Published in final edited form as:

Proteomics Clin Appl. 2008 July ; 2(0): 1025–1035. doi:10.1002/prca.200780137.

Urinary proteomic analysis of chronic allograft nephropathy

Edmond O’Riordan^{1,2}, Tatyana N. Orlova², Natalia Mendeleev², Daniel Patschan², Rowena Kemp¹, Praveen N. Chander³, Rena Hu², Gang Hao⁴, Steven S. Gross⁴, Renato V. Iozzo⁵, Veronica Delaney², and Michael S. Goligorsky²

¹ Department of Renal Medicine, Salford Royal Foundation Trust, Salford, UK

² Department of Medicine, Renal Institute and Division of Nephrology, New York Medical College, Valhalla, NY, USA

³ Department of Pathology, New York Medical College, Valhalla, NY, USA

⁴ Department of Pharmacology, Weill-Cornell Medical Center, New York, NY, USA

⁵ Department of Pathology, Anatomy and Cell Biology and the Cellular Biology and Signaling Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA

Abstract

The pathogenesis of progressive renal allograft injury, which is termed chronic allograft nephropathy (CAN), remains obscure and is currently defined by histology. Prospective protocol biopsy trials have demonstrated that clinical and standard laboratory tests are insufficiently sensitive indicators of the development and progression of CAN. The study aim was to determine if CAN could be characterized by urinary proteomic data and identify the proteins associated with disease. The urinary proteome of 75 renal transplant recipients and 20 healthy volunteers was analyzed using surface enhanced laser desorption and ionization MS. Patients could be classified into subgroups with normal histology and Banff CAN grades 2-3 with a sensitivity of 86% and a specificity of 92% by applying the classification algorithm Adaboost to urinary proteomic data. Several urinary proteins associated with advanced CAN were identified including α 1-micro-globulin, β 2-micro-globulin, prealbumin, and endorepellin, the antiangiogenic C-terminal fragment of perlecan. Increased urinary endorepellin was confirmed by ELISA and increased tissue expression of the endorepellin/perlecan ratio by immunofluorescence analysis of renal biopsies. In conclusion, analysis of urinary proteomic data has further characterized the more severe CAN grades and identified urinary endorepellin, as a potential biomarker of advanced CAN.

Keywords

Chronic allograft nephropathy; Kidney; Transplant; Urine

Correspondence: Dr. Edmond O’Riordan, H4 Renal, Hope Hospital, Salford Royal Foundation Trust, Stott Lane, Salford, M6 8HD, UK, edmond.o’riordan@srft.nhs.uk, **Fax:** 144-161-2065086.

The authors have declared no conflict of interest.

1 Introduction

Chronic allograft nephropathy (CAN) is now the commonest cause of loss of function in a transplanted kidney after death with a functioning graft [1]. The term is a collective one, used to describe a constellation of atrophic and chronic histological changes in renal allografts. The severity is graded according to the Banff '97 criteria (used in this study) by the degree of tubular atrophy and interstitial fibrosis [2]. Clinically, CAN presents as a slow inexorable rise in the serum creatinine (as renal function declines) usually associated with some degree of proteinuria. Clinical guides such as serum creatinine are not sensitive indicators of disease progression [3, 4]. Recent observational studies have given a clearer picture of the natural history of the disorder and associated risk factors in the setting of typical immunosuppressive regimens [5]. Unfortunately, the current histological grading systems predict progression of CAN and individual graft survival poorly, therefore newer techniques that improve the predictive accuracy of graft survival will be crucial for the reduction in patient number and study time required to design practical prospective therapeutic trials [6].

Proteinuria, hypertension, and tubulointerstitial disease have long been recognized as the key risk factors in progressive renal function loss in native kidney disease. Proteinuria has also been shown to track progression of CAN even in cases where the renal function remained stable [4, 7]. Newer techniques in MS now permit the profiling of the urinary proteome and identification of individual species. The aims of this study were (i) to determine if analysis of the urinary proteome using SELDITOF MS is helpful in distinguishing and classifying those patients who have biopsy-proven CAN as defined by the Banff classification, (ii) to identify the protein(s) that best characterize the severity of CAN, and (iii) determine if tissue expression parallels the urinary abundance profile of such protein(s).

