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ORIGINAL ARTICLE

Functional and morphologic evaluation of kidney proximal tubuli and correlation with renal allograft prognosis

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Keywords
chronic allograft nephropathy, kidney transplantation, proximal tubular function, retinol-binding protein, surrogate marker, tubulointerstitial injury.

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Summary

Renal transplant patients with stable graft function and proximal tubular dysfunction (PTD) have an increased risk for chronic allograft nephropathy (CAN). In this study, we investigated the histologic pattern associated with PTD and its correlation with graft outcome. Forty-nine transplant patients with stable graft function were submitted to a biopsy. Simultaneously, urinary retinol-binding protein (uRBP) was measured and creatinine clearance was also determined. Banff's score and semi-quantitative histologic analyses were performed to assess tubulointerstitial alterations. Patients were followed for 24.0 ± 7.8 months. At biopsy time, mean serum creatinine was 1.43 ± 0.33 mg/dl. Twelve patients (24.5%) had uRBP ≥1 mg/l, indicating PTD and 67% of biopsies had some degree of tubulointerstitial injury. At the end of the study period, 18 (36.7%) patients had lost renal function. uRBP levels were not associated with morphologic findings of interstitial fibrosis and tubular atrophy (IF/TA), interstitial fibrosis measured by Sirius red or tubulointerstitial damage. However, in multivariate analysis, the only variable associated with the loss of renal function was uRBP level ≥1 mg/l, determining a risk of 5.290 of loss of renal function ($P = 0.003$). Renal transplant patients who present PTD have functional alteration, which is not associated with morphologic alteration. This functional alteration is associated to progressive decrease in renal function.

Introduction

Histologic abnormalities in chronic allograft nephropathy (CAN) may be observed from the third post-transplant month and are characterized mainly by interstitial fibrosis and tubular atrophy. A protocol biopsy study observed that typically deterioration in renal function in CAN appears after the histologic abnormalities were already present. The histologic characteristic of CAN associated more closely with subsequent decrease in renal function is interstitial fibrosis [1].

In recent years, the role of the tubulointerstitial compartment in the progression of chronic renal disease has become more evident [2–4]. The measurement of urinary low molecular weight proteins can be used in transplant patients to identify proximal tubular dysfunction (PTD), and for early detection of patients with high risk of chronic renal disease [5–8].

We have previously observed among heart transplant patients with adequate and stable renal function that those with high levels of urinary retinol-binding protein (uRBP) have a threefold higher risk for development of

chronic renal failure after 5 years of follow-up [7]. Likewise, in renal transplant patients with good and stable renal function, we and other authors have also shown that the presence of PTD – in our study, assessed by high levels of uRBP – after the 3rd post-transplantation month is associated with a sixfold higher risk of developing CAN within 5 years [8]. However, in these previous studies, morphologic abnormalities of the tubulointerstitial compartment and tubular epithelial cells, which might correlate with high levels of uRBP, were not investigated.

In this study, renal transplant patients with stable renal function were submitted to protocol kidney biopsies and measurements of uRBP. During the follow-up period, renal allograft function was sequentially assessed and correlated with uRBP and histologic features. In this manner, we aimed to identify the morphologic characteristics correlated with PTD (high levels of uRBP).

Material and methods

Subjects

Forty-nine renal transplant patients with stable renal function (mean serum creatinine 1.43 ± 0.33 mg/dl) with a median post-transplant time of 105 days (30–690 days) were submitted to a biopsy. We considered such of those patients with stable renal function i.e. those who presented stable serum creatinine levels during three consecutive measurements at 15 days of interval each, and complete absence of any clinical dysfunction such as drug toxicity, acute rejection or infection. Only those patients were enrolled in our study. Kidney biopsies were simultaneously performed along with uRBP measurement and renal function assessment (serum creatinine and creatinine clearance estimated by the Cockcroft-Gault formula) during the period from April 2001 to January 2004. Participants from two Transplant Centers, Hospital do Rim e Hipertensão and Albert Einstein Hospital, were informed of the research objective and signed the informed consent, which was approved by the Ethics Committees of both hospitals. The medical team was the same at both transplant centers. Forty-four patients completed 1 year of follow-up and 31 patients were followed for at least 2 years. Patients were followed for 24.0 ± 7.8 months.

