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CLINICAL STUDY

IgA nephropathy: association of C4d with clinical and histopathological findings and possible role of IgM

Cihan Heybeli[1], Mehtat Unlu[2], Serkan Yildiz[3], Caner Çavdar[3], Sulen Sarioglu[2], and Taner Camsari[3]

[1]Department of Internal Medicine, Dokuz Eylul University School of Medicine, İzmir, Turkey, [2]Department of Pathology, Dokuz Eylul University School of Medicine, İzmir, Turkey, and [3]Department of Nephrology, Dokuz Eylul University School of Medicine, İzmir, Turkey

Abstract

Background: In patients with IgA nephropathy (IgAN) lectin and alternative pathways of the complement can be activated. Our aim was to analyze the association of glomerular and extraglomerular C4d staining—the representative of lectin pathway—with demographic, clinical and histopathological findings in primary IgAN patients. *Design*: Seventy-three patients were enrolled and after re-evaluation 37 of them were included in this study. Biopsies were analyzed for staining with anti-C4d primary monoclonal antibody by immunohistochemistry. Patients were classified as positive and negative groups based on their glomerular C4d deposition. Groups were compared for their baseline clinical and histopathological findings. *Results*: Sixteen (43.2%) of 37 patients were C4d-positive. Glomerular C4d-staining was associated with more severe proteinuria (2906 mg/day vs. 1091 mg/day; $p = 0.002$), lower GFR (54.87 mL/min vs. 95 mL/min; $p = 0.023$), higher blood pressure ($p = 0.022$), more severe endocapillary hypercellularity ($p < 0.001$) and more severe tubular atrophy ($p < 0.01$). Mesangial IgM deposition was found to be associated with glomerular C4d staining and nephrotic range proteinuria. *Conclusions*: Glomerular C4d deposition was found to be associated with more unfavorable histopathological and clinical findings at the time of diagnosis. Association of mesangial IgM deposition with the activation of lectin pathway is a novel finding. Mesangial IgM deposition in our patients may reflect the genetic heterology of IgAN between diverse populations. However, since these data are about association, a cause-and-effect about IgM and IgAN cannot be proven solely with these findings.

Keywords

C4d, complement, IgA nephropathy, IgM, lectin pathway

History

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Introduction

Being the most common glomerulonephritis world-wide, immunoglobulin A nephropathy (IgAN) is diagnosed according to the immunofluorescence findings of kidney biopsy. There is a wide spectrum of clinical findings both at the time of diagnosis and in follow-up.[1–5] Although IgAN used to be known as a benign condition, in a 20-year follow-up one-third of patients and in a 30-year follow-up almost half of the patients progress to end-stage renal disease (ESRD).[6,7]

It is important to distinguish patients who have unfavorable renal survival since they may benefit from immunosuppressive therapy. Lower GFR, higher degree of proteinuria and higher systolic blood pressures were associated with worse prognosis as higher histologic grade was.[8,9] In the last decade after Roos et al. has shown that C4d, the representative of lectin and classic pathways of the complement system was associated with worse clinical findings at the time of diagnosis,[10] Espinosa et al. demonstrated the role of that marker as a new prognostic factor in IgAN.[11] Then, a multicenter retrospective cohort study showed that C4d is an independent risk factor for the development of ESRD;[12] this was recently supported by another single-center study.[13] Association of glomerular C4d with more unfavorable clinical and histopathological findings was shown in another preliminary study recently.[14] Lastly, several immunohistologic biomarkers were studied to assess their predictive values for disease progression.[15] Here, we wanted to evaluate patients with IgAN in our institution for C4d staining and analyze the associations between both clinical and histopathological findings with C4d staining.