2 Materials and methods

2.1 Patients and urine samples

Patients were recruited at time of transplantation at Westchester Medical Center and followed prospectively from December 2002 to October 2005. Fresh mid-stream urine samples were collected at routine clinic visits and at the time of biopsy. Urine samples were also collected from 20 healthy volunteers. Urine was stored on ice, and multiple aliquots frozen at -80°C within 4 h of voiding. The urinary proteome has been shown to be stable during that period by Schaub *et al.* [8]. Samples were thawed just once prior to the analysis as repeated freeze/thawing has been shown to destroy certain proteins [9]. No protease inhibitor was used. Contemporaneous clinical data, including blood pressure and serum creatinine, were abstracted from the patient record. All patients completed a written consent form permitting both urine and data collection in compliance with HIPAA (Health Insurance Portability and Accountability Act). The Institutional Review Board for clinical trials involving human subjects approved the study protocol.

Immunosuppressive protocols were tailored to individual patients as deemed clinically appropriate but usually included a calcineurin inhibitor (tacrolimus (trough level 5–10

ng/mL) or cyclosporin (trough level 50–150 ng/mL)), rapamycin (trough level 10–15 ng/mL)) mycophenolate mofetil (1–2 g/day), and prednisone tapered from 120 to 10 mg/day by 4 wks post-transplant.

2.2 Sample processing and MS

Sample processing and analysis were performed as previously described [10]. In brief, samples were denatured using CHAPS and Urea in a Tris buffer. The denatured sample was then incubated with chip-specific binding buffer (Table 1) for 1 h, washed, and deposited on the chip surface with a Sinapinic acid matrix. SELDI-TOF MS was performed using four different chip types (CM10, a weak cation exchange surface, Q10 a strong anion exchange surface, IMAC 30, a metal affinity binding surface, and H50 which binds hydrophobic proteins). Analysis was performed on a PBS II ProteinChip™ reader (CIPHERGEN®). The read conditions were: detector sensitivity = 7, laser shots *per spot* = 130, detector voltage = 2850. Calibration was performed using all-in-one-peptide and all-in-one-protein standards (CIPHERGEN). Two reference samples were used *per chip* for spectral quality control [11]. Samples were batch analyzed using a filter setting of 0.2 times the expected peak and a peak signal 4 times the noise. Baseline subtraction was performed on all spectra and all data were TIC normalized.

Protein purification and identification were performed as follows. One milliliter of urine was dialyzed in a 2 L bath of deionized water for 48 h at 4°C. Sample was concentrated in PEG 20 000 in a 3500Mr cutoff dialysis membrane (Spectropor). The concentrated protein mixture was then run on a 10–20% gradient Tricine gel (Invitrogen) at 120 V for 1 h. Staining was performed using CBB, the protein band identified and excised. Gel bands were destained and in-gel trypsinolysis performed. Briefly, gel bands were reduced by 10 mM DTT and alkylated by 50 mM iodoacetamide. Then gel bands were washed three times with 50:50 water/ACN. After dehydration by ACN, trypsin was added (Promega) and digestion performed at 37°C overnight. Peptides were extracted by 50:50:0.1 water/ACN/trifluoroacetic acid, concentrated, and subjected to subsequent LC-MS analysis. Capillary LC-MS/MS (μ LC-MS/MS) was used to obtain protein ID information. The experiments were performed on an 1100 series LC/MSD XCT plus IT mass spectrometer (Agilent). The mobile phases were 0.1% formic acid (solvent A) and 0.1% formic acid in 100% ACN (solvent B). An 8 μ L aliquot of peptide mixture was injected by an autosampler onto a $0.3 \times \text{mm}^2$ Zorbax 300SB-C18, 5 μ M particle size loading column at a flow rate of 10 μ L/min and subsequently separated on $0.075 \times \text{mm}^2$ Zorbax 300SB-C18, 3.5 μ M particle size analytical column at a flow rate of 0.3 μ L/min with a gradient of 15–40% solvent B for 50 min and 40–85% solvent B for 30 min. Mass spectra were acquired in automated data dependent MS/MS mode, consisting of a precursor MS scan followed by MS/MS scans on the four most intensive ions. Fragment amplitude was 1.15 V, scan rate was 13 000 *m/z/s*, capillary voltage is 3500 V, and skimmer voltage was 40 V.

The raw MS/MS spectra were used to identify peptides by database search using Spectrum Mill software (Agilent). Raw data were processed first to extract MS/MS spectra that can be assigned to at least two b or y ion series, and these “good” spectra were searched against the Swiss-Prot database. The important parameters for searching are: minimum matched peak

intensity of 50%, precursor mass tolerance of 2.0 Da and product mass tolerance of 0.6 Da. The number of maximum missed cleavages allowed was two and no peptide modification was considered. The threshold used for the identification is a Spectrum Mill score greater than 10 and a SPI % (the percentage of assigned spectrum intensity out of total spectrum intensity) greater than 70%. All matched MS/MS spectra were manually inspected to ensure that the majority of prominent peaks could be assigned to y/b ions, and sufficient number of positive residues to support multiple charged ions.

