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ORIGINAL ARTICLE

Clinicopathological relevance of granular C4d deposition in peritubular capillaries of kidney allografts

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Summary

While linear C4d staining in peritubular capillaries (PTC) is established as a marker of antibody-mediated rejection, the significance of a distinct granular C4d deposition pattern has not yet been clarified. In this study, 329 renal allograft recipients who underwent indication biopsies were analysed for immunohistochemical C4d staining characteristics. Fifty-six (17%) recipients showed granular C4d in PTC, without any relationship to conventional risk factors and morphological features of rejection. We found a strong association with long-term overall graft survival (7-year survival: 41% vs. 66% in granular C4d-negative subjects, $P = 0.001$), which was mainly driven by a greater risk of mortality [hazard ratio: 3.12 (95% confidence interval: 1.23–7.94); $P = 0.02$]. Granular C4d was associated with delayed graft function [39% vs. 22% (C4d-negative subjects), $P = 0.007$], higher 1-year serum creatinine [median 2.1 (interquartile range: 1.7–2.6) mg/dl vs. 1.6 (1.3–2.0) mg/dl, $P = 0.001$] and a trend towards worse death-censored graft survival ($P = 0.07$). In support of a role of capillary immune complex formation, granular C4d was associated with electron-dense deposits in PTC basement membranes, which were occasionally accompanied by focally distributed capillary IgG deposits. In conclusion, our study suggests clinical relevance of detecting capillary granular C4d deposition. Our results point to a pathogenetic role of alloimmune-independent immune complex deposition.

Introduction

Deposits of classical complement activation products, most prominently C4 split product C4d, can be detected in various compartments of kidney allografts. While C4d staining along peritubular capillaries (PTC), a footprint of alloantibody-triggered complement activation, is accepted as a diagnostic marker of antibody-mediated rejection (AMR) [1–5], the relevance of atypical staining patterns, including C4d deposits in glomeruli, arterioles or tubuli, is less well established [6,7]. Few studies have shown the occasional finding of a distinct pattern of finely granular staining in PTC [7,8]. In a previous study, we found that, in contrast to linear staining in PTC, glomeruli or arterioles, this pattern did not coincide with features of AMR [7]. In its morphological appearance, granular PTC staining is reminiscent of a C4d pattern earlier described for kidney biopsies obtained in patients with systemic lupus erythematosus (SLE) [9,10] or scleroderma renal crisis [11], and even control subjects [11]. Remarkably, in native kidney disease, granular C4d was found to be associated with a more severe clinical course [9,11]. While in SLE this pattern may reflect capillary immune complex deposition [9], morphological studies in patients with scleroderma have failed to detect immune deposits, which has led to the speculation of direct endothelial injury as a trigger of complement activation [11].

In kidney transplantation, the relevance of granular C4d staining has not yet been defined. This study aimed at investigating clinicopathological characteristics associated with this staining pattern. We reviewed the long-term follow-up of a large cohort of 329 renal allograft recipients who had been subjected to indication biopsies. Considering the more severe clinical course earlier described for native kidney disease, we investigated whether granular C4d staining predicts poor clinical outcomes also in transplant recipients. To get first insights into the pathogenesis of this particular pattern of capillary complement activation, we performed in-depth immunohistochemical and ultrastructural studies.

Patients and methods

Study design and patients

This retrospective study was approved by the local institutional ethics committee. All kidney allograft recipients transplanted at our centre during a 4-year period (January 1999 and December 2002) were considered for inclusion. A total of 722 patients, 329 recipients met the following inclusion criteria: (i) at least one indication biopsy performed for unexplained graft dysfunction and/or proteinuria and (ii) availability of adequate biopsy material for immunohistochemical re-evaluation. Baseline data are provided in Table 1. Patients were followed until 2010 (median follow-up 98 months). We recorded date of patient death and graft loss, occurrence of delayed graft function (DGF) defined as the need for dialysis for at least 1 week post-transplantation, serum creatinine (for nonparametric analysis, patients on dialysis were considered as having a serum creatinine of 8 mg/dl) and protein excretion at 1 year (patients on dialysis were not included). Significant proteinuria was defined as a protein/creatinine ratio above 500 mg/g, and, if not available, as a protein excretion above 0.5 g/l or 0.5 g/24 h and/or a positive dipstick urinalysis, respectively.

Biopsies

For C4d staining, 603 archived transplant biopsies were available. Biopsies had been performed after a median of 16 days [interquartile range (IQR): 8–40 days, range: 1–2248 days], with 89% of the samples obtained <6 months post-transplantation.

For ultrastructural analysis, we selected 20 biopsies obtained from 20 different recipients after a median of 246 days. Ten specimens were selected according to the finding of granular C4d deposits in PTC. The other 10 samples were obtained from C4d-negative recipients matched for recipient gender and age, diagnosis of acute T-cell-

Table 1. Baseline variables in relation to granular C4d staining in PTC.