Subjects and methods

Our study was approved by the Ethics Committee of our institution. Seventy-three patients who were diagnosed as IgAN between January 2003 and December 2013 were enrolled in this retrospective study. Patients who were younger than 18 years of age and who had a disease which may be respected to cause IgAN (lymphomas, Behçet's

Address correspondence to Dokuz Eylul, University School of Medicine, Balcova, İnciralti, Mithatpasa Street, İzmir, Turkey. Tel: +90 2324123766; Fax: +90 2324123785; E-mail: heybelic@hotmail.com

disease, seronegative spondyloarthropathies, lymphomas, cirrhosis, solid-organ malignancies, inflammatory bowel diseases, celiac disease and infections with HIV, HBV, HCV, CMV and tuberculosis) were not included. Thereafter, 45 patients were identified as primary IgAN and kidney specimens of these were included in histopathological analysis. Renal biopsy specimens were evaluated with hematoxylin–eosin, Masson's trichrome, periodic acid schiff and methenamine silver stained sections by light microscopy and staining with antibodies against immunoglobulin G (IgG), immunoglobulin A (IgA), immunoglobulin M (IgM), complement 3c, C1q, kappa and lambda for immunofluorescence. Presence of at least three non-sclerotic glomeruli was determined as a requirement to be able to assess glomerular C4d staining. So that 37 patients were established as to be eligible for the study. Medical records of patients were analyzed retrospectively.

Histopathological parameters

Mesangial hypercellularity, endocapillary hyperproliferation, segmental sclerosis and tubular atrophy (MEST) score was analyzed according to Oxford criteria. Crescent formation was classified as cellular and fibrotic. Results of immunohistochemical staining for IgG, IgA, IgM, C3c, C1q, kappa and lambda were classified as negative or positive.

C4d staining

Three-micrometer sections of formaldehyde-fixed renal tissue were stained in Benchmarck Ultra machine using ultra view universal Dab detection kit. Antigen retrieval was performed in ultra CCl solution for 64 min in 95 °C. Primary monoclonal anti-C4d antibody (Cell Marque®, SP91 clone, Rocklin, CA) was applied and incubated for 32 min.

Biopsies were categorized as positive or negative based on glomerular C4d immunohistochemical staining. Patients were classified as ''positive'' if >25% of the glomeruli were positive for C4d. Staining was defined as ''global'' when >50% of mesangial area was affected and as ''segmental'' otherwise. Assessment for tubular epithelium and interlobular capillaries was also performed.

After immunohistochemical staining patients were classified as positive and negative groups based on their glomerular C4d staining-status. Positive and negative groups were compared for their demographic (patient age and sex), clinical (degree proteinuria, serum creatinine level, creatinine clearance, presence of macroscopic hematuria, presence of hypertension or use of anti-hypertensive medicine, use of immunosuppressive, need for renal replacement therapy) and histopathological (C4d staining in glomerular and extraglomerular sites of kidney; Oxford classification system criteria; mesangial immunoglobulin depositions; presence of crescent formation) findings. We calculated the estimated GFR (eGFR) according to modification of diet in renal disease (MDRD) study equation.

Statistical analysis

Chi-square and Fisher's exact tests were used to compare qualitative values. Results are expressed as mean ± standard deviation. A p-value of <0.05 was considered to be statistically significant. For numerical data analysis, Chi-square and Fisher's exact test were used; for variant analysis, the Mann–Whitney U test and correlation analysis were used. Calculations were performed using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) 16.0 version.

Results

Baseline characteristics of patients are presented in Table 1. After histopathological analysis of kidney biopsy specimens of 45 patients who were diagnosed as primary IgAN, eight were excluded due to insufficient non-sclerotic glomeruli for C4d staining. Of remaining 37 patients, 11 were females. Sixteen patients were C4d positive (43.2%). Four of women patients and 12 of men were in glomerular C4d-positive group. Glomerular C4d staining was global in four patients. In addition to glomerular C4d staining in 16 patients, tubular C4d staining was observed in four patients, while interlobular arterial C4d staining was shown in eight patients (Figure 1). Peritubular capillary involvement for C4d staining was not observed in any patient.

Other histopathological analyses

When patients were evaluated based on Oxford classification, 28 (75.7%) were M1, 17 (45.9%) were E1, 27 (73%) were S1, 15 (40.5%) were T0 and 12 (32.4%) were T1. Crescent formation was observed in 12 (32.4%) patients, while 7 of them were cellular in type.

