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ORIGINAL ARTICLE

Antineutrophil cytoplasmic antibody-associated glomerulonephritis with immunoglobulin deposition

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Abstract

Background Antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis is commonly classified as pauci-immune glomerulonephritis; however, some cases have granular immunoglobulin deposition along the glomerular capillary. The pathogenesis of immune deposits is poorly studied.

Methods Of 66 patients diagnosed with ANCA-associated glomerulonephritis on renal biopsy, cases with immunoglobulin deposition along the glomerular capillary were identified and their clinicopathological characteristics were analyzed. We also performed myeloperoxidase (MPO) and double immunofluorescence (IF) stainings to determine the presence of immune complex antigens.

Results Granular IgG deposition, IgG plus IgM deposition, and IgM deposition were observed in 15 (22.1%), 8 (11.2%), and 17 (25.0%) cases, respectively. In cases with granular IgG deposition, MPO-IgG double IF staining revealed co-localization of MPO and IgG. In cases with granular IgM deposition, MPO-IgM double IF staining did not co-localize. By electron microscopy, subepithelial deposition as well as intramembranous, subendothelial, and mesangial deposition was detected in the patients with IgG deposition. In addition, renal survival curves were not significantly different between the immunoglobulin deposition and non-deposition groups.

Conclusions Granular IgG and/or IgM deposition was observed in 60.6% of patients with ANCA-associated glomerulonephritis. In cases with IgG deposition, electron-dense deposits (EDDs) were observed at various sites in the glomerulus, and MPO and IgG immunocomplex deposition was frequently observed along the glomerular capillary. With IgM deposition, EDDs were not obvious in the glomerular basement membrane, and MPO and IgM immunocomplex was not detected. These data suggest differential mechanism between IgG deposition and IgM deposition.

Keywords ANCA · Immunoglobulin · Immunocomplex · MPO · Histopathology

Introduction

Microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA) are known as the antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) because ANCA is involved in the pathogenesis of all these conditions. Myeloperoxidase (MPO) and proteinase 3 (PR3) are the major ANCA antigens [1, 2]. MPO-ANCA-associated vasculitis is more common in Japan. On the basis of immunopathological findings, crescentic glomerulonephritis can be classified into the following three major categories: immune complex glomerulonephritis, anti-glomerular basement membrane (anti-GBM) glomerulonephritis, and pauci-immune glomerulonephritis. Pauci-immune glomerulonephritis is characterized by mild or absent glomerular tuft staining for immunoglobulins and/or complement using immunofluorescence (IF), with few or no electron-dense deposits

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(EDDs) observed by electron microscopy. ANCA-associated glomerulonephritis has been described as pauci-immune necrotizing or crescentic glomerulonephritis.

However, several studies have shown both immunoglobulin and complement deposition in ANCA-associated glomerulonephritis. Ronco et al. performed a retrospective study of 43 patients suffering from polyarteritis nodosa (PAN) and GPA [3]. IgG and C3 deposition was detected in 9 and 55% of patients with GPA, respectively. Neuman et al. reported that 18% of patients with ANCA-associated nephritis showed substantial deposition of immunoglobulin, in the mesangium and/or along the glomerular basement membrane, in addition to severe proteinuria [4]. Haas et al. have reported immune complex deposition using electron microscopy in 54% of renal biopsies in cases of crescentic glomerulonephritis associated with positive ANCA [5].

ANCA-associated glomerulonephritis with immune deposition is often regarded as being superimposed on membranous nephropathy (MGN), post-infectious glomerulonephritis, or IgA nephropathy [6–8]. When immunoglobulin and complement are detected along the capillary wall, there may be coexistence of primary MGN with a secondary form induced by MPO-ANCA-associated glomerulonephritis. Hanamura et al. reported that MPO may form immune complexes [9], and transient immune complex deposition has been observed in an animal model of ANCA-associated glomerulonephritis involving rats injected with human MPO [10]. However, the immune complex antigens in ANCA-associated glomerulonephritis have not been sufficiently examined.

Here, we evaluated IgG and IgM using IF staining and localization of EDDs in the patients with ANCA-associated glomerulonephritis. In addition, we examined the relationship between MPO and immunoglobulin deposition by double IF staining using MPO antibodies.