	Granular C4d in PTC		
Baseline variables*	Yes (n = 56)	No (n = 273)	P value
Recipient data			
Age, years, median (IQR)	50 (41–58)	50 (39–58)	0.6
Female gender, n (%)	20 (36)	102 (37)	0.8
Body mass index, median (IQR)	26 (23–31)	24 (22–27)	0.003
Kidney disease underlying ESRD			
Immune complex glomerulonephritis, n (%)	6 (11)	27 (10)	0.9
Lupus nephritis, n (%)	1 (2)	4 (2)	0.6
Prior peritoneal dialysis, n (%)	12 (21)	31 (11)	0.04
Prior haemodialysis, n (%)	48 (86)	237 (87)	0.8
Duration of prior dialysis, months, median (IQR)	37 (20–57)	35 (14–58)	0.5
Diabetes mellitus II, n (%)	7 (13)	33 (12)	0.9
Diabetes mellitus I, n (%)	1 (2)	7 (3)	0.6
Active or past smoker, n (%)	27 (56)	101 (42)	0.07
Left ventricular systolic dysfunction, n (%)	9 (16)	16 (6)	0.009
Coronary heart disease, n (%)	10 (18)	53 (20)	0.8
Peripheral arterial disease, n (%)	8 (14)	31 (12)	0.6
Cerebrovascular disease, n (%)	5 (9)	23 (9)	0.5
History of malignant disease, n (%)	4 (7)	15 (6)	0.4
Hepatitis B core antigen antibody positive, n (%)	2 (4)	14 (6)	0.4
Hepatitis B surface antigen positive, n (%)	1 (2)	1 (0.4)	0.3
Hepatitis C antibody positive, n (%)	8 (16)	20 (9)	0.1
Transplant-related data			
Living donor transplantation, n (%)	4 (7)	29 (11)	0.3
HLA mismatch (A, B, DR), number, median (IQR)	3 (2–3)	3 (2–4)	0.02
Cold ischaemia time, hours, median (IQR)	14 (8–21)	13 (8–19)	0.5
Donor age, years, median (IQR)	52 (44–59)	48 (39–58)	0.1
CDC panel reactivity >10%, n (%)	17 (30)	71 (26)	0.5
CMV IgG, recipient negative/ donor positive, n (%)	7 (14)	36 (15)	0.7
Initial immunosuppressive therapy, n (%)			
Depleting antilymphocyte antibody	10 (18)	40 (15)	0.5
Interleukin-2 receptor antibody	3 (5)	20 (7)	0.4
Cyclosporin A	53 (95)	254 (93)	0.7
Tacrolimus	2 (4)	15 (6)	0.4
mTOR inhibitor	2 (4)	20 (7)	0.2

CDC, complement-dependent cytotoxicity; CMV, cytomegalovirus; ESRD, end-stage renal disease; IQR, interquartile range; mTOR, mammalian target of rapamycin; PTC, peritubular capillaries.
*For studied variables, complete data were available for at least 84% of the included subjects.

mediated rejection and the extent of chronic allograft injury as reflected by a sum score of Banff chronic indices (cg, ci, ct, cv).

Histomorphology

Histological analysis was performed on two-micrometre sections of formalin-fixed, paraffin-embedded archival biopsies. Sections were stained with haematoxylin and eosin, periodic acid–Schiff reagent, silver methenamine and acid fuchsin/orange G. Histological lesions were scored according to the Banff scheme [12,13].

Immunohistochemistry

For C4d staining, sections were deparaffinized; antigen retrieval was performed by pressure cooking for 5 min at 1 bar in Tris/EDTA buffer (pH 9.0), and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Background staining was blocked using Ultra V Block (UltraVision LP Large Volume Detection System: HRP Polymer, Thermo Scientific). After incubation with polyclonal rabbit anti-C4d reagent (Biomedica, Vienna, Austria), the first antibody was bound by the peroxidase-labelled polymer complex and was visualized using 3-amino-9-ethylcarbazole chromogen. C4d staining along PTC was graded as no, minimal (estimated <10% of specimen), focal (10%–50%) or diffuse (>50%) staining.

For IgG, IgM, IgA, C1q or C3 staining, sections were incubated with appropriately pretitred primary antibodies, followed by biotinylated secondary antibodies and detection by a streptavidin–peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase reaction product was visualized by diaminobenzoline and H_2O_2 or 3-amino-9-ethylcarbazole.

Immunohistochemical slides were reviewed by two pathologists (H.R., N.K.) blinded to the clinical and serological data.

Electron microscopy

Segments of biopsy cores were fixed in a mixture of 4% formaldehyde and 0.1% distilled glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Tissue was postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer and stained with 1% aqueous uranyl acetate. Specimens were embedded in epoxy resin (Serva electrophoresis, Heidelberg, Germany). Ultrathin sections were prepared on an Ultracut-E Ultramicrotome (Reichert–Jung, Vienna, Austria). Final staining was carried out with uranyl acetate in methanol and lead citrate, and sections were analysed with the JEOL JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan). Eleven to 19 PTC were studied per biopsy specimen. Basement membrane (BM) multilayering was classified as being absent if no or only focal splitting of BM was found, as low grade if three to four BM layers (with more severe lesions only occasionally present), and as high grade if five and more layers were observed.

