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C4d Glomerular Deposits and Disease Progression in Native Idiopathic Membranous Nephropathy

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Abstract

Introduction: Since 1989 when Kusunoki et al. described C4d renal deposits in native idiopathic membranous nephropathy (nIMN) their role in disease progression has not been clarified yet. Recent studies have identified C4d glomerular staining as a marker of negative progression of renal function in a primary glomerular disease like IgA nephropathy. We have retrospectively analysed 15 consecutive formalin-fixed paraffin-embedded kidney biopsies from patients with nIMN (7F, 8M) performed in our Unit from October 1995 to February 2011.

Methods: Kidney sections were stained using polyclonal rabbit IgG anti-human C4d antibodies. Normal renal tissue was obtained from heart-beating braindead donors before kidney harvesting. Positive control biopsy was a humoral kidney rejection with intense C4d staining. Data are expressed as M ± SD.

Results: Ten (5F, 5M) of 15 nIMN kidney biopsies showed global and diffuse C4d glomerular capillary staining (C4d+). At 6-month follow-up (C4d+ 31 ± 26 months, C4d− 29 ± 31 months; P = NS) we observed a significantly higher 24-hour urinary protein excretion rate (UPr) in C4d+ (P = 0.0051 vs. C4d−), and a significantly lower MDRD eGFR (P = 0.0337 vs. C4d− at diagnosis) when compared with data at disease presentation.

Conclusion: Our data suggest that C4d glomerular capillary deposits in nIMN with a follow-up longer than 6 months might be a negative prognostic factor for both UPr and eGFR. We are aware that our study has limitations like the relatively short term follow-up and the little number of biopsy analysed. Nevertheless, the association between increasing UPr, worsening of renal function and glomerular deposits of C4d in nIMN patients might deserve reporting and eventually confermation by further investigations.

Background

Increasing evidence from animal studies support the hypothesis that progression of renal failure in glomerular diseases is related by local complement activation [1]. Interstitial vascular C4d vascular deposits have been described in acute and chronic antibody-mediated rejection (ABMR) [2,3]. C4d is a surface-bound split product of inactive C4b obtained from classical or lectin complement pathways. To the date, receptor for C4d has not been identified and its biological function is unknown [4]. C4d positive staining (C4d+) has also been reported in normal human kidneys [5], primary [6-8] and secondary [9,10] nephropathies. The weak segmental C4d+ glomerular staining observed in normal kidney biopsies possibly results by local complement activation involved in the physiological clearance of IgM-containing immune complexes [5]. A study on C4-deficient guinea-pig showed increased IgM glomerular deposits when compared to non C4d-deficient mammalian kidneys [11]. Human diseases studies suggest that C4d+ mesangial staining in patients with IgA nephropathy (IgAN) could identify individuals with a worse long-term prognosis [6]. In lupus nephritis (LN) glomerular C4d+ staining has been associated with higher risk of developing thrombotic microangiopathy [12]. Interstitial peritubular C4d+ capillary deposits in LN appear to identify intense immunological disease activity [13].

Native idiopathic membranous nephropathy (nIMN) is an established antibody-mediated glomerular disease. A recent work has identified the M-type phospholipase A2 receptor (PLA2R), a 185-kD glomerular glycoprotein as the target antigen in nIMN [14].

PLA2R is a podocyte protein expressed in normal glomeruli and

PLA2R antibodies are mainly of IgG4 isotype [14]. Experimental models have clarified that local injury is mediated by *in situ* immune complexes and complement activation [15]. However, IgG4 do not activate complement or very little only [16]. It may support the hypothesis that IgG4 act altering podocyte architecture and barrier function [14] suggesting that IgG subclasses other than IgG4 activate complement in nIMN binding PLA2R and/or other glomerular antigen(s).

Glomerular C4d deposits in nIMN sections has already been documented and often are used as positive control for C4d staining of renal grafts in order to support a diagnosis of ABMR in renal transplanted patients [17]. In absence of ABMR with stable renal function C4d deposits in graft glomerular capillaries appear to be associated with late (> 6 months) graft failure [18], but it is unknown the meaning of early and late outcome of nIMN with C4d+ glomerular staining.