Association between glomerular C4d staining and other baseline histopathological analyses

Glomerular C4d staining was associated with endocapillary hypercellularity ($p<0.001$), interstitial fibrosis/tubular atrophy ($p<0.001$) and mesangial IgM deposition ($p=0.006$). Glomerular depositions of immunoglobulins and complements in glomerular C4d positive and negative groups are as shown in Table 2. Association of glomerular C4d with mesangial hypercellularity score, segmental sclerosis score and crescent formation were not statistically significant. Statistical significance was not found when analysis was done for cellular and fibrotic crescent types, separately (Table 3).

Association between glomerular C4d staining and baseline clinical findings

Statistically significant association of positive glomerular C4d was observed in the presence of hypertension, higher eGFR and more severe proteinuria at the time of diagnosis (Table 4).

C4d staining in extraglomerular sites

In four patients tubular epithelial staining with C4d was observed. This was not statistically significant with any clinical or histological criteria. In eight (21.6%) patients interlobular arterial C4d staining was shown. Although glomerular C4d staining was not associated with cellular crescent formation, interlobular arterial C4d staining was associated with both cellular crescent formation ($p=0.027$) and an E1 score of endocapillary hypercellularity ($p=0.014$).

Table 1. Basic characteristics of all included patients at the time of diagnosis.

Variable	Gender	Age	eGFR (mL/min/1.73 m^2)	Proteinuria (mg/day)	Hypertension (≥140/90 mmHg)	MEST score[a]	Mesangial C4d-stain	Interlobular arterial C4d stain	Tubular C4d stain	Mesangial IgM/IgG stain[b]
n1	Male	36	73	700	No	0/0/1/0	Negative	Negative	Negative	Yes/yes
n2	Male	39	102	864	No	0/0/1/0	Negative	Negative	Positive	Yes/no
n3	Female	20	138	2290	No	0/0/0/0	Negative	Negative	Negative	No/no
n4	Male	34	85	2450	No	1/0/1/1	negative	Negative	Negative	No/no
n5	Male	63	54	1300	Yes	1/0/1/1	Negative	Negative	Negative	No/yes
n6	Male	32	19	4000	Yes	1/1/1/2	Negative	Positive	Negative	Yes/yes
n7	Male	31	206	540	No	1/0/0/0	Negative	Negative	Negative	No/no
n8	Male	21	90	731	No	1/0/1/0	Negative	Negative	Negative	No/no
n9	Male	25	78	1511	No	1/1/1/1	Negative	Negative	Negative	No/no
n10	Male	52	39	100	Yes	1/1/1/1	Negative	Negative	Negative	No/no
n11	Female	29	113	1990	Yes	1/1/1/1	Negative	Negative	Negative	No/yes
n12	Male	23	57	420	No	1/0/0/0	Negative	Negative	Negative	No/no
n13	Female	33	45	930	No	1/1/1/0	Negative	Negative	Negative	Yes/yes
n14	Female	39	85	290	Yes	0/0/0/0	Negative	Negative	Negative	No/no
n15	Male	67	29	540	No	1/1/1/1	Negative	Negative	Negative	No/no
n16	Male	50	78	100	No	0/0/0/0	Negative	Negative	Negative	No/yes
n17	Female	29	107	157	No	0/0/0/0	Negative	Positive	Negative	No/no
n18	Male	28	65	30	Yes	0/0/1/0	Negative	Negative	Negative	No/no
n19	Male	49	114	1340	No	1/0/1/0	Negative	Negative	Negative	No/no
n20	Female	25	82	2500	No	1/1/0/0	Negative	Negative	Negative	Yes/yes
n21	Female	34	82	134	No	0/0/0/0	Negative	Negative	Negative	No/no
n22	Male	51	9	4352	Yes	1/1/1/2	Positive	Positive	Negative	Yes/yes
n23	Male	54	40	8380	Yes	1/1/1/2	Positive	Positive	Negative	No/no
n24	Male	25	146	4400	No	1/1/1/2	Positive	Negative	Negative	Yes/yes
n25	Female	31	78	1800	No	1/1/1/1	Positive	Negative	Negative	Yes/no
n26	Male	24	81	2900	Yes	1/1/1/1	Positive	Negative	Negative	Yes/no
n27	Female	71	19	900	Yes	1/1/0/1	Positive	Positive	Positive	Yes/yes
n28	Male	55	101	2338	Yes	1/1/0/0	Positive	Negative	Negative	No/no
n29	Male	63	52	1744	No	1/1/1/1	Positive	Positive	Negative	No/no
n30	Male	53	12	5960	No	1/1/1/2	Positive	Negative	Positive	Yes/no
n31	Male	30	54	1100	Yes	1/0/1/2	Positive	Negative	Negative	Yes/no
n32	Male	50	32	5500	Yes	1/1/1/2	Positive	Positive	Negative	No/no
n33	Male	44	30	2000	Yes	0/0/1/2	Positive	Negative	Positive	Yes/yes
n34	Male	59	47	975	Yes	1/1/1/1	Positive	Positive	Negative	No/no
n35	Male	38	53	4643	Yes	1/0/1/2	Positive	Negative	Negative	Yes/no
n36	Female	34	89	2000	No	1/1/1/1	Positive	Negative	Negative	Yes/no
n37	Female	48	35	558	Yes	1/1/1/2	Positive	Negative	Negative	Yes/no

