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ORIGINAL ARTICLE

C4d peritubular capillary staining in chronic allograft nephropathy and transplant glomerulopathy: an uncommon finding

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Summary

The true incidence of positive C4d staining in the peritubular capillaries of biopsies with chronic allograft nephropathy (CAN) and transplant glomerulopathy (TGP) remains controversial. We retrospectively reviewed all transplant biopsies performed at Saint Louis University Hospital between June 2002 and May 2004. We examined the incidence of positive C4d staining in the peritubular capillaries of biopsy specimens with pure CAN with or without features of TGP. We identified 54 biopsies in 43 patients showing CAN. The average age was 46 ± 13 years. The average creatinine at the time of biopsy was 308 ± 211 μmol/l (3.5 ± 2.4 mg/dl). Twenty (37%) biopsies exhibited features consistent with TGP. Only two biopsies had positive C4d staining in the peritubular capillaries. The C4d positive biopsies were from two different patients; one patient had donor specific antibodies (DSA) against HLA class 1 at the time of biopsy and the other patient had no detectable DSA. None of the TGP biopsies showed peritubular C4d staining. C4d staining of the peritubular capillaries appears to be rare in patients with pure CAN with and without TGP features.

Introduction

Chronic allograft nephropathy (CAN) is a descriptive term for histologic lesions that encompass atherosclerosis, glomerulosclerosis, interstitial fibrosis, and tubular atrophy [1–5]. It is characterized by proteinuria and progressive decline in glomerular filtration rate (GFR) [6]. CAN remains the leading cause of kidney allograft failure [7]. The term 'transplant glomerulopathy' (TGP) initially coined by Zolliger *et al.* [8] refers to a constellation of histological, ultrastructural and immunofluorescence findings that have been well characterized in recipients of kidney transplantation. TGP is characterized by reduplication of the glomerular basement membrane with sub-endothelial accumulation of electron-lucent material and a negative immunofluorescence microscopy [3,9–11]. The histologic features characteristic of TGP are present in about 15% of biopsies with CAN [12]. TGP manifests itself with increasing proteinuria and progressive decline in GFR and it is clinically indistinguishable from CAN.

The pathogenesis of CAN and TGP is still poorly defined. It is generally thought to involve both immunologic and nonimmunologic factors [1,13–17]. It has been suggested that the immune pathobiology of this disease entity may involve alloantigen-independent and alloantigen-dependant factors [1]. The complement split product C4d, a component of the classical complement pathway, covalently binds to the vascular wall endothelium and has been recently recognized as a marker of acute humoral rejection. In this regard the presence of C4d peritubular capillary staining (PTC) is indicative of a humoral allo-immune mediated process. To investigate the extent of allo-immune activity in allografts with CAN and TGP, we

examined the incidence of C4d positivity is biopsies of patients with CAN and TGP.

Methods

We retrospectively reviewed medical records and examined kidney transplant biopsy specimens of patients diagnosed with CAN or TGP at Saint Louis University Hospital between June 2002 and May 2004. Biopsies with any degree of concomitant tubulitis or vasculitis were excluded. Data collection sheets were designed to collect pertinent information from medical records. Nephropathology reports were reviewed and biopsy specimens were examined for each patient. The nephropathology examination included light microscopy, electron microscopy and immunofluorescence staining for IgA, IgG, IgM, C1q, C3, C4 and C4d. CAN was defined according to the Banff 97 working classification of renal allograft pathology [18]. Features of CAN were identified on light microscopy. TGP was defined as the thickening of the capillary wall in at least three loops as a result of the widening of the subendothelial space by abnormal basement membrane material, and the formation of a new layer(s) of basal lamina [9,10]. Features of TGP were confirmed with characteristic electron microscopy features and absence of immune complexes on immunofluorescence staining. The estimated GFR was calculated according to the four variable formula used in the Modification of Diet in Renal Disease Study [19, 20]. The GFR $= 186 \times (\text{plasma creatinine})^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female}) \times (1.21 \text{ if African–American})$. The study was approved by the institutional review board at Saint Louis University.

Immunofluorescence microscopy

Biopsy sections were stained with a three-step immunofluorescence technique [21]. Four-micrometer frozen sections were incubated in 100 μg/ml avidin D. Sections were washed with PBS and excess avidin was bound by adding 10 μg/ml d-biotin. We used a mouse monoclonal anti-C4d antibody (clone 10–11; Biogenesis, Sandown, NH, USA). The antibody was applied for 30 min following which the sections were washed in PBS and incubated sequentially first with biotinylated horse anti-mouse IgG (1:100) (Vector Laboratories, Burlingame, CA. USA) and after washing in PBS then with FITC–streptavidin (1:50) (Biomeda, Foster City, CA, USA), each for 30 min. Endothelial cells were detected with biotinylated *Ulex europaeus* agglutinin-I (Ulex lectin; Vector Laboratories, Burlingame, CA, USA), which binds to α-L-fucose (in blood group substance O) [22]. Biopsies from patients with membranous nephropathy as well as biopsies from patients with previously documented positive C4d in PTC served as positive controls in each individual biopsy specimen examined. In all biopsy results the concurrent positive control was verified to be adequately positive.