Materials and methods

Patients

Study subjects included 66 consecutive patients diagnosed with ANCA-associated glomerulonephritis by renal biopsy at Tokyo Women's Medical University Hospital, Saiseikai Kurihashi Hospital, Toda Chuo General Hospital, and Tokyo Women's Medical University Yachiyo Medical Center, between 1998 and 2013. This study was conducted in accordance with the Declaration of Helsinki. The Research Ethics Board of Tokyo Women's Medical University approved the study protocol (approval number 3556). Patients with anti-GBM and immune complex-mediated diseases, such as systemic lupus erythematosus,

post-infectious glomerulonephritis, or IgA vasculitis, were excluded. The clinicopathological diagnosis of each vasculitis was established using definitions adapted by the Chapel Hill Consensus Conference on the Nomenclature of Systemic Vasculitis. All patients were MPO-ANCA positive. Among these 66 cases, 35 were men and 31 were women, with a mean $\pm$ SD age at diagnosis of 61.2 ± 13.9 years (range 25–85 years). At the time of diagnosis, all cases were MPO-ANCA positive and one case was also PR3-ANCA positive. We assessed blood pressure, albumin (Alb), creatinine (Cre), eGFR, CRP, urinary protein (g/gCre), U-RBC, IgG, IgM, IgA, CH50, WBC, and hemoglobin (Hb) at the time of renal biopsy. We applied the Birmingham Vasculitis Activity Score (BVAS2003) to evaluate the extent of disease activity [11]. Twenty-nine of the 66 cases had been treated prior to renal biopsy.

Renal biopsy examination

All 66 renal biopsies were reviewed using light microscopy; IF for IgG, IgA, IgM, C3, C4, IgG subclass, and MPO, and electron microscopy. IF data were classified into the following four staining intensities: $-$, $\pm$, 1+, and 2+. We compared light microscopy data and clinical characteristics between the patient groups in which granular immunoglobulin deposition was along the glomerular capillary (deposition group) and the patient group in which deposition was absent (non-deposition group). Cases with $-$ or $\pm$ IF intensities were considered as having no deposition, and only cases with 1+ or 2+ intensities were included in the deposition group. Patients with granular IgG deposition were further differentiated into IgG subclasses. In the patients with IgG and/or IgM deposition, we investigated the localization and extent of deposition by electron microscopy. The extent of deposition was measured on a scale from $-$ to 3+, where $-$ and + were considered to be insignificant and 2+ and 3+ were considered to be significant. Furthermore, we determined the localization of immunoglobulin deposition and MPO antigens using double IF staining. Frozen sections of biopsies from cases with IgG depositions were incubated with mouse anti-human mc MPO antibody (Santa Cruz) for 60 min and then with Alexa Fluor 568-labeled goat anti-mouse IgG antibody (Molecular Probes) for 60 min; the samples were then incubated in diluted normal rabbit serum ($\times 10$) for blocking and were further incubated in fluorescein isothiocyanate-labeled rabbit anti-human IgG antibody for an additional 60 min. Biopsy samples from cases with IgM depositions were similarly tested as cases with IgG depositions, but FITC-labeled rabbit anti-human IgM antibody was used during the last step. The sections were then observed using a fluorescence microscope. As a

control group, we performed double IF staining on samples from patients with primary MGN. Electron microscopy was performed as per the standard clinical routine. The quantity of EDDs in mesangial, subepithelial, intramembranous, and subendothelial locations was graded according to a scale from 0 to 3: 0, absent; 1, a deposit located in 1–3 mesangial regions or glomerular capillaries; 2, more deposits than grade 1, but involving fewer than 50% of the mesangial regions or glomerular capillaries; 3, deposits in >50% of mesangial regions or glomerular capillaries.

Follow-up

All patients received intravenous and/or oral methylprednisolone therapy with or without immunosuppressants after diagnosis, according to the attending physician's judgement. Renal function was assessed at follow-up every 1–3 months after the initiation of therapy. ESRD was defined as requiring permanent renal replacement therapy. We assessed the development of ESRD during follow-up. We compared renal survival between the deposition group and non-deposition group, and between the IgG deposition group, IgG + IgM deposition group, and IgM deposition group.

Statistical analysis

The Mann–Whitney U test and independent samples t test were applied for correlation of rank and continuous variables, respectively, and the Chi-square test was used to compare proportions. Renal survival rates from the day of renal biopsy were calculated using the Kaplan–Meier estimate of a survival curve. Differences between the curves were tested with the log-rank test. p values <0.05 were considered to be significant.

