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Peritubular Capillary C4d Deposition in Chronic Allograft Dysfunction

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Peritubular capillary (PTC) C4d staining represents a marker for acute humoral rejection, however, the impact of positive staining on chronic allograft dysfunction has received little attention. Ninety-three renal allograft biopsies from 93 patients were selected from a total of 174 renal allograft biopsies, which were obtained 6 months or more after transplantation (median: 89 months). Fresh frozen renal tissue was stained with monoclonal antibody against C4d. Sixteen of 93 biopsies showed C4d staining in PTC. C4d staining was positive in 40% of acute rejection cases (n=15) and 21% of chronic rejection cases (n=24). When the samples were divided according to C4d positivity, the C4d (+) group had a higher proportion of acute rejection than the C4d (-) group. However, no significant difference was observed between the two groups in terms of the prevalence of chronic rejection. Degrees of histological injury including tubulitis, interstitial inflammation and interstitial fibrosis were not significantly different between C4d (+) and C4d (-) groups. However, the 2-year graft survival rate after biopsy was lower in the C4d (+) group than in the C4d (-) group (24.8% versus 59.0%, p=0.1255). C4d staining in PTC is associated with late acute rejection, but not with chronic rejection based on conventional morphologic criteria in patients with chronic allograft dysfunction.

Key Words: Acute rejection, C4d, chronic allograft dysfunction, peritubular capillary, renal transplantation

INTRODUCTION

C4d is a degradation product of classic complement pathway component C4 and remains at the site of complement activation by covalent binding to tissue.[1] In renal transplantation, C4d staining in peritubular capillaries (PTCs) has been reported to be a marker of acute humoral rejection.[2-4] Diffuse linear staining was observed in the acute rejection of ABO-incompatible grafts[5,6] and in patients with specific anti-donor antibodies.[7] Moreover, C4d positivity has been associated with higher serum creatinine levels and an inferior graft survival.[8-10]

In contrast to the association between C4d positivity and acute humoral rejection, the significance of positive C4 staining is controvertial in patients with chronic rejection. C4d was reported to be positive in chronic rejection and to be related to poor graft survival; however, no differential histological features were reported between C4d (+) and C4d (-) in chronic rejection.[8] Moreover, Nickeleit et al.[11,12] reported that C4d staining is present in other conditions of chronic allograft dysfunction, and suggested that C4d staining in a background of chronic tubulointerstitial fibrosis implies an acute/active rejection episode rather than it being a marker of chronic rejection. The aim of this study was to examine the prevalence and significance of C4d staining in PTCs in patients with chronic allograft dysfunction.

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MATERIALS AND METHODS

Case selection

Ninety-three of 174 renal allograft biopsy samples obtained from July 2000 to July 2003 were selected for this study. The inclusion criteria were

as follows: 1) Renal transplantation was performed at the Yonsei University Medical Center; 2) Renal allograft biopsy was performed 6 months or more after transplantation; 3) The number of glomeruli exceeded four by light microscopy; and 4) that fresh renal tissue was left for C4d staining. Renal tissue was obtained, using an 18-gauge biopsy gun under ultrasound guidance. The indications for biopsy were creatinine elevation in 65.6%, proteinuria of ≥ 1 g/24 h in 30.1%, and isolated hematuria in 4.3%. Fifty-five patients received cyclosporine- or tacrolimus-based double immunosuppression, and 38 patients received triple immunosuppression.

Renal histology

Renal tissue was routinely examined by light, immunofluorescent, and electron microscopy. For light microscopy, formalin-fixed, paraffin-embedded 3 μm sections were stained with hematoxylin-eosin, periodic acid-Schiff (PAS), aldehyde fuchsin orange G, and periodic acid-methenamine silver. At least 6 PAS-stained sections were examined to evaluate renal histology. The percentages of global and segmental sclerosis were calculated, and the degrees of interstitial inflammation, tubulitis, tubular atrophy, interstitial fibrosis, arteriolar hyalinosis, and vascular intimal thickening were scored from 0 to 3 according to the Banff scheme.[13] Chronic rejection was defined as the presence of concentric fibrous intimal thickening of the vascular wall and/or intimal foam cells in addition to tubulointerstitial fibrosis. The presence of tubular atrophy and interstitial fibrosis without characteristic glomerular or vascular lesions was categorized as chronic allograft nephropathy (CAN). Transplant glomerulopathy was characterized by a double contour feature of the glomerular basement membrane and mesangial matrix increase without immune deposits. Chronic cyclosporine nephrotoxicity was defined as the presence of transmural nodular hyaline deposition in arterioles and in interlobular arteries.