Initial immunosuppression regimen included a calcineurin inhibitor associated with steroids and an antiproliferative agent (azathioprine or mycophenolate mofetil). Two patients received anti-lymphocytic antibody preparations for induction therapy. Treatment of acute rejection was based on the Banff 1997 Classification [9] and clinical presentation.

Doses of cyclosporine were adjusted to maintain 12-h trough levels of 200–300 ng/ml for the first 3 months post-transplant; after this period, we subsequently tapered doses to maintain trough levels between 50 and 150 ng/ml. Whenever tacrolimus was utilized, doses were adjusted to attain target whole-blood trough concentrations close to 10 ng/ml for the first 3 months and 5–10 ng/ml thereafter.

Tubular dysfunction analysis

Urinary retinol-binding protein was determined by immunoenzymometric assay, and the upper limit of normal was set at 0.4 mg/l [10]. Urine samples were collected and frozen until determination of RBP. No conservative or special precautions were considered to be necessary as RBP is stable in urine. Concentrations in the samples were calculated by comparison with the standard curve, prepared by using nonlinear regression, usually as a third-degree polynomial. In our study, patients were divided into two groups according to uRBP values, <1 mg/l and ≥1 mg/l.

Renal function

Renal function was measured by 1/serum creatinine and creatinine clearance (estimated by the Cockcroft-Gault formula). It was evaluated at the time of biopsy and again at the end of the study period (after a follow-up of 24.0 ± 7.8 months). Delta of creatinine clearance (Cockcroft-Gault formula) and 1/serum creatinine were calculated by the differences between their values at the end of the follow-up period and creatinine clearance at biopsy time respectively.

Although serum creatinine was not the best method to evaluate renal function in renal transplantation, it has been used in clinical practice and in some data reported from different groups.

Histologic studies

Renal tissue specimens from biopsies were subjected to routine staining (hematoxylin–eosin, Jone's Silver Methenamine, periodic acid Schiff-PAS and Masson's Trichrome), and Sirius red staining (for collagen I and III determination) was assessed under polarized light. All biopsies were analysed by a blinded renal pathologist and using Banff's 1997 Classification and an arbitrary semi-quantitative classification of tubulointerstitial abnormalities. Briefly, a scale ranging from 0 to 3 was used for the semi-quantitative analysis of acute and chronic tubular (atrophy, acute epithelial abnormality and tubulitis) and interstitial abnormalities (inflammation and fibrosis), thereby allowing the calculation of tubulointerstitial injury score. Fibrosis, atrophy and tubulitis were classified according to Banff-97 ranging from 0 to 3. The tubular score

(0–9) was obtained by the sum of tubular changes, the interstitial score (0–6) by the sum of interstitial abnormalities and the total score by adding these two scores (0–15).

Patients were divided into two groups depending on the presence or absence of (IF/TA) in biopsy according to Banff's 1997 Classification.

For Sirius red staining, 0.1 g of Sirius red and 100 ml of saturated picric acid solution were used. Paraffin blocks were prepared into 3-μm thick histologic sections and deparaffinized with xylol and subsequently hydrated with absolute alcohol (4×) and running water. Subsequently, the sections were immersed in saturated picric acid solution for 15 min and then in Sirius red for additional 20 min. Contra-staining was carried out with Harris hematoxylin.

Sirius red: image analysis

Sirius red-stained sections were analysed by an Olympus Bx50 microscope with an Olympus camera attached. Manual shots of the cortex magnified 40× and observed under polarized light were taken of at least five different fields in each slide, and structures such as: glomeruli, subcapsular cortex, large vessels and medulla were excluded. These pictures were digitized (HP scanjet 2400) and then the interstitial volume of collagen in the cortex as compared with the overall cortex area was quantified by morphometry.

For the morphometric analysis, the *Image Processing and Analysis in Java, Image J* software (National Institutes of Health, Bethesda, MD, USA) was used. The result of each image is given in percentages, which means a proportion between the interstitial volume of collagen in the cortex and the total cortical interstitial volume, and then the arithmetic mean of the analysed fields was calculated for each slide. The confidence in reproducibility of interstitial fibrosis volume obtained by the morphometric analysis of the digital image stained with Sirius red was based on previous studies [11].