Results

Among the 66 identified cases, 40 cases (60.6%) showed more than 1+ intensity for granular immunoglobulin along the glomerular capillary on IF staining, and 26 cases (39.4%) showed less than 1+ intensity of immunoglobulin. There were 8 cases (12.1%) with 2+ granular immunoglobulin staining, which was not consistent with a diagnosis of pauci-immune glomerulonephritis. Figure 1 shows the pattern of immunoglobulin deposition. All cases also had variable intensities of C3 staining. The clinical and histological data and comparison between the non-deposition and deposition groups are shown in Table 1. Compared with the non-deposition group ($n = 26$), patients in the deposition group ($n = 40$) were significantly younger ($p = 0.005$), showed higher eGFR ($p = 0.03$), and had higher serum albumin (alb)

($p = 0.02$), lower BVAS ($p = 0.046$), and a high frequency of RLV ($p = 0.04$). There were no differences in urinary protein or serum creatinine levels between the two groups. By light microscopy, there was no thickening of the GBM or spike formation in the deposition group. There were no significant differences in the percentage of active crescentic formation, global sclerosis, interstitial fibrosis, or tubular atrophy (IFTA).

The cases with immunoglobulin deposition were divided into the following three groups: the IgG group included cases with IgG and IgG + IgA deposition ($n = 15$), the IgG + IgM group included cases with IgG + IgM and IgG + IgA + IgM ($n = 8$), and the IgM group included cases with IgA + IgM and IgM ($n = 17$). The clinical and histological characteristics of these three groups are compared in Table 2. There were significant differences in the severity of urinary RBC ($p = 0.007$) and the level of IgM ($p = 0.02$) and CH50 ($p = 0.03$) at the time of biopsy between the three groups.

To reveal whether MPO was the immune complex antigen, we performed MPO staining and double IF staining. There were 13 cases in the IgG deposition group and five cases in the IgM deposition group with positive MPO staining. All cases in the IgG deposition group had MPO staining along the capillary, whereas all cases in the IgM deposition group had little MPO staining. Seven cases were further analyzed with MPO-IgG double IF staining and MPO-IgG localization overlapped in all cases (Fig. 2a). All of the glomeruli observed had co-localization of IgG and MPO, and IgG depositions were seen in both glomeruli with crescentic formations and necrotic lesions and in those without. C3 was co-localized with IgG; however, the location of the C3 and IgM depositions clearly did not overlap. C3 was also co-localized with MPO and IgG on IF. Cases in the IgM group did not show IgM and MPO co-localization (Fig. 2b). In contrast, MPO staining was not observed in the primary MGN controls (data not shown).

In the cases with IgG deposition, we conducted IgG subclass staining. IgG1 was observed in all cases, but the incidence of IgG2, IgG3, and IgG4 varied. 17% of cases showed only IgG1, 28% showed IgG1 + IgG2, 11% showed IgG1 + IgG3, 5% showed IgG1 + IgG4, 11% showed IgG1 + IgG2 + IgG3, 17% showed IgG1 + IgG2 + IgG4 and 11% showed IgG1 + IgG3 + IgG4. In the cases with only IgG1 deposition, both λ and κ staining patterns were observed using IF, thereby ruling out the possibility of monoclonality.

By electron microscopy, subepithelial deposition was most prominent (77.8%) in the cases with IgG group. EDD in other regions such as intramembranous, subendothelial, and mesangial regions were also observed in 44.4, 61.1, and 66.7% of cases, respectively (Fig. 3). There was little EDD observed in any case from the IgM group.