C4 staining

Fresh frozen renal tissue embedded in OCT was cut at 3 μm on a cryostat and stained with monoclonal anti-C4d antibody (Biogenesis, Poole, England, 1:50 dilution) and FITC-conjugated rabbit anti-mouse IgG F (ab)'$_2$ antibody (Dako Cytomation, Glostrup, Denmark, 1 : 10 dilution). Diffuse but not focal staining in PTC was considered positive. Staining of glomerular capillaries, mesangium or arterioles was interpreted as nonspecific. Thirty-eight samples of native kidney (14 cases of minimal change disease, 5 cases of benign hematuria, 14 cases of lupus nephritis, 4 cases of proliferative glomerulonephritis and one acute tubular necrosis case) served as controls.

Clinical parameters

Donor type, type of original renal disease, biopsy indication, time interval from transplantation to biopsy, serum creatinine levels and 24h proteinuria at the time of biopsy, duration of follow-up, and the cause of graft failure were retrieved from biopsy requisition forms and/or clinical records.

Statistical analysis

Results are expressed as means ± standard deviation (SD). Differences in clinical and histological changes in the C4d (+) and C4d (-) groups were evaluated using the Student's t-test and the Chi- square test as appropriate. Actuarial graft survival rates after biopsy were calculated using the Kaplan-Meier method, and comparisons between the two groups were performed using the Log-rank test. A p-value of less than 0.05 was considered significant.

RESULTS

C4d staining and case distribution

C4d staining was positive in 16 of 93 allograft biopsy samples examined (Fig. 1). The C4d (+) rate was 40% in acute rejection, 20.8% in chronic rejection and 11.1% in chronic allograft nephropathy. Both samples with intimal arteritis were positive for C4d staining. Fibrinoid necrosis or neutrophils in PTCs were not present in any sample. The median time interval from trans

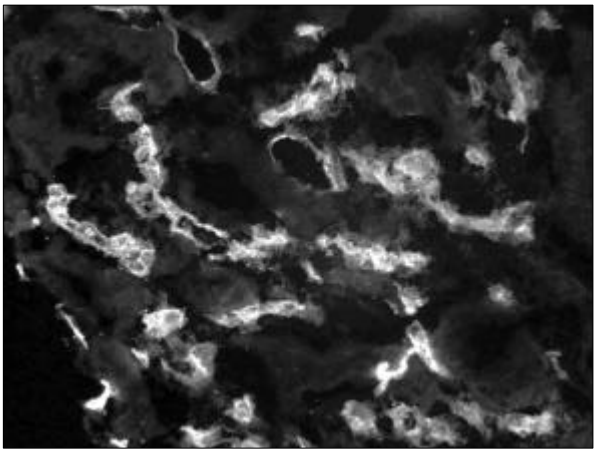

Fig. 1. Diffuse C4d staining along peritubular capillaries (× 400).

plantation to biopsy was shorter in samples with acute rejection than in samples with other diagnoses (66 months versus 97.5 months). Transplant glomerulopathy was observed in 8 samples and 2 of these were C4d (+). C4d staining was occasionally observed in glomerular capillaries, but not in the PTCs of native kidneys. When the samples were divided according to C4d positivity, a significant difference was found in the distributions of pathologic diagnoses in the C4d (+) and C4d (-) groups (p=0.039). The C4d (+) group had a higher proportion of acute rejection than C4d (-) group. However, no difference was observed between the two groups with respect to the prevalence of chronic rejection (Table 1).

Comparison of clinical features between C4d (+) and C4d (-) groups

No difference was observed in clinical parameters between the two groups (Table 2).

Comparison of histological injury between C4d (+) and C4d (-) groups

The number of glomeruli were 9 in C4d (+) group and 10 in C4d (-) group. The degree of tubulitis was slightly higher in the C4d (+) group than in the C4d (-) group, but this was not statistically significant. When chronic rejection samples were evaluated, no difference in the severity of histological injury was observed according to C4d staining results (Table 3).