Serological techniques

C1q-binding circulating immune complexes were detected by enzyme-linked immunosorbent assay. Microtitre plates were coated with human C1q (Calbiochem, EMD Biosciences, La Jolla, CA) for 72 h at 4 °C and then blocked with 0.4% bovine serum albumin. After washing, defined concentrations of heat-aggregated (63 °C, 12 min) human IgG (standard) and patient sera (diluted 1:200) were added for 2 h at 4 °C. Plates were then washed and incubated for 1 h at 37 °C with alkaline phosphatase-conjugated goat F(ab)2 anti-human IgG. After another washing step, substrate (*p*-nitrophenyl phosphate at 1 mg/ml) was added, and after 30 min incubation, the reaction was terminated (stop solution: NaOH at 1 M). Absorbance was read at 405 nm. Concentrations were calculated according to standard curves using SOFTmax PRO software (Molecular Devices, Ismaning, Germany). A cut-off value of 50 mg/ml was used to define a positive result. Antinuclear antibodies were assessed according to standard protocols.

Statistics

Chi-square or Fisher's exact tests were used to compare proportions. For comparisons of continuous data, non-parametric tests (Mann–Whitney *U*-test) were applied. For multivariate analysis of risk factors for granular C4d staining, we applied binary logistic regression. Kaplan–Meier analysis was used to calculate graft and patient survival, the Mantel–Cox log-rank test to compare survival between groups. Cox regression analysis was applied to evaluate the independent effect of granular C4d in PTC on all-cause mortality. A two-sided P value <0.05 was considered as statistically significant. Statistical calculations were performed using PASW for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA).

Results

Overall, 603 indication biopsies obtained in 329 kidney transplant recipients (baseline variables, see Table 1) were re-evaluated for C4d staining characteristics. A distinct finely granular C4d staining along PTC was detected in 62 (10%) of the biopsies. Granular C4d deposits were consistently focally distributed affecting less than 50% of PTC. Representative examples of focal granular C4d staining are shown in Figs 1 and 2. There were no statistical

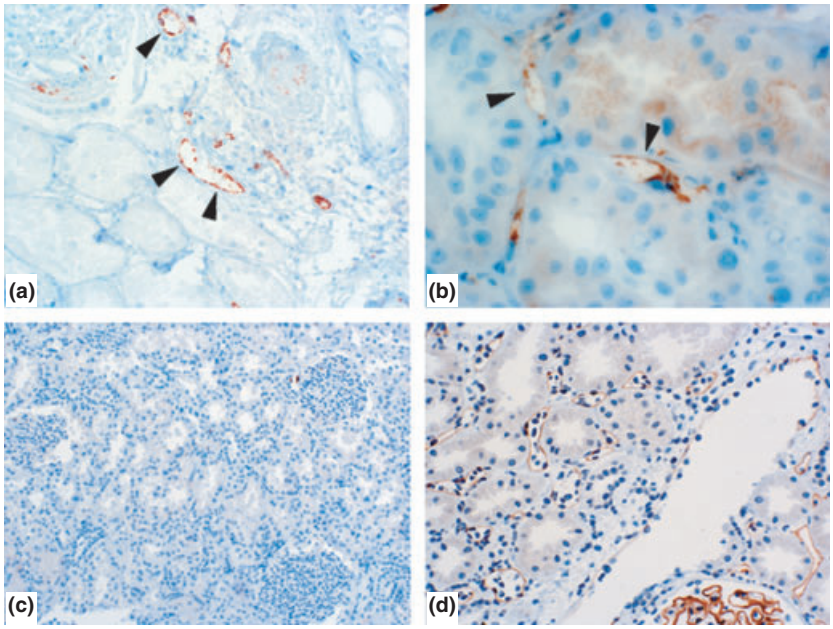

Figure 1 (a) Immunohistochemical staining of a representative renal transplant biopsy sample showing a focally distributed granular pattern of C4d deposition along PTC (arrows). (b) The same specimen showed also capillary IgG deposits in a few PTC (arrows). (c) A corresponding null biopsy performed shortly before reperfusion stained C4 negative. (d) Representative transplant biopsy obtained from a patient with AMR, showing diffuse linear C4d staining along PTC.