Fig. 1 Presence and type of granular immunoglobulin deposits along capillaries
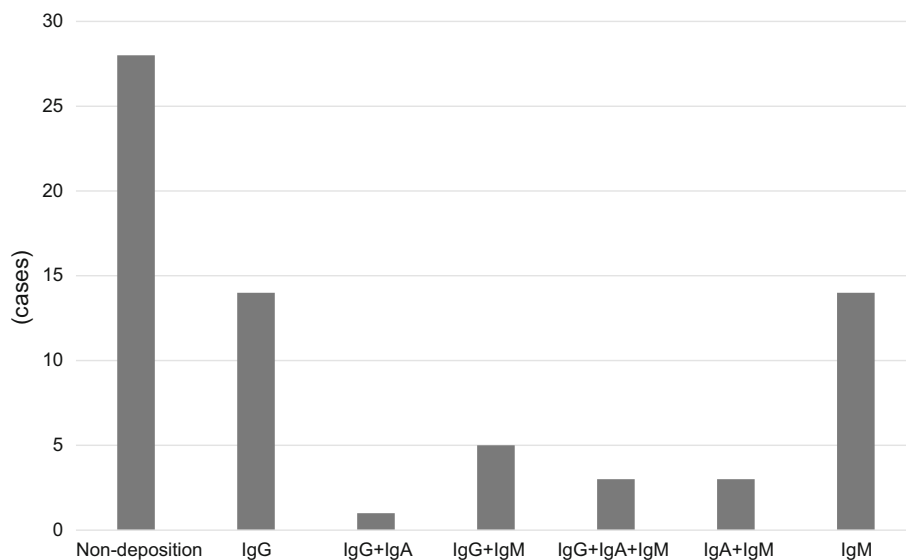
Table 1 Patient characteristics and comparison between the non-deposition and deposition groups

|	Total	Non-deposition group	Deposition group	*p* value
N	66	26	40	
Age	61.2 ± 13.9	67.1 ± 8.7	57.4 ± 15.3	0.005
Female	31	11	20	0.54
SBP (mmHg)	132.9 ± 21.1	131.8 ± 20.9	133.7 ± 21.6	0.72
DBP (mmHg)	75.9 ± 13.8	73.5 ± 15.2	77.5 ± 12.7	0.25
Cre (mg/dL)	3.07 ± 2.16	3.02 ± 1.63	3.11 ± 2.47	0.87
eGFR (mL/min/1.73 m^2)	27.6 ± 22.0	20.5 ± 11.9	32.9 ± 26.1	0.03
CRP (mg/dL)	3.31 ± 5.57	4.87 ± 6.28	2.30 ± 4.87	0.07
UP (g/gCre)	1.8 ± 1.4	1.7 ± 0.9	1.9 ± 1.8	0.56
uRBC (/HPF)	36.4 ± 35.5	34.2 ± 35.1	38.0 ± 36.1	0.68
MPO-ANCA (U/mL)	248.4 ± 230.3	278.1 ± 249.6	228.1 ± 217.1	0.40
PR3-ANCA (*n*)	2	0	2	
Alb (g/dL)	3.0 ± 0.6	2.8 ± 0.6	3.2 ± 0.6	0.02
IgG (mg/dL)	1310.5 ± 444.6	1260.3 ± 444.5	1338.7 ± 449.2	0.55
IgA (mg/dL)	268.0 ± 116.1	280.4 ± 118.2	261.8 ± 116.5	0.60
IgM (mg/dL)	114.7 ± 64.9	93.9 ± 40.6	125.1 ± 72.5	0.12
CH50 (U/mL)	46.6 ± 10.3	48.0 ± 11.5	45.7 ± 9.7	0.47
WBC (/μL)	10361.5 ± 5008.0	10151.9 ± 3723.9	10,497.8 ± 5733.5	0.79
Hb (g/dL)	9.8 ± 1.8	9.3 ± 1.5	10.1 ± 1.9	0.08
BVAS	16.3 ± 5.5	18.1 ± 4.7	15.1 ± 5.7	0.046
RLV	16	2	14	0.04
The time from disease onset until renal biopsy (months)	7.6 ± 13.3	3.8 ± 3.6	9.6 ± 16.8	0.09
Global sclerosis (%)	25.0 ± 20.4	22.5 ± 26.6	26.6 ± 20.4	0.427
Active crescent (%)	31.1 ± 19.9	33.3 ± 17.9	29.6 ± 21.2	0.468
IFTA (%)	38.2 ± 24.9	35.8 ± 22.8	39.6 ± 26.3	0.56

Regarding prognosis, the renal survival time was not significantly different between the immunoglobulin deposition group and non-deposition group. There was no significant difference in the renal survival curves for the IgG, IgG + IgM, and IgM deposition groups (Fig. 4).