Operational definitions

Delayed graft function (DGF) was defined as the need for dialysis during the first week after transplant in the absence of rejection and/or technical problems. We assumed total score ≥1 as an indicator of tubulointerstitial injury in biopsy. We defined graft function loss as any decline of creatinine clearance value between the biopsy time and end of follow-up period.

Statistical analysis

Pretransplant demographic characteristics used for covariate-adjusted analyses included the dialysis time, type of donor (deceased versus living donor), recipient gender, age of the donor, age of the recipient and HLA compatibility among living donors. The post-transplant related variables included DGF, acute rejection, cold ischemia time, type of immunosuppressive medication used, tacrolimus or cyclosporine, mycophenolate mofetil or azathioprine. Laboratorial parameters used were: 1/serum creatinine at biopsy time and at the end of follow-up time, creatinine clearance at biopsy time and at the end of follow-up time; delta of creatinine clearance between biopsy time and at the end of the study period was used to determine graft loss, uRBP <1 mg/l or ≥1 mg/l and calcineurin inhibitors levels. Morphologic variables included were: presence or absence of IF/TA in biopsy, interstitial fibrosis assessed by Sirius red staining, tubular, interstitial and total scores. We assumed total score <1 or ≥1 as a categorical variable, as a marker of tubulointerstitial injury.

Values are presented as mean and standard deviation (SD) and when appropriate, as median and ranges. To evaluate the relationship between quantitative variables, Pearson's correlation coefficients and Spearman's correlation coefficients were calculated. Parametric and nonparametric tests, chi-squared test and Fisher's exact test were performed when appropriate. A multivariate Cox proportional hazards model was used to analyse the relationship among graft loss and the other covariates which were significant in univariate analysis or were well-known to be involved in graft loss. The significance level was 0.05 and the statistical software used was SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Demographic data

Among 49 patients, 31 patients (63.3%) were male subjects and their mean age at enrollment was 38.3 ± 10.5 years. Median post-transplant time at enrollment was 105 days (30–690 days). All patients underwent dialysis therapy prior to transplantation, with median time of 24 months (5–96 months). Thirty-two patients received an organ from living donors (65%). Regarding HLA compatibility (Locus A, B and DR), among living donor transplants, 16 (50%) were HLA identical, 11 (34.3%) haploidentical and 5 (15.6%) unrelated. Mean cold ischemia time was 19.5 ± 7.0 h in transplants from deceased donors. Mean age of donors was 37.2 ± 10.9 years (Table 1). Six (12.2%) patients had DGF and eight (16.3%) patients had acute rejection (AR). Mean cyclosporine and tacrolimus blood trough levels at biopsy time were 169.8 ± 78.8 and 12.0 ± 4.5 ng/ml respectively. One patient presented a histologic diagnosis of cyclosporine nephrotoxicity. It was observed after

Table 1. Demographic characteristics of patients.

	Values
Recipient age (years)	38.3 ± 10.5
Donor age (years)	37.2 ± 10.9
Recipient gender (male/female)	31 (63.3)/18 (36.7)
End-stage renal disease	
Glomerular	16 (33)
Arterial hypertension	9 (18)
Adult polycystic kidney disease	4 (8)
Interstitial	2 (4)
Diabetes mellitus	2 (4)
Urinary tract congenital malformation	1 (2)
Unknown origin	15 (30)
Dialysis time (months)*	24 (5–96)
Type of donor (living/deceased)	32 (65.3)/17 (34.7)
HLA compatibility living donors	
Identical	16 (29.6)
Haploidentical	11 (34.4)
Distinct	5 (15.6)
Cold ischemia time (deceased donors)	19.5 ± 7.0
Delayed graft function	6 (12.2)
Acute rejection	8 (16.3)

*Dialysis time was expressed as median.
Values given in parentheses are in percentages.

540 days after transplantation, when his cyclosporine trough level was 345 ng/ml, serum creatinine was 2.0 mg/dl and uRBP was 0.62 mg/l.