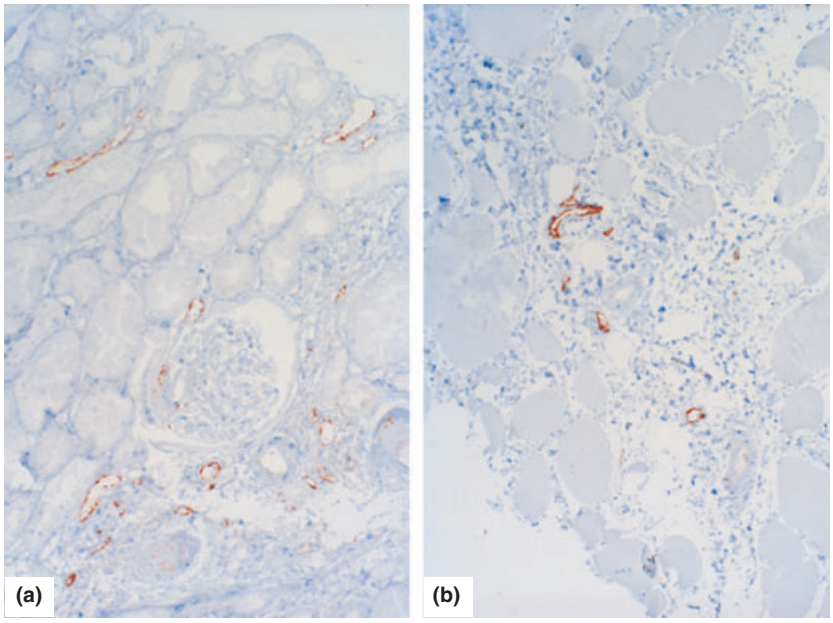

Figure 2 (a) A biopsy sample with focally distributed granular C4d deposition in cortical PTC and (b) a comparable capillary staining in adjacent muscle and connective tissue.

associations of such staining with linear C4d deposition in PTC (an overlap between granular and linear staining was observed in six biopsies) or features of acute rejection (Table 2). Moreover, with the exception of a trend towards more frequent transplant glomerulopathy ($P = 0.06$), the pattern did not coincide with chronic tissue injury (Table 2). Some granular C4d-positive biopsies showed lesions reflecting chronic injury. Many of these biopsies

Table 2. Granular C4d in PTC and features of acute and chronic transplant injury in 603 kidney allograft biopsies.

	Granular C4d deposition in PTC		
Parameter*	Yes (n = 62)	No (n = 541)	P value
Linear C4d in PTC, %	10	11	0.7
Single Banff lesions - Acute			
Glomerulitis, %	9	12	0.5
Tubulitis, %	42	44	0.7
Interstitial inflammation, %	66	62	0.6
Intimal arteritis, %	11	19	0.1
Peritubular capillaritis, %	38	30	0.2
Single Banff lesions – chronic			
Arterial hyalinosis, %	16	12	0.4
Tubular atrophy, %	18	14	0.4
Interstitial fibrosis, %	23	19	0.4
Chronic vasculopathy, %	20	15	0.4
Transplant glomerulopathy, %	6	1	0.06

PTC, peritubular capillaries.
*Of 603 biopsies, at least 474 (78%) specimens fulfilled the adequacy criteria for scoring of individual morphological lesions.

were performed late after transplantation (median 352 days; range: 23–1621 days). Six biopsies, however, were obtained after <6 months. In these cases, corresponding pre-implant biopsies displayed the same lesions in the respective compartments (data not shown). Among archived specimens, two granular C4d-positive biopsy cores were associated with fragments of attached nonrenal tissue. Remarkably, in these cases, a similar capillary staining pattern was observed also in tissue of recipient origin (skeletal muscle) (Fig. 2).

Associations of granular C4d in PTC with patient and graft survival

Patients were divided into two groups: 56 (17%) recipients showing granular C4d deposits in one or more indication biopsies and 273 granular C4d-negative recipients. As shown in Fig. 3, study groups differed significantly with respect to overall graft survival (survival rates at 1, 3 and 7 years in granular C4d-positive versus C4d-negative subjects: 79%, 68% and 41% vs. 85%, 80% and 66%, respectively, P = 0.001). Outcome differences were related to a higher rate of patient death in granular C4d-positive recipients (P = 0.003) (Figure 3). Adjusted multivariate analysis revealed an independent effect of granular C4d in PTC on all-cause mortality [hazard ratio: 3.12 (95% confidence interval: 1.23–7.94); P = 0.02] (Table 3). Furthermore, granular C4d-positive recipients showed a trend towards inferior death-censored allograft survival (Figure 3). Observed clinical associations markedly differed from those for linear capillary C4d staining, a finding associated with poor death-censored graft survival (44% vs. 80% 7-year