Aim of this retrospective study was to analyse changes of daily urine protein excretion and glomerular function in nIMN patients according to the presence of C4d deposits.

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Materials and Methods

From December 1995 to February 2011, 1207 native kidney biopsies have been performed in our Unit. Frozen and formalin-fixed paraffin-embedded kidney samples have been stored for all biopsies. Frozen sections have been tested for routine IF with FITC-conjugated rabbit anti-human IgG, IgA, IgM, C3, C4, fibrinogen, kappa and lambda light chains (Dako, Glostrup, Denmark). Paraffin sections are stained for histopathological evaluation with Harris hematoxylin and eosin, periodic acid-Schiff stain (PAS), methenamine-silver and Masson trichrome. Diagnosis and classification of nIMN glomerular lesions are carried out according to the classic staging by Ehrenreich and Churg as reported by Schawartz [19].

C4d routine staining for transplanted kidney biopsies was introduced in our Unit in May 2007. To date C4d staining has been performed in 10 kidneys from heart-beating braindead donors before transplantation and 97 transplanted renal allografts respectively.

C4d immunohistochemical detection was performed on formalin-fixed, paraffin-embedded kidney sections by indirect immunoperoxidase staining. Four µm thick sections cut from formalin-fixed paraffin-embedded renal tissue were deparaffinized, and endogenous peroxidase activity is blocked by 8-min 1:4, 30% hydrogen peroxide dilution with distilled water. Antigen retrieval was obtained by 13-min 1 bar pressure cooking at 250°C. Primary polyclonal rabbit IgG anti-human C4d antibodies (Biomedica Group, Vienna, Austria) were applied at 1:30 dilution using Dako citomation solution (Dako, Glostrup, Denmark) [20] and incubated for 1 hour at 37°C. Slides were then incubated with En Vision solution (Dako, Glostrup, Denmark) and staining was visualized by 3, 3'diaminobenzidine. Immunoperoxidase reaction was blocked by distilled water and nuclear staining was obtained using Harris haematoxylin.

Negative control renal tissue was obtained from ten (6M, 4F) previously mentioned heart-beating braindead donors (42 ± 12 year-old) before kidney harvesting (Figure 1A). Positive control for C4d staining was renal graft sections from a kidney transplanted patient with ABMR and intense C4d interstitial capillary staining (Figure 1B).

Data are expressed as mean ± standard deviation. Statistical analysis was performed using T-test analysis. The null hypothesis was rejected when the *P*-value was less than 0.05. All analyses were performed using the statistical package Stata 8.0 (Stata Corporation. College Station, Texas 77845 USA, 2003).

Results

Negative control biopsies showed glomerular, vascular and interstitial negative C4d immunohistochemistry (Figure 1A). All fifteen nIMN biopsies tested for C4d were retrieved from Caucasian patients (7M, 8F) at diagnosis and before treatment. Mean age was 53.1 ± 21.9 years. Laboratory, histology and C4d immunohistochemistry findings are summarized in Table 1. Ten of five nIMN kidney biopsies showed glomerular capillary wall C4d deposits. At diagnosis no differences were observed between the two groups regarding age, sex, renal function, proteinuria, and disease stage (Table 1). Treatment induction with *i.v.* cyclophosphamide was administrated in 6 cases (4 C4d$^+$, 2 C4d$^-$). Oral prednisone (3 C4d$^+$, 2 C4d$^-$), prednisone with azathioprine (5 C4d$^+$, 2 C4d$^-$) or cyclosporine A alone (1 C4d$^+$) were given as maintenance therapy (Table 2). nIMN C4d positivity was characterized by fine granular continuous diffuse and global staining of glomerular capillary walls (Figure 1D). Tubules, interstitium and interstitial capillaries resulted always C4d$^-$ (Figure 1).

Data on 24-hour proteinuria (UPr) adjusted for 100 ml MDRD eGFR (Modification of Diet in Renal Disease estimated Glomerular Filtration Rate) [21] and MDRD eGFR have been analysed at diagnosis, after 6 months and at latest follow-up (C4d$^+$ 31 ± 26 months and C4d$^-$ 29 ± 31 months (*P* = NS) for C4d$^+$ for C4d$^-$ nIMNs respectively. Statistically significant higher UPr levels were observed in C4d$^+$ (when compared to C4d$^-$ nIMNs at last follow-up (Table 3). Significantly lower C4d$^+$ MDRD eGFR was found at last follow-up when compared to MDRD eGFR values at diagnosis (Table 3).