2.3 Classification algorithms and statistical analysis

A multiple tree-based classification algorithm, randomForest, permits good visualization of SELDI data, and was used as an initial tool [10, 12]. Briefly, this classifies samples using the intensities of a randomly selected, user defined, number of protein peaks (39 were used in this analysis) to create the branches of a tree with each training sample classifying the last branch. The “out of bag” test set (35%) is then passed down the tree with the last branch deciding its classification. Many trees are created (10 000 in this analysis) with each tree voting for a sample to belong to a particular diagnostic group. This was combined with a different form of classification algorithm, Adaboost, a technique that has also been previously shown to be effective for the classification of SELDI analysis, the purpose being to ensure thorough examination of this complex, high dimensional data [13]. This is a method for combining weak classifiers to create a summary and stronger classifier. The basic principle is that after initial selection of the variable most likely to correctly predict the class of a sample, the samples are reweighted with increase in weight applied to the misclassified samples, the next best classifier variable is then selected. This process is then repeated with summation of the classifiers to create a robust and accurate classifier. An out of bag test set of 35% was used for the Adaboost classification. This classification algorithm required similar group sizes to work well. Ten patients in the Banff grade 0 group had a small degree of interstitial fibrosis and tubular atrophy (up to 5% percent is permissible under the classification). Therefore, 13 of the BANFF group 0 patients were used in the classification algorithm with equal sized random subsamples being selected from the larger patient groups. It is important to note that both of these classification methodologies are distribution free. Variable distribution was assessed by kurtosis, skewness, and the Kolmogorov–Smirnov test. Between groups analysis used either the Student's *t*-test or one-way analysis of variance (ANOVA) for parametric distributions or Mann–Whitney-*U* test for nonparametric distributions. Correlations were assessed using Kendall's Tau (τ) for nonparametric distributions. Differences were considered significant with $p < 0.05$. Analysis was performed using the statistical package for the Social Sciences; randomForest; and Adaboost are available from the R Network for Statistical Computing (www.r-project.org, as packages “RandomForest” and “gbm”).

2.4 ELISA assay for estimation of urinary concentration of endorepellin

High-throughput proteomic techniques such as SELDI are not quantitative. Therefore, a recognized quantitative technique, ELISA, was used to confirm differences in urinary endorepellin between patients groups. An ELISA plate was coated with 1 $\mu\text{g/mL}$ of monoclonal antihuman endorepellin antibody (R&D systems), diluted in PBS and was incubated overnight at 4°C. Blocking was then performed with 1% BSA for 1 h. Urine

samples were diluted 20 times with dilution buffer (0.7% BSA in PBS) and 100 uL of each sample and recombinant endorepellin controls were bound to the plate in duplicate for 1 h at room temperature.

Wells were washed, and 100 uL of 0.8 ug/mL of antihuman goat polyclonal antibody to endorepellin (R&D systems) were incubated for 1 h. Washing was repeated and wells incubated with donkey antigoat HRP conjugated antibody for 40 min.

Color development was with Sure-Blue/TMB (KPL) and reaction was stopped with TMB Stop Solution. Absorbance was read at 450 nm. The urinary endorepellin concentration was calculated from a standard curve. Urinary creatinine was measured with a creatinine assay kit from Cayman. Recombinant Endorepellin was gift from Dr. Iozzo.

2.5 Morphologic and immunofluorescence studies

Seventy-five patients underwent renal transplant biopsy due to a decline in renal function and/or emergence of proteinuria (>1 g/day). All biopsies were evaluated prospectively according to the Banff classification by an experienced renal transplant pathologist (PNC).