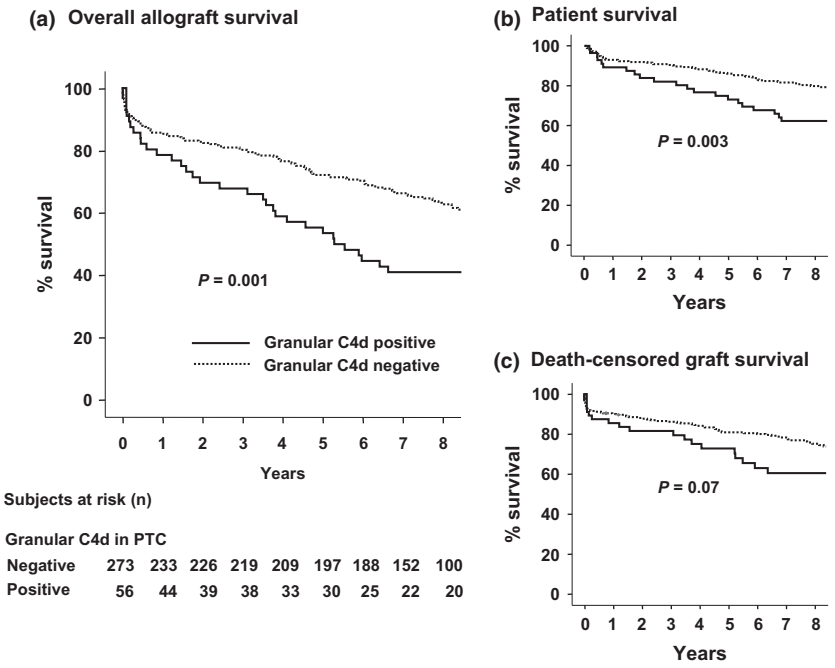

Figure 3 Comparison of biopsied kidney transplant recipients with (n = 56; solid lines) and without (n = 273; dashed lines) granular C4d deposition in PTC regarding (a) overall graft survival, (b) patient survival and (c) death-censored graft survival. The Mantel–Cox log-rank test was used to compare Kaplan–Meier survival between study groups.

Table 3. Independent effect of granular C4d in PTC on all-cause mortality.

Variables*,†	Hazard Ratio	95% Confidence interval	*P* value
Granular C4d in PTC	3.12	1.23–7.94	0.02
Coronary arterial disease	3.76	1.38–10.21	0.009
History of smoking	4.14	1.61–10.68	0.003

PTC, peritubular capillaries.
*Only variables significant in the final model ($P < 0.05$ by Cox regression analysis) are shown.
†For multivariate analysis, covariates were selected for the model if they (i) had either a clinically/biologically plausible relation with the outcome and/or they were (ii) associated with patient survival in unadjusted analysis ($P < 0.2$): recipient age, female gender, body mass index category according to the WHO classification, smoking history, pretransplant comorbid conditions, such as documented coronary heart disease, systolic left ventricular dysfunction, peripheral vascular disease, cerebrovascular disease, diabetes mellitus and hepatitis C infection, duration of pretransplant dialysis therapy, dialysis modality (peritoneal dialysis), donor age, history of prior transplantation, living donation, CDC-PRA>10%, induction therapy with a depleting antibody, linear C4d-positive antibody-mediated rejection, T-cell-mediated rejection and a serum creatinine above 1.5 mg at one year.

graft survival, $P < 0.0001$), without any effect on patient survival (76% vs. 79% 7-year survival, $P = 0.3$).

Associations of granular C4d in PTC with kidney allograft function

We found a significantly higher rate of DGF among patients with granular C4d deposits [22/56 (39%) vs. 59/268 (22%) C4d-negative subjects, $P = 0.007$]. Levels of 1-year serum creatinine were significantly higher in granular C4d-positive subjects (median 2.1 (IQR: 1.7–2.6) vs. 1.6 (1.3–2.0) mg/dl, $P = 0.001$), and there was a trend towards more patients with significant levels of urinary protein excretion at one year (granular C4d-positive versus C4d-negative subjects: 6 of 31 (19%) vs. 18 of 185 (10%) tested patients, respectively, $P = 0.1$).

Risk factors for granular C4d staining in PTC

Univariate analysis revealed that granular C4d-positive patients had a significantly higher body mass index, had documented systolic left ventricular dysfunction more often and were more frequently active or previous smokers (Table 1). Moreover, the incidence of granular C4d staining was higher among patients with a history of peritoneal dialysis. There were no associations with coronary, cerebral or peripheral vascular disease, diabetes mellitus, or a previous malignant disease. Notably, there was no relationship with underlying renal disease including any type of immune complex glomerulonephritis or SLE. With the exception of a lower HLA mismatch among granular C4d-positive subjects, transplant-related variables turned out to be equally distributed between groups (Table 1). As shown in Table 4, binary logistic regression analysis revealed independent effects of previous peritoneal dialysis, body mass index, left ventricular systolic dysfunction and positive hepatitis C antibody status on the occurrence of granular C4d deposits in PTC.

Immunohistochemical and ultrastructural correlates

We performed a detailed morphological analysis of biopsy material obtained from 10 transplant patients selected according to the finding of granular (but not linear) C4d staining and the availability of stored material for retrospective electron microscopy and immunohistochemistry (material was obtained from the same biopsy cores). For comparison, 10 matched C4d-negative control subjects were selected (Table 5).