Notes: [a]Mesangial hypercellularity, endocapillary hypercellularity, segmental sclerosis, tubular atrophy/interstitial fibrosis, respectively.
[b]IgM and IgG, respectively.

There was no significant association between extraglomerular and glomerular C4d staining.

Discussion

In the current study, we showed that glomerular C4d staining is associated with more severe endocapillary hypercellularity and interstitial fibrosis/tubular atrophy. T2 score was an independent risk factor for progression to ESRD in the study of Spanish Glomerular Disease Study Group.[12] It is clearly known that T2 score of tubular atrophy is the strongest histopathologic criteria which predicts progression to end-stage renal failure.[16] In our study, glomerular C4d staining was significantly associated with a score of T2. This reflects the importance of glomerular C4d staining on predicting renal survival.

Our results suggested that patients with glomerular C4d staining clearly had lower GFR, more severe proteinuria and higher rates of hypertension at the time of diagnosis. Thus, confirming the previous studies we have shown that glomerular C4d staining is associated with worse clinical findings and more severe histologic grade.

Of 16 patients with glomerular C4d-positive staining, four were also positive for interlobular arterial C4d staining. However, four patients were also positively stained for interlobular arterial C4d in glomerular C4d-negative group. In glomerular C4d-positive group, three patients' renal biopsies were stained for tubular C4d. In glomerular C4d-negative group number of patients with positively tubular C4d staining was 1. Neither of the data about concurrence of glomerular and extraglomerular C4d were statistically significant. Since the number of patients with extraglomerular staining was small, it would be unreliable to comment on association of glomerular and extraglomerular C4d staining, and role of extraglomerular staining in disease prognosis.

No statistically significant association between gender and glomerular C4d-staining was found. Mean follow-up was 24.3 months and excluding two patients who had ESRD at the time of diagnosis, no patient had ESRD in follow-up. Only three patients were in a worse state of glomerular filtration rate compared to baseline eGFR. This regression in eGFR in three patients was not significant and did not cause to categorize the patients as in a more advanced kidney failure stage.