Perlecan and endorepellin staining were performed on native renal biopsies from “control” patients ($n = 15$), which included patients with hypertensive nephropathy ($n = 2$), FSGS ($n = 3$), diabetic nephropathy ($n = 2$), membranous nephropathy ($n = 2$) and others ($n = 6$), patients with acute rejection ($n = 18$), and patients with CAN of severity Banff grade II or III ($n = 16$). Frozen sections from human renal biopsies were washed with PBS and blocked with 20% goat serum (Sigma) for 1 h at room temperature. After washing in PBS the sections were incubated with a polyclonal rabbit antiperlecan domain V (endorepellin) antibody at a dilution of 1/250 and monoclonal mouse antiperlecan domain III antibody (Zymed), dilution 1/50, at 4°C overnight [14]. Sections were washed three times with PBS before incubation with AlexaFluor™ 594 goat antirabbit antibody (Molecular Probes) and FITC goat antimouse antibody (Jackson ImmunoResearch) for 1 h. Sections were then rinsed with PBS and coverslipped. Quantitative image analysis was performed by digital imaging of the entire section using a TE2000-U microscope (Nikon) equipped with a CCD digital camera (Diagnostic Instruments) and appropriate dichroic mirrors to detect red and green fluorescence and quantifying the intensity of red, green, and yellow (overlap of green and red) fluorescence using the pixel counting function in Photoshop™ (Adobe). Image collection and quantification were performed by an investigator to whom the tissue diagnosis was unknown to exclude observer bias.

3 Results

3.1 Demographics and clinical parameters

Seventy-five patients and 20 healthy volunteers are included in this analysis. All had proteomic analysis of urine and the patient group had detailed histological analysis of biopsy material. The patient demographics are outlined in Table 2. There was an excess of males (male/female ratio 2.6:1) probably due to the increased risk of CAN in male patients [15].

Serum creatinine increased with rising Banff score as expected. The correlation (Kendall's Tau-b (τ)) with the Banff score was strongest for the creatinine level measured at last follow-up, a finding congruent with the previously described correlation between graft outcome and histological findings [16, 17]. Correlations were as follows; creatinine nadir after transplantation, $\tau = 0.25$ ($p < 0.01$), creatinine at biopsy, $\tau = 0.24$ ($p < 0.01$), and creatinine at last follow-up, $\tau = 0.35$, ($p < 0.001$). Serum creatinine was higher at the time of biopsy 3.1 ± 1.7 (biopsies were performed for clinical reasons) and at last follow-up 3.3 ± 2.2 *versus* nadir after transplantation, 1.9 ± 1.0 for all patients. Acute and chronic Banff scores for the patient population are detailed in Table 3. The histological diagnosis for graft dysfunction in the Banff CAN = 0 group included, drug toxicity ($n = 8$), acute tubular necrosis ($n = 6$), acute rejection ($n = 5$), thrombotic microangiopathy ($n = 2$) focal infarction ($n = 1$), and diabetic glomerulosclerosis ($n = 1$). Blood pressure did not differ significantly between the groups ($p = 0.78$) (KS = 0.69, ANOVA) although there was a trend toward higher values in the patients with CAN. Mean patient time from transplantation to biopsy averaged 612 ± 910 days while time from sampling to last follow-up was 203 ± 176 days. Collectively, these data are comparable to other reported series (18–20) and provide the background to the analyses performed.

3.2 Proteomic data visualization

A total of 1222 peaks were detected on the four chip surfaces. There were 381 peaks on the CM10 surface, 347 on H50, 313 on IMAC30, and 181 on Q10. Initially, the distribution of the patients was assessed by multidimensional scaling (MDS) plots of urinary proteomic data created by the randomForest proximity matrix (Fig. 1). The urine samples of healthy subjects form a tight cluster clearly distinct from the urine of transplant patients. There is good separation of those with CAN Banff grade 0 *versus* those with CAN Banff grades 1–3, suggesting there is sufficient structure within the data to identify protein peaks that distinguish the two patient groups.

3.3 Data interrogation using the classification algorithm Adaboost

This section outlines the classification accuracy of the urinary proteome when applied to transplant recipients with and without CAN. Analysis of the urinary proteome of those with normal graft histology (Banff = 0, $n = 13$) *versus* an equal sized sample of randomly selected patients from both the Banff grade 2 and Banff grade 3 subgroups of patients performed well and is outlined in Table 4. The corresponding bar charts depicting the most important protein peaks with the length of the bars representing the degree of significance are shown in Fig. 2.