As shown in Table 5, a major observation was the finding of electron-dense deposits in PTC BM in six of the ten granular C4d-positive specimens. Representative examples are depicted in Fig. 4. In the control group, only one specimen showed such deposits in a single PTC. Deposits were in all cases focally distributed affecting 6 to 16% of analysed PTC. Signs of endothelial cell injury in PTC were observed more frequently in granular C4d-positive ($n = 8$) than C4d-negative specimens ($n = 4$) (not shown). Another remarkable finding was a distinct BM multilayering of PTC in all 10 granular C4d-positive biopsies (diffuse distribution: 5 cases) (Fig. 4). In seven biopsies, BM multilayering was classified as low grade (3 or 4 layers) and in 3 biopsies as high grade (5 or more layers). In control biopsies, BM multilayering was less frequent (3 diffuse and 3 focal cases) with two specimens classified as high grade (data not shown). For three of the granular C4d-positive patients,

Table 4. Risk factors for granular C4d deposition in PTC.

Variable*,†	Odds Ratio	95% Confidence interval	*P* value
Peritoneal dialysis	3.89	1.51–10.05	0.005
Body Mass Index category‡	1.65	1.04–2.61	0.03
Left ventricular systolic dysfunction	3.56	1.18–10.74	0.02
Hepatitis C antibody positive	3.39	1.18–9.68	0.02

PTC, peritubular capillaries.
*Variables significant in the final model ($P < 0.05$ by binary logistic regression analysis) are listed.
†Covariates were selected if they appeared to be imbalanced between granular C4d-positive and C4d-negative subjects ($P < 0.2$) (see Table 1).
‡Body mass index categories were defined according to World Health Organization definitions.

Table 5. Immunohistochemical and electron microscopy results in relation to granular C4d in PTC.

Parameter	Granular C4d in PTC		*P* value
	Yes (n = 10)	No (n = 10)	
Selection/matching criteria			
Linear C4d in PTC, *n*	0	0	
Recipient age, years, median (range)	50.6 (24–70)	52.3 (27–75)	0.9
Female gender, *n*	4	2	0.3
T-cell-mediated rejection, *n*	1	1	0.8
Chronic lesion score, median (range)	3 (0–10)	3 (0–12)	0.5
PTC immunohistochemistry			
IgG*, *n*	4	0	0.04
IgM*, *n*	4	2	0.3
IgA*, *n*	0	0	–
C1q*, *n*	1	2	0.5
C3*, *n*	1	2	0.5
PTC electron microscopy†			
Deposits in PTC BM‡, *n*	6	1	0.03
PTC BM multilayering, *n*	10	6	0.04
BM multilayering score, median (range)	4 (3–5)	3 (0–5)	0.05

BM, basement membrane; PTC, peritubular capillaries.
*Finely granular deposition pattern in 1–5% of PTC.
†Analysis of 11 to 19 PTC per biopsy specimen.
‡Focal distribution of affected PTC (6 to 16% of analysed PTC).

biopsy specimens contained glomerular tissue for ultrastructural analysis. We found electron-dense deposits in glomerular BM and significant BM multilayering in two and one sample, respectively (not shown).

In some cases, granular C4d was associated with a discrete focal pattern of IgG deposition in a few (1–5%) PTC (a representative example is shown in Fig. 1). Peritubular capillary IgG staining was found in four of the C4d-positive, but in none of the C4d-negative specimens (P = 0.04). Notably, in most instances, such staining did not colocalize with areas of C4d deposition. Traces of capillary IgM were observed in six specimens, without any relationship to C4d deposition. None of the included biopsies showed IgA deposits along PTC. Focal peritubular capillary C1q or C3 (1–5% of PTC) was occasionally observed, with no numerical differences between C4d-positive and C4d-negative specimens (Table 5). In granular C4d-positive biopsies, we found occasional segmental glomerular IgM (five specimens) but no IgG staining in the BM of capillary loops and/or in the mesangium. In addition, we noted a focal endothelial IgG signal in a few arterioles (two specimens). IgM staining was exclusively found in hyalinized arterioles (not shown).

For 7 of the 10 index patients, material of a corresponding null biopsy obtained shortly before reperfusion was available. All these biopsies were C4d negative (Fig. 1).