Graft outcome

The 2-year graft survival rate after biopsy was lower in the C4d (+) group than in the C4d (-) group (24.8% versus 59.0%), but this was not statistically significant (Fig. 2). The degrees of tubulitis (0.4 versus 0.4) or interstitial inflammation (1.2 versus 1.1) at the time of biopsy were no different for functioning and failed grafts; however, the degrees of tubular atrophy (2.0 versus 1.3, p=0.001) and interstitial fibrosis (2.0 versus 1.3, p=0.002) were significantly higher in failed grafts.

Table 1. Distribution of Cases in the C4d (+) and C4d (-) Groups

Pathologic diagnosis	C4d (+)		C4d (-)		p-value
	N	%	N	%	
Acute rejection	4	25.0	4	5.2	0.039
Acute rejection + chronic rejection	1	6.3	5	6.5	
Acute rejection + chronic allograft nephropathy	1	6.3	-	-	
Chronic rejection	5	31.3	19	24.7	
Chronic allograft nephropathy	2	12.5	16	20.8	
Cyclosporine nephrotoxicity	1	6.3	15	19.5	
Nonspecific	-	-	9	11.7	
Others*	2	12.5	9	11.7	
Total	16		77		

*8 cases of glomerulonephritis (4 IgA nephropathy, 3 membranous nephropathy and 1 mesangioparoliferative glomerulonephritis), 2 cases of polyomavirus nephropathy and 1 case of diabetic nephropathy. p-value was calculated using the Chi-square test.

Table 2. Comparison of Clinical Manifestations in the C4d(+) and C4d(-) Groups

Clinical manifestations	C4d(+) (N=16)	C4d(-) (N=77)	*p*-value*
Age at transplantation (years old)	34.6 ± 12.3	33.9 ± 10.0	0.827
Age at graft biopsy (years old)	40.8 ± 11.8	40.7 ± 10.9	0.978
Male : Female	12 : 4	59 : 18	0.889
Main immunosuppressive agent, Cyclosporine : FK-506	15 : 1	69 : 8	0.610
Immunosuppressive regimen, Double : Triple regimen	10 : 6	45 : 32	0.764
Donor type, LRD : LURD : CAD+	5 : 10 : 1	41 : 34 : 1	0.248
Degree of HLA mismatch in LRD, 0 haplo mismatch : 1 haplo mismatch	0 : 5	5 : 36	0.220
Degree of HLA mismatch in LURD/CAD	3.45 ± 0.69	3.72 ± 0.74	0.293
ABO blood type, ABO identical : ABO not identical	13 : 3	62 : 15	0.946
Episodes of acute rejection, absent: present	5 : 11 (68.8%)	44 : 33 (42.9%)	0.059
Interval from transplantation to graft biopsy (months) (range)	79.6 ± 45.4 (14-152)	85.5 ± 55.2 (6-269)	0.689
Indication of graft biopsy, increased s-Cr.: proteinuria: hematuria	10 : 1: 5	51 : 3 : 23	0.902
Serum creatinine level at biopsy time (mg/dL)	3.44 ± 3.07	2.55 ± 1.66	0.102
24 hour urinary protein excretion (g/day)	1.89 ± 3.36	1.61 ± 2.53	0.699
Post-biopsy follow-up duration (months)	13.9 ± 6.6	15.2 ± 12.9	0.561

*p-value was calculated by using the student's t-test or Chi-square test.
†LRD : LURD : CAD mean living related donor : living unrelated donor : cadaveric donor (respectively).

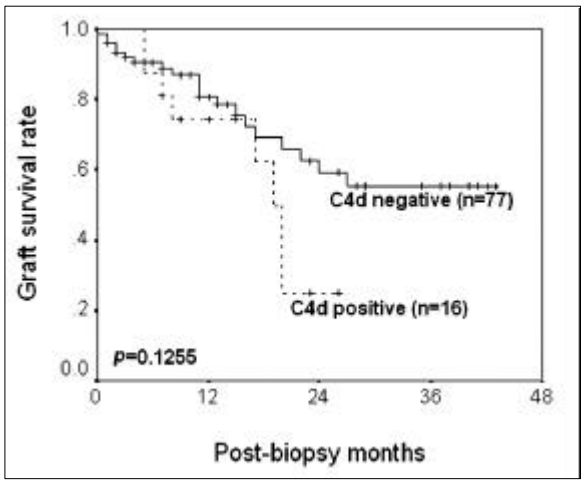

Fig. 2. Graft survival curve in C4d(+) and C4d(-) groups.