3.4 Protein identification

Proteins of masses ~9.9, ~13.9, ~20.9, and ~43.4.0 kDa were selected based on the their utility in distinguishing patients with CAN Banff grade 0 *versus* CAN Banff grade II or III (Figs. 2a and b). The reasons for the selection of these masses included their appearance in both lists and the exclusion of molecular masses that are either difficult to identify using tryptic digest PMF (small molecular masses), or those likely to belong to proteins that are abundant in urine and are elevated nonspecifically in disease, *e.g.*, albumin ~66 kDa. Gel

views of the SELDI data are provided in Supporting Information. Protein identification was performed using MS/MS, as detailed in Section 2. The identified proteins were β 2-microglobulin, prealbumin, α -2 micro-globulin/bikunin precursor (AMBP), and a C-terminal perlecan fragment, as detailed in Table 5. As the first three candidates lacked specificity for, or had been previously identified as markers of CAN, we focused attention on perlecan, a ubiquitous basement membrane heparan sulfate [18]. This candidate seemed to be especially relevant due to the recent demonstration that the C-terminal domain V, endorepellin, has antiangiogenic properties [19]. Therefore, the abundance and distribution of perlecan and endorepellin were further investigated to determine if changes in tissue expression paralleled the proteomic findings.

3.5 ELISA detection of endorepellin

A quantitative analysis of urinary endorepellin was required to confirm this as a novel biomarker of CAN. An ELISA was developed and urinary endorepellin measured in samples from healthy volunteers ($n = 8$) and patients with either Banff grade 0 or 1 ($n = 19$) and patients with Banff grade 2 or 3 ($n = 17$). There was no statistical difference between mean urinary endorepellin/creatinine ratios for healthy volunteers (0.0026 ± 0.0017) and renal transplant recipients with CAN grades 0 and 1 (0.00545 ± 0.005) ($p = 0.283$, Mann–Whitney). However, there was significantly increased levels of urinary endorepellin in those with more advanced grades of CAN (Banff grades 2 and 3) (0.016 ± 0.014) *versus* those with normal histology or low grade CAN (Banff grades 0 and 1) (0.00545 ± 0.005) ($p < 0.01$, Mann–Whitney) (Fig. 3). Urinary endorepellin concentration did not correlate with serum creatinine ($p = 0.7$, Kendall's tau). The sensitivity, specificity, positive, and negative predictive values were respectively, 65, 79, 73, and 71%. The area under the curve for the receiver operator characteristic curve is 0.774 (Supporting Information).

3.6 Tissue expression

Tissue expression of perlecan and its C-terminal fragment were determined using antibodies raised against domain III (whole molecule) and the domain V (endorepellin). Representative images are shown in Fig. 4. The results showed a remarkable overlap between perlecan and endorepellin staining in control biopsies without CAN. In contrast there was a significant increase in the ratio of domain V staining to domain III staining (red to green) in patients with CAN compared with patients experiencing acute rejection, or disease control ($p < 0.01$, Kruskal–Wallis) (Fig. 4). The linear staining pattern observed in uncomplicated control biopsies was changed in CAN, as it was in acute rejection: staining has become punctated and usurated. In CAN biopsies, endorepellin distribution exceeded that of the perlecan parent molecule, suggesting that the former was diffusing from the basement membranes (Fig. 5).

4 Discussion

CAN is receiving increasing attention as acute rejection becomes a less prevalent clinical entity [20]. As diagnostic techniques improve, graft disease that would previously have been labeled as CAN is now being attributed to etiological based diagnoses such as antibody mediated rejection (C4d positive, anti-donor antibody positive) or viral nephropathy [20–

22]. Unfortunately, at the time of writing, no clearly defined cause can be attributed to the majority of cases of CAN. The current hypothesis is that it represents heterogeneous conditions, representing common histological endpoints of disease driven by a combination of genetic, immunological, and environmental stimuli (such as recurrent disease, drug toxicity, hypertensive, and hyperfiltration mechanisms) underlying each case to a variable extent [23, 24].

The work of both Nankivell *et al.* [5] and Seron and Moreso [7] suggest that CAN increases quickly during the first 3 months and thereafter more slowly. In a serial protocol biopsy study they demonstrated that CAN grade 1 is present in 94.2% by 12 months and increases to 100% by year 5, whereas CAN grade 2 or 3 is present in 24.7% by 12 months, 65.9% by year 5, and 89.8% by year 10 [5]. These data suggest the rate of progression varies widely. Schwarz *et al.* [3] in a recent large study noted a seven-fold increase in CAN in the first 6 months, but clinical risk factors for CAN had low statistical significance and a low risk factor score for CAN. Thus, one may consider that the histological appearance of CAN is neither linear nor uniform and that although histological changes predict functional decline, the degree of correlation is variable, particularly overtime.