Detection of anti-donor antibodies

Anti-donor HLA antibody was determined only in patients with positive C4d in PTC using complement-dependant cytotoxicity assay. Both anti-human globulin-enhanced T-cell and standard complement-dependent cytotoxic B-cell assays were used. Donor T and B cells were isolated with immunomagnetic beads at transplantation and stored at −70 °C. Serum samples were collected from patients at the time of biopsy. Patients' sera were added to the T- and B-cell trays for 30 min at 37 °C. The trays were then washed three times with 10 μl of PBS and spun in a centrifuge for 1 min at $400 \times g$. One microliter of AHG was then added to each T-cell tray. After 1 min 5 μl of complement was added to each well of the trays. The B and T-cell trays were then incubated for 1 h. Ethidium bromide, acridine orange working solution and 2.5% India ink solution was added to the trays and the results were viewed under fluorescence microscopy.

Results

We identified 54 biopsies in 43 patients; 34 with pure CAN and 20 with additional features of TGP. The average age was 46 ± 13 years; 27 (63%) were white people, 14 (33%) were African–Americans and two (4%) were Asians. Twenty-six (60%) patients were males and 17 (40%) were females. Thirty-two (74%) patients were diabetic, and 23 (53%) patients were hypertensive. The underlying kidney disease was diabetic nephropathy in 18 (42%) patients and hypertensive nephrosclerosis in 12 (28%) patients. Three patients (7%) had focal segmental glomerulosclerosis, two patients (4.6%) had lupus nephritis, two patients (4.6%) had Alport syndrome, two patients had membranoproliferative glomerulonephritis (4.6%), one (2.3%) patient had adult polycystic kidney disease, one (2.3%) patient had congenital small kidneys, one (2.3%) patient had chronic reflux nephropathy, and one patient had thrombotic microangiopathy (Table 1). The average time from date of transplant to date of biopsy was 5 ± 2.4 years. The average creatinine at the time of biopsy was 308 ± 211 μmol/l (3.5 ± 2.4 mg/dl). The average estimated GFR at the time of biopsy was 26 ± 12.7 ml/min/1.73 m^2. The demographic characteristics of the study population are presented in Table 1. The immunosuppressive regimen at the time of biopsy consisted of cyclosporin, mycophenolate mofetil and prednisone in 31 (57%) patients. Fifteen (28%) patients were on tacrolimus, mycophenolate mofetil and prednisone. The

Table 1. Demographic characteristics of the study population.

Average age	46 ± 13 years
Race	
White people	27 (63)
African–American	14 (33)
Asian	2 (4)
Diabetes	32 (74)
Hypertension	23 (53)
Etiology of underlying chronic kidney disease	
Diabetic nephropathy	18 (42)
Hypertensive nephrosclerosis	12 (28)
Focal segmental glomerulosclerosis	3 (7)
Lupus nephritis	2 (4.6)
Alport syndrome	2 (4.6)
MPGN	2 (4.6)
Adult polycystic kidney disease	1 (2.3)
Congenital small kidneys	1 (2.3)
Chronic reflux nephropathy	1 (2.3)
Thrombotic microangiopathy	1 (2.3)
Average time from transplant to biopsy	5 ± 2.4 years
Average creatinine	308 ± 211 μmol/l (3.5 ± 2.4 mg/dl)
Average estimated GFR	26 ± 12.7 ml/min/1.73 m^2

Values are given as *n* (%) and mean ± SD.

Table 2. The immunosuppressive regimen of patients at the time of biopsy.

Immunosuppressive regimen at time of biopsy	Number of patients (%)
Cyclosporin, mycophenolate mofetil, prednisone	31 (57)
Tacrolimus, mycophenolate mofetil, prednisone	15 (28)
Cyclosporin, azathioprine, prednisone	3 (5.6)
Sirolimus, mycophenolate mofetil, prednisone	3 (5.6)
Azathioprine, prednisone	2 (3.8)

immunosuppressive regimen in the remaining patients is reported in Table 2.