DISCUSSION

C4d staining in PTC is now regarded as a component of acute humoral rejection. The diagnostic criteria for acute humoral rejection include, C4d deposition in PTC, histologic evidence of acute injury, and the presence of circulating antidonor antibodies. If only two of the three criteria are present, the finding is recommended to be 'suspicious for', not diagnostic of, acute humoral rejection.[14,15] However, we could not apply this criteria to make a diagnosis, since the antidonor antibody test has not been performed routinely in patients that have undergone an allograft biopsy. Instead, we evaluated the significance of C4d staining in PTCs in renal allograft biopsies, in which a diagnosis had been made by conventional light microscopic criteria alone.

In this study, a relationship was observed between acute rejection and C4d staining. Acute rejection as a whole showed 40.0% C4d positivity, and the positive rate reached 100% for vascular rejection cases. However, morphologic features

Table 3. Degrees of Histological Injury in the C4d (+) and C4d (-) Groups

Histological injury score	C4d (+) (N=16)	C4d (-) (N=77)	*p*-value*
Number of glomeruli	9 ± 3.3	9.8 ± 4.0	0.500
Transplant glomerulitis	0	0. ± 0.4	0.566
Transplant glomerulopathy	0.4 ± 1.0	0.2 ± 0.7	0.368
Tubulitis	0.9 ± 1.2	0.3 ± 0.7	0.062
Tubular atrophy	1.4 ± 0.8	1.5 ± 1.0	0.688
Interstitial inflammation	1.5 ± 1.1	1.0 ± 0.8	0.157
Interstitial fibrosis	1.5 ± 0.8	1.5 ± 1.0	0.881
Arteriolar hyalinosis	1.1 ± 1.0	1.3 ± 1.1	0.366
Vascular wall thickening	0.4 ± 0.5	0.6 ± 0.7	0.488

*p-value was calculated using the student's t-test.

could not predict the presence of humoral rejection. Fibrinoid necrosis of arteries or neutrophilic infiltrate in PTCs was not present in any of the C4d(+) cases. Mauiyyedi et al.[7] reported that 65% of C4d(+) patients had neutrophils in PTCs and that 25% had fibrinoid necrosis of arteries.

With regard to chronic rejection, the C4d(+) rate was not different to those of CAN or chronic cyclosporine nephrotoxicity. Furthermore, degrees of tubular atrophy and interstitial fibrosis were not different according to C4d positivity. The association between C4d staining and acute, but not chronic rejection in patients with chronic allograft dysfunction, supports the notion of Nickeleits' et al. that C4d staining implies acute/active rejection in grafts with a chronic tubulointerstitial lesion.[12] However, we could not completely exclude the possibility of a low prevalence of chronic humoral rejection or sampling error. Another problem is in the interpretation of chronic humoral rejection, in which morphologic changes are more difficult to demonstrate by routine histology than in acute humoral rejection; basement membrane lamellation of PTCs could be only demonstrated by electron microscopy.[16-18] Transplant glomerulopathy, which is regarded as a glomerular lesion of chronic rejection, should be differentiated from other forms of immune-complex mediated glomerulonephritis. In the study, glomerular immune complex deposition was excluded by immunofluorescence and electron microscopy, however, PTC changes could not be thoroughly examined. C4d was not stained in control biopsies, which is in accord with a previous study of lupus nephritis.[19]

Survival rate was shorter in the C4d(+) group than in the C4d(-) group. We are uncertain whether this difference was attributed to a higher percentage of acute rejection in the C4d(+) group. Furthermore, it is unclear whether C4d(+) patients should be treated more aggressively than C4d(-) patients irrespective of the pathologic diagnosis. Since PTC nourishes the cortical tubulointersitium, PTC perturbation may cause ischemic injury to the surrounding tubulointerstitium and affect long-term graft survival.[20,21] Alternatively, the probability of repeated humoral rejection with C4d deposition might be higher in the C4d(+) group than in the C4d(-) group, and cumulative injury may occur.

Summarizing, our results do not support the notion that C4d is a marker for chronic rejection. Instead, C4d positivity was associated with late acute rejection in patients with chronic allograft dysfunction. More data will be needed upon the long-term effects of C4d on graft outcome to understand its significance in chronic allograft dysfunction.

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