Renal function assessment

Mean of 1/serum creatinine values were 0.73 ± 0.18 mg/dl and 0.77 ± 0.15 mg/dl at biopsy time and at the last assessment respectively (Table 2). There was a slight improvement of creatinine clearance in the overall group that finished the follow-up (60.2 ± 12.5 to 65.2 ± 14.2 ml/min). The median of delta of creatinine clearance during the follow-up was 3.3 ml/min (from −51.10 ml/min to 36.50 ml/min) and the median of delta of 1/serum creatinine was 0.05 mg/dl (from −0.40 to 0.34 mg/dl). Based on their delta of creatinine clearance, 18 (36.7%) patients had a loss in renal function during the follow-up period, including one patient who lost the graft during follow-up. Among these 18 patients who experienced reduction in renal function, the median of delta of creatinine clearance was −6.6 ml/min (−51.10 to −0.40 ml/min).

All patients were screened for proteinuria, and if they were positive, we then tested for albuminuria. In our study, only one patient had proteinuria at enrollment (1.62 g/l). This patient was biopsied 95 days after transplantation, his uRBP was 0.03 mg/l and he had a good renal function (sCr = 1.6 mg/dl and ClCr = 79.1 ml/min).

Tubular function assessment

Among 49 patients, the median value of uRBP was 0.24 mg/l (0.01–9.98 mg/l). Twelve (24.5%) patients had uRBP values ≥1 mg/l (median: 1.70 min: 1.04 – max: 9.98 mg/l) and 37 (75.5%) patients presented values of uRBP <1 mg/l (median: 0.16 min: 0.01 – max: 0.94 mg/l).

Histologic findings on biopsy performed at enrollment

Among 49 protocol biopsies, 23 (47%) were normal according to the 1997 Banff's Classification. One patient had subclinical rejection and another had borderline changes. Of the 49 patients, 5 (10%) had morphologic findings compatible with IF/TA. During the follow-up period, one patient lost the allograft as a result of CAN, 27 months after transplantation. In patients with morphologic findings of IF/TA, the median time after

Table 2. Laboratorial data of 49 patients studied.

	Mean	SD*	Median	Min	Max
1/Serum creatinine at biopsy time (mg/dl)	0.73	0.18	0.66	0.45	1.11
1/Serum creatinine at the end of follow-up (mg/dl)	0.77	0.15	0.74	0.56	1.11
Creatinine clearance at biopsy time* (ml/min) (*N* = 49)	60.2	12.5	59.4	35.2	94
Creatinine clearance at the end of follow-up (ml/min)† (*N* = 48)	65.2	14.2	62.0	37.5	98.2
Delta of 1/serum creatinine (mg/dl)	0.03	0.15	0.05	−0.40	0.34
Delta of creatinine clearance (ml/min)	3.71	15.2	3.30	−51.1	36.5
uRBP level at biopsy time (mg/l)	0.83	1.67	0.24	0.01	9.98
Cyclosporine level at biopsy time (ng/ml) (*N* = 33)	169.7	78.8	151	47	369
Tacrolimus level at biopsy time (ng/ml) (*N* = 14)	12	4.5	10.5	7.40	24.9

*,†Creatinine clearance was estimated by Cockcroft-Gault formula.

transplant when the biopsy was performed was 180 days (90–690 days) vs. 92.5 days (30–540 days) in patients without IF/TA ($P = 0.047$).

Taking into consideration the semi-quantitative analysis of tubulointerstitial injuries, 61.2% of the biopsies had no tubular changes, 34.7% had no interstitial abnormalities and 33% had neither interstitial nor tubular changes.

Collagen I and III are important components of interstitial fibrosis. Therefore, we assessed them by quantitative measurement of interstitial volume in the cortex, using Sirius red staining. The mean value of Sirius red staining was 4.1 ± 2.1%. This correlated positively with interstitial ($r = 0.313$, $P = 0.03$) and total scores ($r = 0.33$, $P = 0.023$).

