
C-**F**: Representative images of the influence of albumin on the ANCA-induced netting neutrophils. IgG (5 mg/mL) from a normal control was made as control (C). MPO-ANCA containing IgG (5 mg/mL) from one patient combined with 40 g/L albumin (**D**), with 25 g/L albumin (**E**) and with no albumin (F) induced the increasing levels of NETs.
G: Measurement of the levels of free DNA in the supernatants of ANCA-induced netting neutrophils. Alb: albumin; ANCA: antineutrophil cytoplasmic antibody.

of ANCA-MPO binding by ceruloplasmin which is the physiological inhibitor of MPO (22). The pathogenic role of ANCA has been confirmed by many studies (23-25). ANCA-induced activation of the neutrophils has been proved to be able to initiate vasculitis in animal model (26). We found albumin could inhibit the ANCA-induced respiratory burst and the NETs formation of neutrophils in a concentration dependent manner *in vitro*. We think this may at least partially explain the results achieved from the clinical data.

There are no significant differences for most clinical and laboratory baseline characteristics between patients with light and nephrotic hypoalbuminaemia. We speculate the higher CRP level in patients with nephrotic hypoalbuminaemia might be able to explain this phenomenon partially. A growing number of studies demonstrate that CRP is not only a positive acute phase reaction protein in inflammation, but also a regulator of complement. CRP can bind factor H and down-regulate the activation of alternative complement passway which is crucial in the pathogenesis of AAV (27-29). So the increased CRP might partially offset the enhanced pathogenicity of ANCA in patients with nephrotic hypoalbuminaemia.

Several limitations of this study should be mentioned. First, this is a retrospective study and treatment regimens which might influence the evaluation of the patients' outcome were not controlled strictly. Second, all patients involved in this study were from nephrology department and patients without obvious kidney injury were not included. Therefore, selection bias could not be excluded. Third, since the AAV with positive PR3-ANCA is rare in Chinese, only MPO-ANCA positive patients were included, so the results of the current study need to be further validated in patients with positive PR3-ANCA.

In conclusion, serum albumin have an inhibitory effect on the binding between ANCA and its antigen. The incidence of hypoalbuminaemia in AAV with kidney involvement is high but is not caused by heavy proteinuria. Hypoalbuminaemia is correlated with the high inflammation level and poor prognosis of AAV. The existence of hypoalbuminaemia should be considered as a reflection on the severity of AAV and therapy targeting hypoalbuminaemia might benefit patients.

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