Using the 97 Banff classification, the severity of CAN ($n = 54$) was divided into three grades: grade I (mild, $n = 7$, 13%); grade II (moderate, $n = 15$, 28%); and grade III (severe, $n = 32$, 59%). Only two biopsies had positive C4d staining in PTC (Fig. 1). The C4d positive biopsies were from two different patients. The first patient was a 23-year-old African–American male with deceased donor kidney allograft maintained on cyclosporine, mycophenolate mofetil and prednisone immunosuppressive regimen. The patient had donor-specific antibody against HLA class 1 at the time of biopsy. The second patient was an 18-year-old White female with deceased donor allograft maintained on tacrolimus, mycophenolate mofetil and prednisone immunosuppressive regimen. This patient had no detectable donor-specific antibodies. The

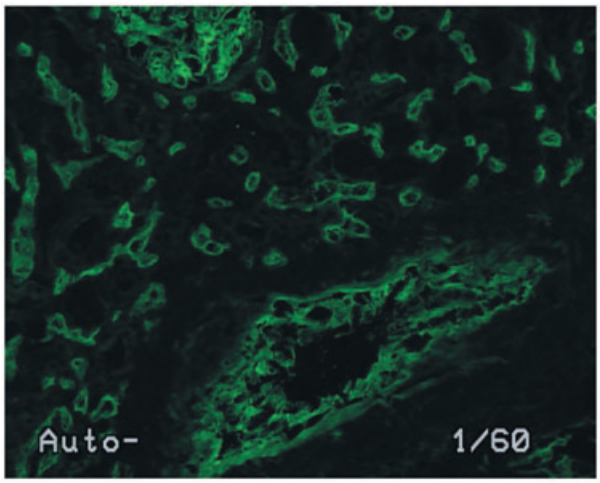

Figure 1 Representative photomicrograph of positive C4d in PTC.

remaining 32 biopsies with CAN had negative C4d staining in PTC (Fig. 2a and b). None of the patients with TGP had any PTC C4d staining on their biopsy (Fig. 3a and b).

Discussion

Our results suggest that C4d staining in PTC in patients with CAN and TGP is very rare. We examined 54 biopsies in 43 patients; 34 with CAN and 20 with TGP and found that only two biopsies with CAN were positive for C4d in PTC. All specimens were examined with a concurrent positive control. To ascertain the internal validity of the results, we also examined the incidence of PTC C4d staining in biopsy specimens of acute rejection during the same period of time (June 2002 to May 2004). We identified 55 biopsies with acute rejection. Twenty (47%) biopsies were positive for C4d in the PTC. These results are in general agreement with the results published by Mauiyyedi *et al.* [23].

C4d staining in PTC is now considered a reliable marker of humoral allograft rejection [24,25]. The significance of this complement split product in the pathobiology of CAN and TGP is less well defined. Regele *et al.* [26] examined C4d deposition in PTC of biopsies obtained from 213 patients with CAN and determined that 34% of the biopsies were positive for C4d deposits in PTC. The investigators found that morphologic features of CAN (with the exception of tubular atrophy) were not associated with C4d deposits in PTC [26]. They interestingly reported a strong association between C4d positivity and morphologic changes characteristic of TGP [26]. Nickeleit *et al.* reported that C4d staining in PTC was not significantly linked to the morphologic lesions that

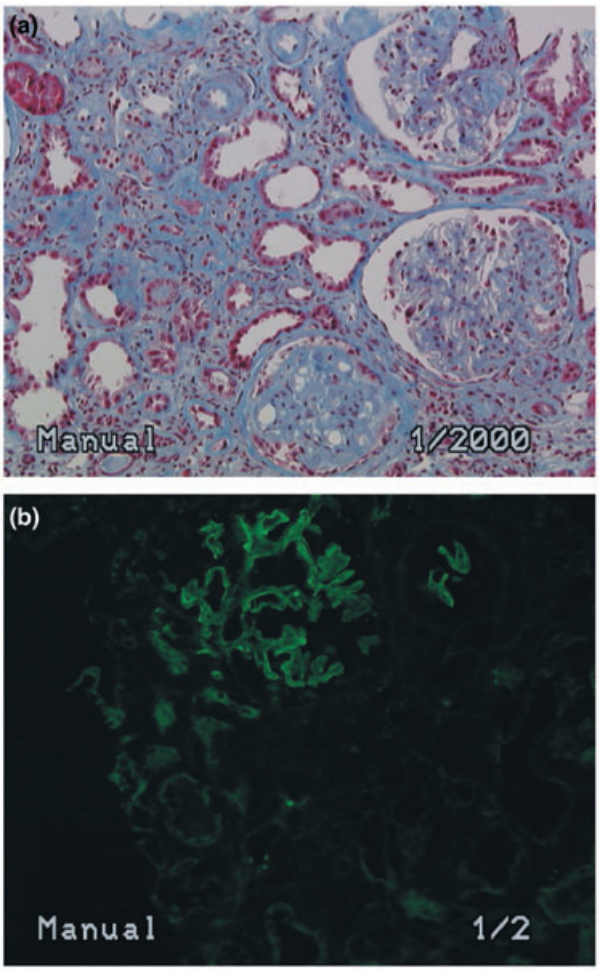

Figure 2 Representative photomicrograph of biopsies with CAN. (a) Findings on trichrome staining; (b) immunofluorescence microscopy showing negative C4d staining in PTC.