Table 2 Clinical and histological characteristics of the IgG, IgG plus IgM, and IgM groups

	IgG	IgG + IgM	IgM	*p* value
N	15	8	17	
Age	57.1 ± 11.9	58.9 ± 14.2	56.9 ± 18.8	0.95
Female	5	4	11	
SBP (mmHg)	141.0 ± 19.5	126.1 ± 19.8	130.7 ± 23.3	0.22
DBP (mmHg)	81.1 ± 12.9	75.6 ± 8.3	75.1 ± 14.2	0.38
Cre (mg/dl)	3.16 ± 2.18	2.85 ± 2.26	3.19 ± 2.94	0.95
eGFR (mL/min/1.73 m^2)	31.2 ± 27.5	33.7 ± 32.2	34.1 ± 22.8	0.18
CRP (mg/dL)	1.01 ± 1.53	4.28 ± 9.19	2.50 ± 3.90	0.30
UP (g/gCre)	2.2 ± 2.3	1.2 ± 0.9	1.9 ± 1.6	0.45
uRBC (/HPF)	34.7 ± 32.4	71.3 ± 36.0	24.8 ± 30.3	0.007
MPO-ANCA (U/mL)	212.5 ± 216.8	262.5 ± 229.7	225.3 ± 224.3	0.87
PR3-ANCA (*n*)	1	0	1	
Alb (g/dL)	3.3 ± 0.5	3.0 ± 0.6	3.2 ± 0.6	0.58
IgG (mg/dL)	1193.7 ± 341.2	1469.4 ± 573.7	1419.5 ± 468.3	0.32
IgA (mg/dL)	262.8 ± 89.0	331.1 ± 151.8	220.3 ± 109.8	0.13
IgM (mg/dL)	83.9 ± 45.2	141.6 ± 76.9	160.0 ± 76.5	0.02
CH50 (U/mL)	50.5 ± 29.9	38.7 ± 8.3	45.1 ± 7.9	0.03
WBC (/μL)	10,456.7 ± 5816.4	11,816.3 ± 8488.1	9913.5 ± 4215.7	0.75
Hb (g/dL)	10.0 ± 2.1	10.2 ± 1.9	10.21 ± 1.7	0.96
BVAS	15.1 ± 5.0	12.6 ± 6.1	15.9 ± 63	0.15
RLV	5	3	6	
Global sclerosis (%)	26.7 ± 20.9	27.3 ± 19.0	26.3 ± 21.7	0.99
Active crescent (%)	41.1 ± 23.2	14.7 ± 11.0	26.5 ± 18.0	0.002
IFTA (%)	42.7 ± 25.5	35.6 ± 30.6	38.8 ± 26.2	0.826

Fig. 2 Immunofluorescent staining. **a** Double immunofluorescence with anti-MPO antibodies and anti-IgG antibodies. Merger of immunofluorescence demonstrated co-localization of MPO and IgG. **b** Double immunofluorescence with anti-MPO antibodies and anti-IgM antibodies showed little staining for MPO and no co-localization
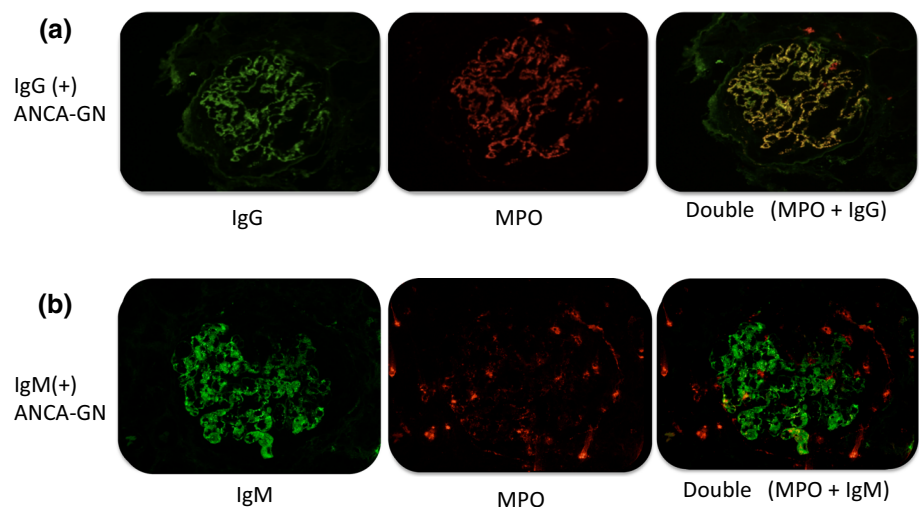
Discussion