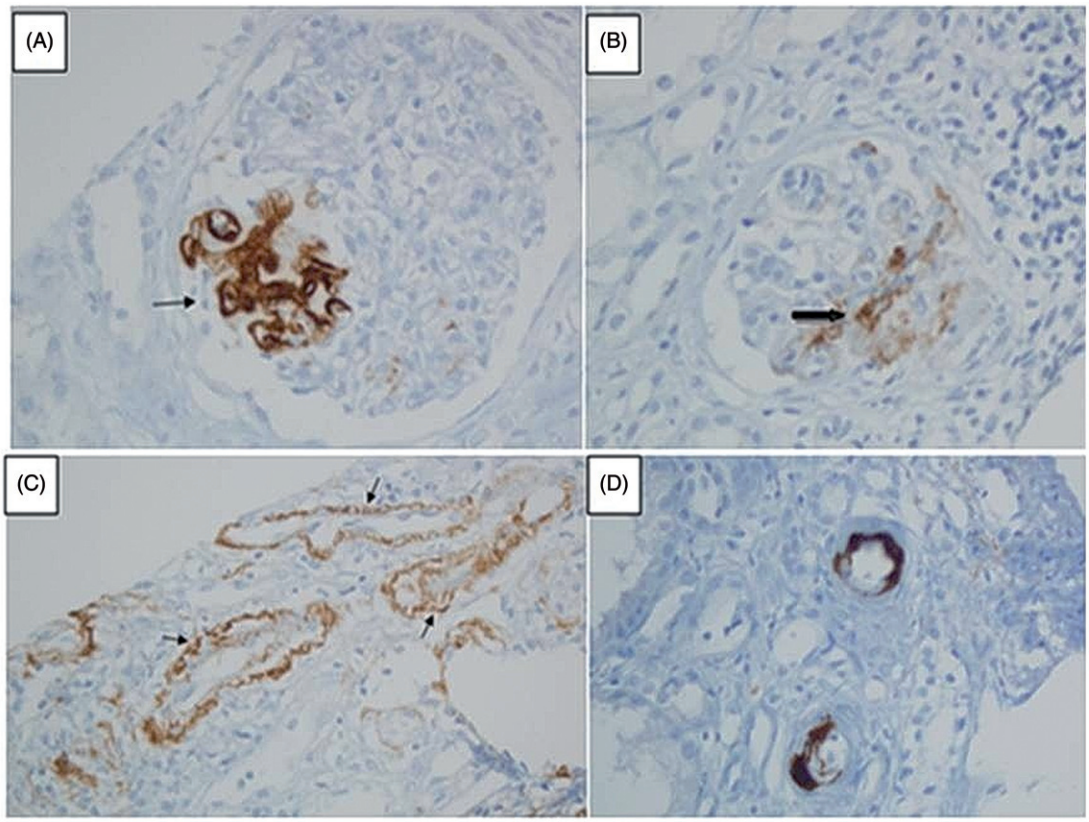


Figure 1. C4d staining in different sites of kidney biopsy specimens of patients with primary IgAN. (A) C4d staining in a segmentally sclerotic mesangial area. (B) C4d staining in another mesangial area with no sclerosis. (C) C4d staining in renal tubular epithelium. (D) Interlobular arterial C4d staining (stained with anti-C4d monoclonal antibody; magnification ×20).

Table 2. Glomerular depositions of immunoglobulins and complement molecules in glomerular C4d positive and negative groups.

C4d staining	IgG (+)[a]	IgA ≥ 3(+)	IgM (+)[b]	C3c ≥ 3(+)	C1q
Positive ($n = 16$)	4	6	11	7	0
Negative ($n = 21$)	7	9	5	2	0
p	0.723	1	0.006	0.024	

Notes: [a]Number of patients with positive mesangial IgG deposition in mesangial C4d positive and negative groups.
[b]Number of patients with positive mesangial IgM deposition in mesangial C4d positive and negative groups.

Table 3. Association between glomerular C4d staining and baseline histopathological analyses.

Variable	Total ($n = 37$)	C4d-positive ($n = 16$)	C4d-negative ($n = 21$)	p-Value
Oxford class				
M0/M1	9/28	1/15	8/13	0.0565
E0/E1	20/17	3/13	17/4	<0.001[a]
S0/S1	10/27	2/14	8/13	0.137
T0/T1/T2	15/12/10	1/6/9	14/6/1	<0.001/ 0.024/<0.001[a]
Crescent	12	6	6	0.565[b]
Cellular	7	5	2	0.202[b]
Fibrotic	5	1	4	0.364
Mesangial IgM	16	11	5	0.006[c]

Notes: [a]Glomerular C4d positive staining was associated with more severe endocapillary hypercellularity and interstitial fibrosis/tubular atrophy score and this was statistically significant. Severity of other Oxford classification criteria was not associated with glomerular C4d-staining.
[b]Crescent formation was not associated with glomerular C4d staining even when analyzed for cellular and fibrotic crescents, separately.
[c]Mesangial IgM deposition was associated with glomerular C4d staining and this was statistically significant.

We think that since IgAN is usually an indolent disease, this may count for this result. Thus, we could not analyze the effect of complement staining on renal survival.