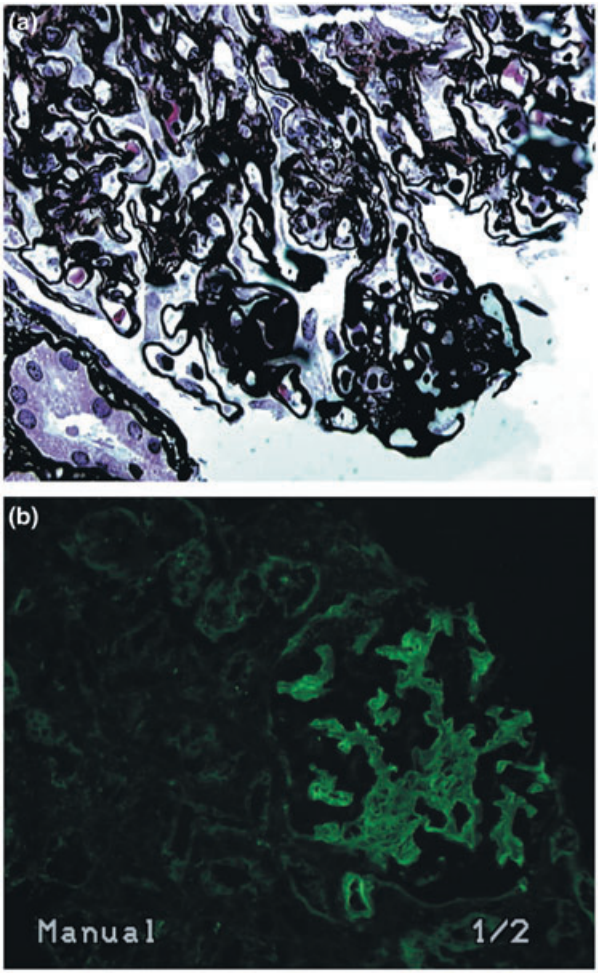

Figure 3 Representative photomicrograph of biopsies with transplant glomerulopathy. (a) Findings on silver staining; (b) immunofluorescence microcopy showing negative C4d staining in PTC.

characterize inactive chronic rejection, such as sclerosing transplant vasculopathy, glomerulopathy or cyclosporin/tacrolimus-induced alterations [27]. Mauiyyedi *et al.* [24] examined 38 biopsies with CAN and found that 61% of biopsies had positive C4d staining in PTC. The investigators found that the majority of patients with positive C4d in PTC had anti-donor HLA antibody (15 of 17; 88%) and none of the C4d-negative CAN tested had anti-donor antibody [24]. Mauiyyedi *et al.* [24] concluded that a substantial fraction of CAN is antibody mediated.

Vongwiwatana *et al.* [28] reported C4d deposition in PTC of 25% of 24 patients with TGP. Horita *et al.* examined nine biopsy specimens with TGP and found that all the patients had positive C4d staining in PTC [29]. Most recently Sijpkens *et al.* [30] reported that PTC C4d staining was present in only four of the 11 patients with TGP. Nickeleit *et al.* [27] reported that positive C4d staining in PTC was present in 57% of patients with TGP but found no significant correlation between the accumulation of C4d and the histologic changes of TGP. Akalin *et al.* [12] found that PTC C4d staining was not significantly different in patients with CAN 7/11 (64%) when compared with patients with CAN and TGP 4/5 (80%). The findings by Nickeleit and Akalin suggest that humoral allo-reactivity may not be a central feature of TGP. The discrepancy between the above reports, however, merits further investigation to elucidate the significance of C4d staining in this setting, the extent of allo-immune mediated factors in the pathogenesis of CAN and TGP, and the potential clinical utility of this marker.

CAN and TGP may in fact be heterogeneous in nature and this heterogeneity may in part be a result of the different immunosuppressive regimens used in induction and maintenance, the different immunologic characteristics

of each individual patient, the patient comorbidities and the demographic characteristics of the patient population. Another possible explanation is the inter-center variability in C4d immunostaining. In addition, it has been reported that C4d staining could change from negative to positive and vice versa within days to weeks [27]. Thus, C4d positivity in PTC is highly time dependant and this indeed may contribute to the variability in reported literature.

In conclusion, C4d staining in PTC appears to be rare in patients with pure CAN or TGP. The findings suggest that allo-immune activity in patients with CAN and TGP in our institution appears to be scant. Whether CAN, and particularly TGP is mediated through a humoral allo-immune mechanism of injury remains to be determined.

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