In this study, we found that 40 of 66 cases (60.6%) that were diagnosed with MPO-ANCA-associated glomerulonephritis had immunoglobulin deposition along the glomerular capillary. In ANCA-associated glomerulonephritis, immunoglobulin deposition in the glomeruli is considered to be infrequent and minimal [12, 13]. We observed a higher incidence of Ig depositions than that reported in other papers; however, differences in the evaluation of staining intensity might have played a role. In most papers, the staining intensity was evaluated from 1+ to 3+, whereas we evaluated it from ± to 2+. Thus, some cases that would have been considered as pauci-immune in other papers could have been included in our data and used in our calculations. In addition to cases with
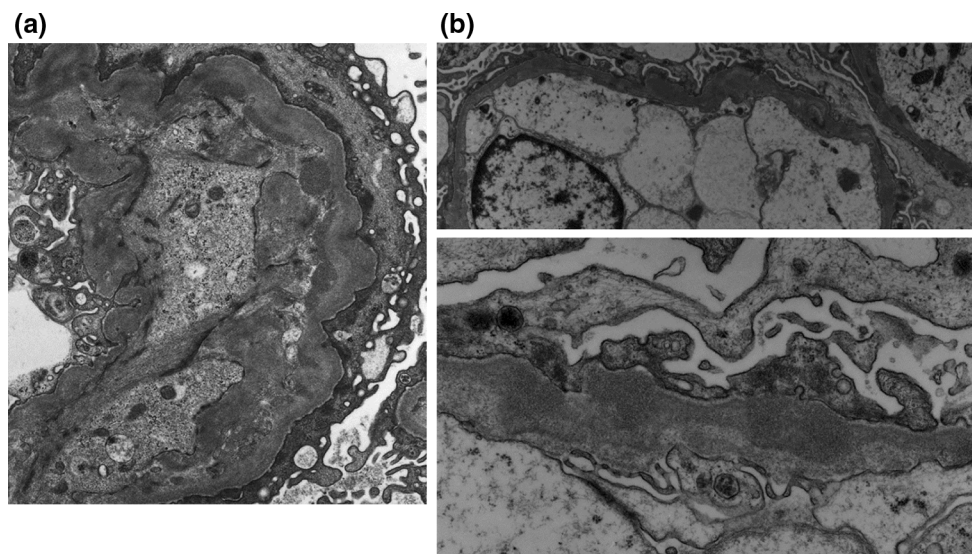
Fig. 3 Electron micrographs. **a** Subendothelial and mesangial deposition. **b** Subepithelial and intramembrane deposition
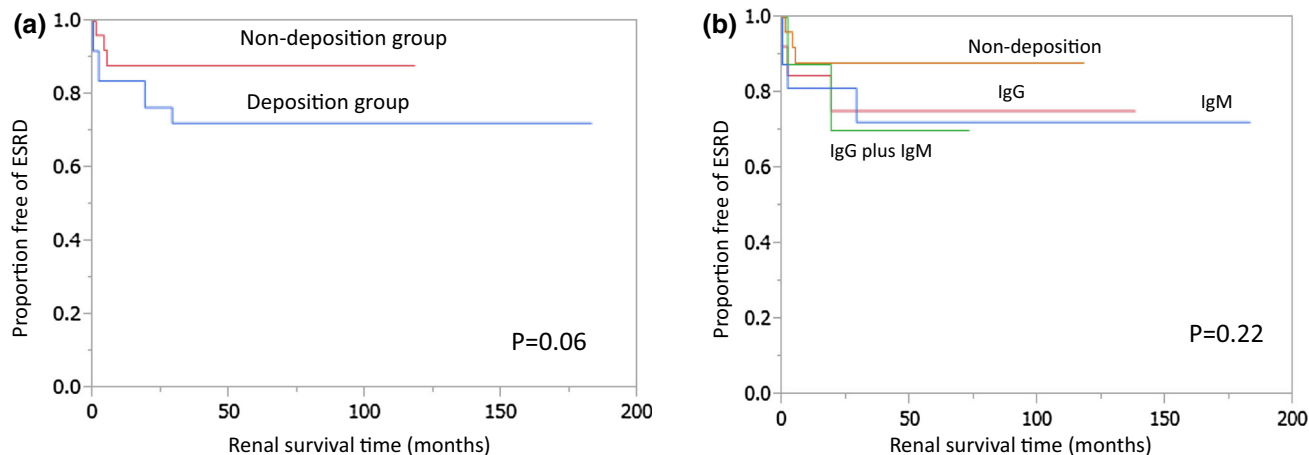

Fig. 4 Kaplan–Meier curves showing probability for end-stage renal failure requiring dialysis. **a** Cumulative renal survival for 66 patients in the non-deposition group or the deposition group. **b** Cumulative renal survival in the IgG, IgG + IgM, and IgM groups

global depositions in the GBM, cases with focal depositions that did not cover 50% of the GBM, a characteristic of concurrent MGN, were also included.