Discussion

We have described glomerular capillary C4d deposits in 66% of our nIMN kidney biopsies. C4d$^+$ nIMNs appeared to be associated to a) a significant increase of UPr during a follow-up longer than 6 months when compared to C4d$^-$ patients; b) a significant reduction of C4d$^+$ MDRD eGFR has been observed at last follow-up (31 ± 26 months) when compared to values at diagnosis. Although in experimental membranous nephropathy the role of complement for podocyte injury is essential [22] it is questioned in nIMN because the human disease is characterized mainly by IgG4 rather than IgG3 deposits [23]. It is known that IgG4 subclasses do not or weakly activate complement. Nevertheless, our data showed that Cd4$^-$ nIMN patients had a more stable UPr and glomerular function when compared to C4d$^+$ nIMN patients. Little is known on C4d$^+$ at a biological level, but an interesting ultrastructural study has been recently carried out on transplant glomerulopathy [24]. It revealed that C4d$^-$ endothelium after C4d deposition shifted from normal fenestrated to a continuous structure with expansion of mesangial matrix and late podocyte fusion. It appears consistent with our clinical finding in C4d$^+$ nIMN patients of a relatively slow reduction of renal function associated with an increase of UPr excretion. We are aware that our results have limitations; we know that they show the outcome of a retrospective study with a small biopsy series, and a relatively short term follow-up. Nevertheless, this
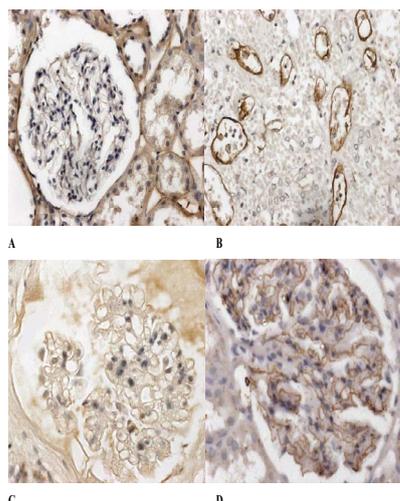

Figure 1: Immunoperoxidase staining of formalin-fixed, paraffin-embedded kidney sections using polyclonal rabbit IgG anti-human C4d antibodies.**A**, C4d negative control: Normal renal tissue obtained from heart-beating braindead donor before kidney retrieval; **B**, C4d positive control: Interstitial vascular C4d immunoperoxidase staining in antibody-related acute renal graft rejection; **C**, C4d negative immunoperoxidase staining of biopsy from nIMN (native Idiopathic Membranous Nephropathy) patient; **D**, C4d positive immunoperoxidase staining of biopsy from nIMN patient showing fine granular diffuse staining of glomerular capillary walls

Patient No.	C4d	Age	Sex	SCr	eGFR	Proteinuria	nIMN
	pos/neg	years	M/F	mg/dl	ml/min	g/day	stage [20]
1	pos	67	M	2.12	31	35.40	III
2	pos	68	F	0.94	59	6.41	II
3	pos	24	F	0.80	148	6.62	I
4	pos	24	M	0.75	129	2.16	II
5	pos	68	F	0.97	57	2.45	III
6	pos	52	M	0.88	91	0.64	I
7	pos	67	M	1.20	60	10.31	III
8	pos	17	F	0.48	166	0.01	II
9	pos	78	F	1.34	37	33.88	II
10	pos	24	M	0.85	111	8.42	III
11	neg	49	F	0.88	66	1.01	II
12	neg	39	M	0.73	120	2.69	III
13	neg	40	F	1.50	39	6.11	III
14	neg	28	F	2.20	27	2.87	II
15	neg	68	M	1.90	35	10.72	II