Interestingly, we could not show an association between glomerular C4d deposition with another predictor of worse prognosis, crescent formation. Results were still statistically insignificant when analyzed for cellular and fibrous crescents, separately. However, it is known that cellular immunity has a major role on crescent formation rather than the complement system.[17] On the other hand, we found that interlobular C4d staining is associated with cellular crescent formation and both were associated with only one criteria of Oxford classification, endocapillary hypercellularity. We speculate that this reflects the endothelial damage, the first hit necessary for the formation of crescents.

An interesting result of our study was about the association of mesangial IgM deposition with glomerular C4d staining which is a novel finding. In the study of Espinosa et al.[11] the number of patients with mesangial IgM deposition was very small and there was no patient with mesangial IgM deposition in C4d positive group. This is important because although IgM is known to activate the classic pathway of the complement system as IgG does, it also has the potential to activate the lectin pathway both *in vitro* and *in vivo*.[18]

Table 4. Association between glomerular C4d staining and baseline clinical findings.

Variable	Total ($n = 37$)	C4d-positive ($n = 16$)	C4d-negative ($n = 21$)	p-Value
Presence of macroscopic hematuria	11	2	9	0.071
Presence of hypertension	17	11	6	0.022[a]
eGFR (MDRD; mL/min)				
Mean	70.8	54.9	82.9	0.023[b]
Minimum	9	9	19	
Maximum	206	146	206	
Proteinuria (mg)				
Mean	1847	2906	1091	0.02[c]
Minimum	30	558	30	
Maximum	8380	8380	4000	
<1 g/day	16	3	13	
>1 g/day	21	13	8	

Notes: [a]Association of glomerular C4d staining and the presence of hypertension which was defined as blood pressure ≥140/90 mmHg or use of anti-hypertensive medicine was statistically significant.
[b]Glomerular C4d positive group had a lower baseline eGFR than negative group.
[c]Glomerular C4d positive group had a more severe proteinuria than negative group at the time of diagnosis.

Mesangial IgM deposition was associated with both scores of E1 and T2, and with the presence of nephrotic syndrome. Although this difference on IgM deposition between our data and previous study results may be due to technical issues in detecting IgM, paucity of patients with mesangial IgM deposition in previous studies may also be the consequence of geographical differences. There is no data to claim a role for IgM in disease pathogenesis. Speculatively, IgM may aggravate detrimental effects of lectin pathway in kidney which was started by IgA1 immune complexes in circulation. In a GWAS performed by Kiryluk et al.[19] newly identified seven single-nucleotide polymorphisms were differently distributed between ethnicities. Dissimilar results between previous studies and ours for IgM deposition could be a result of various candidate gene actions between diverse patient populations. On the other hand, since our results reflect an association, a cause-and-effect about IgM and IgAN cannot be proven solely with these findings.

Compared to monoclonal antibody, using polyclonal antibody is an inexpensive and quick way on recognizing multiple epitopes on an antigen. However, monoclonal antibodies are more specific. One of our aims was to analyze the deposition of C4d in regions other than mesangium. Polyclonal antibody for C4d may lack the signal in these areas.[20]

It is still unknown whether there is a direct role of lectin pathway on pathogenesis of IgAN or it is only a result of the main cause. We think it would be wisely to assess immunoglobulin deposition and C4d staining in origins of antigen presentation like intestinal tissue layers, although classical pathway may also be activated in these cites. This may render new opinions in pathogenesis of IgAN. Moreover, secondary IgAN patients were excluded in previous studies. In forthcoming studies including those patients and analyzing their C4d staining status would show possible different effects of different facilitators of IgAN.

In conclusion, glomerular C4d staining is shown to be associated with worse clinical and histopathologic findings once again in a different geographical region. Association of mesangial IgM deposition with glomerular C4d staining is a new finding. Since IgM has the potential to activate lectin pathway, the discrepancy of results between previous studies and ours should be re-evaluated in forthcoming studies. IgAN has a two-sided pathway in its pathogenesis: alternative and lectin pathways of the complement system. In these well-defined ways, studying the role of mesangial IgM deposition, effects of secondary causes of IgAN on lectin pathway and the role of complement staining in antigen-presentation sites like intestinal tissue layers would construct new avenues on this two-sided pathway.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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