The combination of MGN and ANCA-related crescentic glomerulonephritis has been reported in several cases [14–17]. In this study, all cases in the IgG group showed co-localization of IgG and C3. However, the clinical findings showed that the range of proteinuria levels was from 0.12 to 7.02 g/gCr with a mean of 2.2 ± 2.3 g/gCr. 8 cases showed proteinuria in the nephrotic range, and 6 cases showed a maximum of up to 1 g/gCr. Spike and overlying membrane formations were not seen, and some cases did not have deposition coverage over 50% nor staining intensity ≥2+, the diagnostic characteristics for MGN concurrent with ANCA-associated glomerulonephritis [7, 8]. Even if there is a possibility of MGN

concurrent with ANCA, secondary MGN is more likely than primary from IgG subclass and distribution of electron-dense deposits. We observed that MPO was also co-localized with IgG and C3 from IF double staining. From this, we hypothesize that IgG and MPO are forming an immunocomplex. IgM depositions did not show co-localization with MPO or electron-dense deposits. We suspect insudation of IgM rather than a formation of an immunocomplex.

Kawashima et al. reported that MPO and MPO-positive cells were present along the glomerular capillary walls and also detected MPO, IgG, and C3 deposition along the glomerular capillary walls in the early phase of severely damaged glomeruli during the active phase of MPO-ANCA-associated glomerulonephritis, and also they suggested that MPO released from neutrophil as well as

immune complexes composed of MPO and anti-MPO antibodies may be associated with pathogenesis of glomerular injury [18, 19]. Recently, Kassenblock et al. reported that neutrophil extracellular traps contain both MPO and nuclear fragments in the chromatin fiber and are released from ANCA-stimulated neutrophils, resulting in glomerular capillary necrosis during ANCA-associated glomerulonephritis [19]. In our study, IgG deposition was observed in seemingly normal glomeruli that were not damaged nor changed, and there was no significant difference between crescent formation and sclerotic glomeruli between the deposition and non-deposition groups.

Some papers report immunocomplex formation only during the early phases of the disease. An animal model for MPO-ANCA-associated glomerulonephritis showed that in situ immune complexes of MPO and anti-MPO antibodies only appeared in the early phase of ANCA-associated glomerulonephritis [10]. In clinical studies, immune complexes of MPO and anti-MPO antibodies appeared during early-phase disease as well [18]. In the present study, there was no significant difference in the time from disease onset until renal biopsy between the immunoglobulin group and the non-immunoglobulin group. Re-biopsy was not performed in our study; however, it is required to elucidate whether immune complexes persist or disappear over the course of the disease.

Yu et al. reported that patients with ANCA-associated vasculitis with immune complexes showed more proteinuria and glomerular cellularity compared with patients with pauci-immune vasculitis [20]. Haas et al. also reported that the presence of deposits on electron microscopy was associated with higher median levels of proteinuria and a higher percentage of crescentic formation [5]. In our study, proteinuria was not significantly different between the immunoglobulin group and the non-immunoglobulin group; however, proteinuria in some patients with heavy subepithelial deposition was in the nephrotic range, thus proteinuria levels may depend on the degree of subepithelial deposition.

In conclusion, granular IgG and/or IgM deposition was observed in 60.6% of patients with ANCA-associated glomerulonephritis. In cases with IgG deposition, electron-dense deposits were observed at various sites in the glomerulus, and MPO and IgG deposition were co-localized along the glomerular capillary. These depositions were also co-localized with C3; therefore, immunocomplex was suspected. According to clinicopathological findings in the deposition group, immunocomplex may not cause glomerular damage. With IgM deposition, EDDs were not obvious in the glomerular basement membrane, and MPO and IgM immunocomplex was not detected. These data suggest differential mechanism between IgG deposition and IgM deposition.

Further investigation is required to elucidate the role of immune complex deposition in the development of ANCA-associated vasculitis.

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