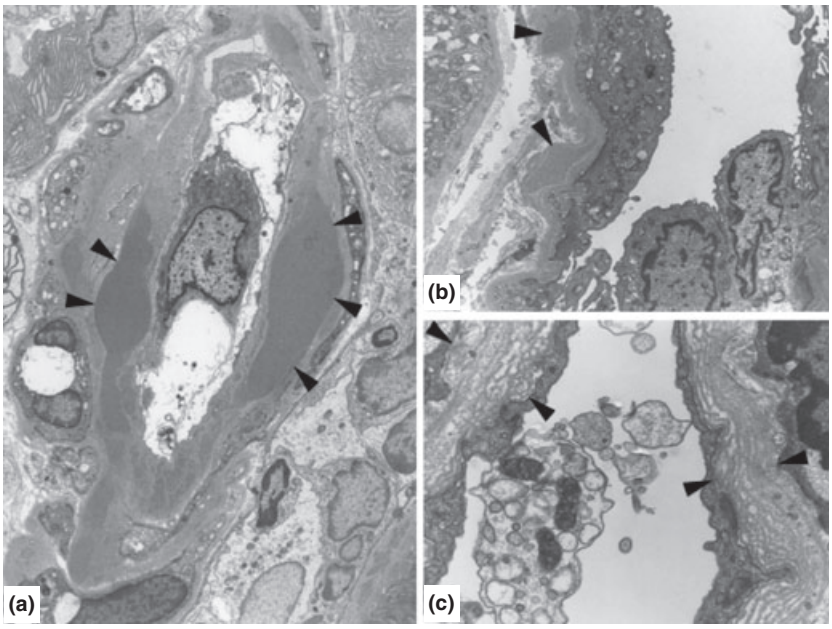

Figure 4 Electron microscopic views of PTC in granular C4d-positive biopsy specimens. Representative specimens obtained from two different patients (a, b) showing PTC with electron-dense deposits in the BM (arrows), and (c) in the second case, a PTC with profound BM lamellation (arrows).

Granular C4d is not associated with circulating immune complexes and antinuclear antibodies

Considering earlier studies showing capillary immune complex deposits in systemic autoimmune diseases [9], we investigated whether there could be a relationship between granular C4d and the presence of circulating immune complexes or antinuclear antibodies. For 83 patients, serum samples obtained both before transplantation and at the time of indication biopsy were available for retrospective analysis. The number of patients with detectable circulating C1q-binding immune complexes was equally distributed ($P = 0.6$) between C4d-positive [pretransplantation: $n = 3$ (21%), at the time of indication biopsy: $n = 1$ (7%)] and C4d-negative patients [pretransplantation: $n = 18$ (27%), indication biopsy: $n = 8$ (12%), $P = 0.5$]. Moreover, we found no significant difference regarding proportions of ANA-positive samples (titre ≥1:40) between patients with and without granular C4d deposits (before transplantation: 4 (29%) vs. 14 (20%), $P = 0.4$; at the time of indication biopsy: 1 (7%) vs. 12 (17%), $P = 0.3$).

Discussion

In this study, we investigated the relevance of capillary granular C4d staining in kidney transplant biopsies, a hitherto ill-defined atypical complement deposition pattern. A cardinal finding was a significant association with clinical outcomes including allograft function and patient survival. Morphological studies suggested a role of classical complement activation triggered by capillary immune complexes.

In its morphological appearance, granular C4d was readily distinguishable from linear C4d deposition, a footprint of capillary alloantibody binding [1–5]. In contrast to linear staining, granular C4d was not associated with morphological features or risk factors for rejection. Remarkably, univariate analysis revealed an even inverse correlation with the number of HLA mismatches. This and the observation of granular C4d deposits also in nonrenal recipient tissue suggested a role of nonalloimmune complement activation.

As in previous studies of our group [3,14], C4d staining was carried out by immunohistochemistry, a technique discussed to be prone to false-positive staining. In earlier reports, granular C4d deposition was suggested to reflect C4d-positive plasma in capillaries producing artefacts that interfere with the interpretation of C4d staining [8]. However, our ultrastructural studies argue against nonspecific staining and suggest a role of immune complex formation. A remarkable observation was that granular C4d was accompanied by focally distributed electron-dense deposits in the BM of PTC. Moreover, we found a discrete capillary IgG binding in some of the granular C4d-positive specimens. Staining for IgM or complement components C1q or C3 was not or only occasionally observed, without any apparent association with C4d. Notably, the deposition of immune deposits was not restricted to PTC. For example, electron-dense deposits were occasionally found also in glomerular BM. However, while in some biopsies, glomerular capillary and/or mesangial IgM was detected, immunohistochemistry failed to demonstrate glomerular IgG staining. Interpreting our failure to detect a clear colocalization of IgG with C4d staining, it is important to note, that in AMR, where linear C4d in PTC is known to tightly associate with circulating antidonor alloantibodies, immunomorphological studies have, for unknown reason, failed to detect significant amounts of Ig or other complement products in the specific location of PTC [15,16].

There are several lines of evidence suggesting that the peritubular capillary bed may serve as site for deposition of immune complexes. In a rodent model, injection of endothelium-binding concanavalin A followed by anticoncanavalin A IgG was demonstrated to cause transient immune complex and complement deposition in PTC [17]. Moreover, clinicopathological studies have demonstrated immune complex-mediated classical complement activation along PTC in SLE [9,10]. Li *et al.* [9] reported a distinct granular peritubular capillary C4d staining in approximately 7% of patients diagnosed with lupus nephritis, which was accompanied by granular IgG staining and electron-dense deposits in the BM of PTC. Interestingly, in SLE, granular C4d in PTC was associated with higher autoantibody levels, more proteinuria, and higher disease scores [9]. In our present study, we were unable to demonstrate associations with antinuclear autoantibodies and underlying renal disease, including SLE. Notably, some of our patients showed significant levels of circulating immune complexes before and after transplantation, however, without any association with granular C4d deposits in PTC. Thus, speculating a role of immune complex-mediated capillary complement activation, one candidate mechanism could be the in situ formation of immune deposits.