Table 1: General and laboratory findings of 15 nIMN patients tested for kidney C4d immunohistochemistry.nIMN, native Idiopathic Membranous Nephropathy; No., number; M, male; F, female; SCr, serum creatinine; eGFR, MDRD eGFR (Modification of Diet in Renal Disease estimated Glomerular Filtration Rate) [22]; proteinuria, adjusted for 100 ml MDRD eGFR: average from 3 consecutive days sampled at the time of hospitalization; pos, C4d positive immunoperoxidase staining; neg, C4d negative immunoperoxidase staining

Patient No.	C4d (pos/neg)	Induction	Maintenance
1	pos	oral prednisone 1 mg/Kg/day for 1 month	prednisone 5 mg/day
2	pos	i.v. methylprednisolone 1 g for 3 consecutive days	prednisone 10 mg/day
3	pos	i.v. methylprednisolone 1 g for 3 consecutive days	prednisone 10 mg/day
4	pos	i.v. methylprednisolone 1 g for 3 consecutive days	prednisone 5 mg/day + cyclosporine 300 mg/day
5	pos	i.v. cyclophosphamide 1 g / month for 6 months	prednisone 5 mg/day + azathioprine 50 mg/day
6	pos	i.v. methylprednisolone 1 g for 3 consecutive days	prednisone 5 mg/day + azathioprine 50 mg/day
7	pos	i.v. cyclophosphamide 1 g / month for 6 months	prednisone 12.5 mg/day + azathioprine 100 mg/day
8	pos	i.v. methylprednisolone 1 g for 3 consecutive days	prednisone 5 mg/day
9	pos	i.v. cyclophosphamide 1 g / month for 6 months	prednisone 12.5 mg/day + azathioprine 100 mg/day
10	pos	i.v. cyclophosphamide 1 g / month for 6 months	cyclosporine 125 mg/day
11	neg	none	none
12	neg	i.v. cyclophosphamide 1 g / month for 6 months	prednisone 12.5 mg/day + azathioprine 100 mg/day
13	neg	i.v. cyclophosphamide 1 g / month for 6 months	prednisone 12.5 mg/day + azathioprine 100 mg/day
14	neg	i.v. methylprednisolone 1 g for 3 consecutive days	prednisone 5 mg/day
15	neg	i.v. methylprednisolone 1 g for 3 consecutive days	prednisone 10 mg/day

Table 2: Treatment details of 15 nIMN patients tested for C4d immunohistochemistry on renal biopsy. nIMN, native Idiopathic Membranous Nephropathy; No., number; pos, C4d positive immunohistochemistry; neg, C4d negative immunohistochemistry; induction, induction therapy at diagnosis; maintenance, maintenance therapy during follow-up.

nIMN patients		Proteinuria, g/day; M ± SD		
	No	At diagnosis	6-month follow-up	Last follow-up
C4d positive	10	10.63 ± 13.10	12.98 ± 24.48	4.75 ± 3.10[*]
C4d negative	5	4.68 ± 3.85	3.87 ± 3.60	1.34 ± 1.15
nIMN patients		MDRD eGFR, ml/min; mean ± standard deviation		
	No	At diagnosis	6-month follow-up	Last follow-up
C4d positive	10	88.9 ± 47.5	74.5 ± 52.4	56.20 ± 28.91[**]
C4d negative	5	57.4 ± 37.9	71.0 ± 48.6	43.7 ± 41.0

Table 3: Proteinuria and renal function in ten C4d negative and five C4d positive nIMN patients.Proteinuria, daily proteinuria adjusted for 100 ml MDRD eGFR (Modification of Diet in Renal Disease estimated Glomerular Filtration Rate) [22]; [*]$P = 0.0051$ vs. C4d positive nIMN patients at last follow-up; MDRD eGFR, [**]$P = 0.0337$ vs. C4d positive at diagnosis.

is not the first time that C4d renal deposits are associated with negative disease progression. As previously described for IgAN [6] also for nIMN we have revealed that C4d immunohistochemistry is a valuable marker of disease worsening. In our opinion the demonstration of glomerular capillary wall C4d deposits might justify a more aggressive immunosuppressive regimen as already standardized in clinical transplantation. It is auspicable that larger nIMN series with longer follow-up would confirm our finding that glomerular C4d+ is a reliable clinical predictive marker of nIMN progression.

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