Correlation of demographic, laboratory and histologic features with PTD

The only demographic characteristic of patients associated with uRBP value was HLA compatibility. Patients with higher compatibility had lower values of uRBP (Table 3). There was no association between uRBP levels and the use of immunosuppressive drugs or the blood trough levels of calcineurin inhibitors. Neither tacrolimus nor cyclosporine trough levels were correlated with uRBP levels (Pearson's correlation: $r = 0.014$, $P = 0.961$ and $r = 0.019$ and $P = 0.916$ respectively).

Table 3. Association between uRBP levels and demographic characteristics (univariate analysis).

49 patients	RBPu <1 mg/l *N* = 37	RBPu ≥1 mg/l *N* = 12	*P*-value
Female gender	15	3	0.494
Male gender	22	9	0.494
Living donor	23	9	0.503
Deceased donor	14	3	0.503
HLA matching			
Identical	13	3	0.031
Haploidentical	9	2	
Unrelated and deceased	1	4	
Post-transplant time at enrollment (month)	32.2 ± 25.2	34.0 ± 20.4	0.625
Receptor age	37.0 ± 10.4	42.0 ± 10.5	0.162
Donor age	37.7 ± 11.0	35.4 ± 11.0	0.812
Dialysis time (month)	32.2 ± 25.2	34.0 ± 20.4	0.525
DGF	5	1	0.540
Acute rejection	7	1	0.660

Data as time at enrollment. Age and dialysis time were expressed as mean with their respective standard errors.

In patients who had subclinical rejection and borderline changes, the values of uRBP were 2.01 mg/l and 0.94 mg/l, respectively. In patients with morphologic features of IF/TA, the median of uRBP value was 0.20 (0.04–5.8 mg/l) and 0.26 mg/l (0.01–9.98 mg/l) in patients without CAN ($P = 0.835$). Two (40%) patients with CAN had uRBP ≥1 mg/l and three (60%) had uRBP <1 mg/l ($P = 0.584$). During the follow-up period, one patient lost the allograft as a result of CAN, where this patient had a uRBP level of 1.04 mg/l.

In patients with total score ≥1, the median of uRBP values was 0.22 (0.01–9.98), similar to the values obtained in patients with total score <1 (0.39, 0.01–1.89, $P = 0.76$). In patients with total score ≥1, six (18%) patients had uRBP values ≥1 mg/l, and 27 (82%) patients had uRBP values ≤1 mg/l, ($P = 0.17$). The median of Sirius red was 3.8% (1.55–10.9) in patients with uRBP <1 mg/l, and 2.8% (1.33–9.75) in patients with uRBP ≥1 mg/l, ($P = 0.33$).

In general, there was no association among levels of uRBP and tubulointerstitial scores, interstitial fibrosis assessed by Sirius red, and the histologic presence of IF/TA. Thus, no morphologic abnormality of the tubulointerstitial compartment was associated with PTD as assessed by uRBP (Table 4).

Correlation of demographic, laboratory, and histologic features with graft loss

For the 18 patients who lost graft function, Cox-multivariate analysis was performed to evaluate the variables that were independently associated with loss. Variables which were statistically significant in univariate analysis and classically related with a long-term worse prognosis were included. Among those variables, uRBP ≥1 mg/l was the only variable associated with loss of renal function, deter-

Table 4. Association between uRBP and morphologic markers (univariate analysis).

	RBPu <1 mg/l *N* = 37	RBPu ≥1 mg/l *N* = 12	*P*-value
IF/TA at biopsy (yes)	3	2	0.584
Sirius red (morphometry)	3.8 (Min: 1.55–Max: 10.9)	2.83 (Min: 1.30–Max: 9.75)	0.333
Tubular score (0–9)	0 (Min: 0–Max: 4)	0 (Min: 0–Max: 3)	0.968
Interstitial score (0–6)	1 (Min: 0–Max: 6)	0.5 (Min: 0–Max: 3)	0.436
Total score (0–15)	1 (Min: 0–Max: 9)	0.50 (Min: 0–Max: 5)	0.458

Tubulointerstitial scores and Sirius red were expressed in median.
IF/TA, interstitial fibrosis and tubular atrophy.

Table 5. A Cox multivariate analysis to evaluate the variables independently associated with graft loss.