Analysis of baseline variables revealed statistical associations with several characteristics not related to alloimmunity, such as previous peritoneal dialysis, left ventricular dysfunction or positive hepatitis C antibody status. We have no plausible explanation for the associations computed in multivariate analysis, even though one may argue that the observed relationship with a positive hepatitis C antibody status could be in some accordance with a possible role of immune complex deposition. Indeed, in an earlier study, hepatitis C and mixed cryoglobulinemia were described to be associated with deposition of C4d in skin biopsies, presumably as a consequence of capillary immune complex deposition [18].

Interpreting our study results, it is important to note that granular C4d in PTC may not necessarily indicate immune

complex-mediated disease. For example, in a recent study, Batal *et al.* [11] described a very similar pattern of PTC staining in a subset of patients with scleroderma suffering from a more aggressive clinical course. In some contrast to lupus nephritis, C4d staining was not associated with immune deposits. Interestingly, the authors reported occasional detection of minimal C4d deposits in PTC also in normotensive and hypertensive controls, which led to the speculation that direct endothelial injury, for example because of hypertension or drugs, could have triggered complement activation [11].

In this respect, it was an interesting observation that the incidence of DGF was nearly twofold higher in patients with granular C4d deposits. In a recent study, Castellano *et al.* [19] demonstrated transient capillary deposition of focal C4d after 30 min ischaemia in a swine model of ischaemia/reperfusion injury. The same authors demonstrated focal capillary C4d deposits in 40% of transplant patients subjected to biopsy for DGF 7–15 days after transplantation, in the absence of any histological rejection features [19]. These data may be in some contrast to a clinical study, showing only occasional C4d staining in perioperative biopsies, which in all cases was associated with subsequent AMR [20]. In our study, all studied pre-implant biopsies stained C4d negative. This observation may argue against the formation of granular deposits before organ donation or during cold perfusion, but may not exclude a role of reperfusion as a trigger of complement activation. In this context, it is important to note that in DGF patients, granular C4d-positive biopsies were performed after a median of 19 days (range 8–587 days), frequently many months post-transplantation. Moreover, there were many granular C4d-positive recipients showing immediate graft function.

A remarkable observation was that granular C4d staining was associated with greater risks of long-term patient and graft loss, with a 25% difference in 7-year overall graft survival. In contrast to linear C4d staining, where survival differences were related to inferior death-censored graft survival, granular C4d-positive recipients experienced a greater risk of mortality. This effect remained significant in multivariate analysis and was independent of potentially confounding risk factors for death. While we have no plausible explanation for the greater risk of mortality, differences in graft function and trends towards more frequent proteinuria and worse death-censored graft survival can be speculated to result from distinct structural lesions detected in granular C4d-positive specimens. Deposits in BM of PTC were associated with endothelial cell injury and PTC BM multilayering, even in biopsies performed shortly after transplantation. Similar ultrastructural lesions were earlier reported to occur also in the course of AMR, already early after transplantation [21–23]. Our observation of a trend towards more frequent transplant glomerulopathy in granular C4d-positive biopsies may be in some accordance with the previously described finding of early PTC BM multilayering in AMR being associated with structural lesions including transplant glomerulopathy late after transplantation [22,23].

There are several limitations of this study including its retrospective design and the lack of serial protocol biopsies. Accordingly, the results of our study may be influenced by a bias of selecting patients with graft dysfunction, and there is a lack of information regarding incidences of granular C4d and clinical associations in stable recipients. Moreover, our study was limited to the use of immunohistochemical C4d staining on paraffin-embedded formalin-fixed biopsy specimens. Compared with immunofluorescence, this technique may be less sensitive and produce subtle differences regarding patterns of C4d deposition [8]. As frozen sections were not routinely collected at our unit, we were unable to directly compare the two different techniques. In this context, however, it has to be pointed out that in patients with SLE, the pattern of granular C4d in PTC was also detectable by immunofluorescence [9]. Considering the widespread use of immunofluorescence techniques, there will be the need for prospective studies comparing atypical C4d staining patterns on frozen versus paraffin sections to clarify whether there are staining differences related to the processing of biopsy samples or the use of specific reagents (polyclonal versus monoclonal anti-C4d antibodies), respectively.

This study, the first investigating the relevance of a hitherto ill-defined atypical granular C4d staining in kidney transplantation, provides strong evidence for clinical relevance of this pattern. We suggest that granular C4d in PTC may result from alloimmune-independent complement activation possibly triggered by immune complex deposition. Our data may provide a valuable basis for future in-depth studies designed to include larger cohorts and a systematic prospective sampling of biological material to clarify the mechanisms underlying this particular pattern of complement deposition.

Authorship

ZK, NK, HR and GAB: designed study, performed study, collected data, analysed data, wrote the paper. VN, GJZ, MW and GB: analysed data, wrote the paper. KP and LM: analysed data.

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