Variable	*P*-value	Exp (B)	Lower 95% CI	Upper 95% CI
RBP	0.003	5.290	1.794	15.622
IF/TA	0.105	3.842	0.756	19.514
Acute rejection	0.562	1.593	0.331	7.679
DGF	0.628	1.775	0.175	18.003
Living donor	0.744	0.817	0.243	2.743

mining a risk of 5.290 of graft function loss, $P = 0.003$ (Table 5).

Discussion

Diagnostic methods that can identify early those patients with greater risk of allograft loss must be actively pursued. We have previously observed that PTD is an early marker of renal function deterioration in heart and kidney transplant recipients [7,8].

As tubulointerstitial abnormalities are strong predictors of renal prognosis, in this study, functional and structural abnormalities of the interstitium and tubular epithelial cells in kidney transplant recipients with stable renal function were investigated.

In our study, tubulointerstitial injury was present in 67% of biopsies, although of a mild degree. The collagen deposition was observed especially in areas of greater tubulointerstitial injury, however this deposition was generally weak as compared with other studies. In addition to having stable renal function, the majority of patients included were not under immunologic or toxic aggressions as may be observed by the adequate levels of calcineurin inhibitors at the time of biopsy, the low incidence of DGF, acute rejection and by the large number of HLA-identical donors.

Although most of our patients had good and stable renal function, with low degree of morphologic alterations, we detected an incidence of 24.5% of PTD, i.e. levels of uRBP ≥1 mg/l. Nevertheless, high levels of uRPB did not correlate with morphologic findings compatible with IF/TA, collagen deposition or with tubulointerstitial injuries. These results suggest that high levels of uRBP are more likely to reflect a functional disorder of tubular epithelial cell which precedes the structural changes.

The absorption of low molecular weight proteins by proximal tubule is calcium-dependent, and involves high energy expenditure. They are endocytosed when they reach the luminal portion of the tubular cell via endocytic receptors, called megalin and cubilin. Subsequently these proteins are dissociated from their receptors by endosomal acidification and transported to the lysosomes where they are degraded and the resulting aminoacids cross the contraluminal membrane and return to circulation. Megalin and cubilin return to the apical membrane. Therefore, changes in any of the steps involving the absorption of these proteins might induce the loss of these proteins in the urine before any established morphologic change occurs [12–15].

In renal transplantation, specifically, potential injuries to tubular epithelial cell might be related with ischemic-reperfusion injury, acute rejection, CNI-induced nephrotoxicity and infections, such as cytomegalovirus or polyomavirus. Nankivell *et al.* showed that the natural history of CAN is determined by two phases of tissular injury: the first one being in early phase post-transplantation, when tubulointerstitial alterations occur rapidly and intensely, where these are related with ischemic and immunologic insults. The morphologic feature of the second phase is vascular alteration, being a result mainly of CNI nephrotoxicity [16].

Teppo *et al.* had previously presented that increased urinary excretion of alpha-1-microglobulin at 6 months after transplantation was associated with urinary excretion of transforming growth factor-beta 1 and indicated poor long-term renal outcome [6]. In our study, patients with PTD at the time of biopsy, despite adequate renal function, developed renal function deterioration during the follow-up period.

In this study, high levels of uRBP were the only variable associated with long-term graft loss in the multivariate models. In addition, patients with high levels of uRBP had no morphologic change as compared with patients with normal uRBP. It is possible that during an insult, when at the same time the PTD occurs, the tubular epithelial cell is also stimulated to produce molecules involved in renal fibrogenesis such as TGF-β1 [6]. Moreover, as previously shown, this growth factor is involved in several intracellular processes, including the ability to inhibit protein endocytosis mediated by megalin and cubilin receptors [17].

Thus, the presence of tubular functional abnormalities without morphologic changes, suggests that early intervention therapy such as discontinuation or replacement of calcineurin inhibitors based on uRBP levels might lead to prevention of progression to chronic allograft dysfunction in such patients.

Authorship

ACCM and APSF: designed research/study. ACCM: performed research/study. SH and ABP: contributed important reagents. ACCM and AFFO: collected data. ACCM, NOSC, LARM, and MFF: analysed data. ACCM, NOSC, and APSF: wrote the paper.

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