Nankivell *et al.* [25] demonstrated that persistent proteinuria at 3 months was significantly associated with subsequent decline in renal function with an odds ratio of ~16 for the development of CAN during follow-up. Legendre *et al.* provided further confirmation of the importance of proteinuria in a study of patients who had neither acute rejection nor CAN and were biopsied at 3 months and 2 years. CAN was present in 25% at 3 months and at 50% at 2 years with proteinuria of 130 ± 6 mg/day in the group that progressed compared with 50 ± 3 mg/day in those that remained stable [4].

Despite the established relationship between proteinuria and CAN and the ability of both to predict failing graft function, a robust clinical biomarker for the Banff CAN grade that would avoid the need for biopsy has proved elusive. This is in part due to the adjacent nature of the Banff classes, as evidenced by the ability of the proteomic data to identify group classifiers when using subgroup analysis comparing the subset of Banff CAN grade 0 with no histological abnormality to Banff CAN II. Undoubtedly, other factors play a role and we believe the most important of these are (i) the possibility of a sampling error resulting in the misclassification of the samples used to build the classifier with a resultant impact on classification accuracy, (ii) the likelihood that the urinary proteome becomes deranged before the appearance of any histopathological abnormality, and (iii) the heterogeneity of the etiologic factors leading to tubulointerstitial scarring in the graft. Sampling error in renal transplant biopsies is inevitable with the error rate for CAN estimated at ~12% and is difficult to avoid [26]. Its effect is mitigated given the large study population, and the algorithms used here are among the most resistant to misclassification of samples used for training.

Prealbumin (transthyretin), AMBP, and b2-micro-globulin were identified as potential markers of CAN, however, these molecules have been previously identified as markers of tubular injury in other renal diseases reducing their validity as biomarkers of CAN or had previously been linked with progressive renal allograft dysfunction [27–36]. In contrast, the

identification of multiple C-terminal (domain V) fragments of the abundant basement membrane heparan sulfate proteoglycan, perlecan, represented a novel finding in CAN and confirmation of the increased urinary excretion was sought using ELISA. Perlecan has been identified by several investigators using MS approaches to map the normal urinary proteome, and is one of the most abundant urinary proteins [37–39]. Perlecan stabilizes the supramolecular structure of basement membranes partly *via* domain V binding to fibulin-2 and laminin–entactin–nidogen complex. This component also provides crosslinking to the fibrillar components and serves as a depot for growth factors like basic FGF. Perlecan is secreted by endothelial cells, is expressed in all basement membranes, and its deficiency in mice results in lethality on embryonic day 10.5, when vasculogenesis occurs [18, 40, 41]. In transgenic mice with heparan sulfate-deficient perlecan, smooth muscle cells showed enhanced proliferation *in vitro* and intimal proliferation *in vivo* [42]. Perlecan has been found to be atheroprotective, whereas atherogenesis is believed to be linked to the loss of perlecan [43].

The domain V, C-terminal fragment (amino acids 3787–4391) has potent antiangiogenic properties, providing it with a potential role in the development of a relatively avascular scar tissue as a part of the process leading to progressive renal failure (19). Notably, the terminal laminin-like globular domain (LG3) of ~ 25 kDa can be generated by a specific proteolytic cleavage performed by BMP-1/Toll-like family of metalloproteinases, ubiquitous enzymes that are involved in tissue remodeling and inflammation (44). Thus, it is possible that endorepellin and LG3 might be released by a concerted action of various proteases thereby allowing the diffusion of powerful bioactive proteins within the renal parenchyma and in the urine. Disruption of the basement membrane has been linked with progressive loss of renal allograft function (45). Also notable is that the endorepellin LG3 is a compact globular protein with a highly negative charge (*pI* ~ 5) (44). As such, LG 3 could bypass the anionic filter provided by the glomerular basement membrane. Endorepellin has been shown to disrupt actin stress fibers and focal adhesions *via* interaction with $\alpha 2\beta 1$ integrins, thus leading to capillary pruning (14). Appearance of this fragment in the urine of patients with CAN occurred at the expense of the parent molecule, perlecan, as demonstrated by the results of immunohistochemical analysis and ELISA. The loss of perlecan demonstrated in this study may contribute to the development of the vasculopathy central to CAN, and the ensuing imbalance of perlecan/endorepellin may result in increased capillary pruning with subsequent scar formation.

These studies, together with our findings of endorepellin by proteomic analysis suggest a possible mechanism by which LG3/endorepellin/perlecan may be involved in the progression of fibrosis and vasculopathy.

This study is limited by the cross-sectional nature of the data and that renal biopsy was performed in patients with clinical need. A further source of potential bias in this study is that not all urine specimens on which proteomic analysis was performed subsequently had endorepellin assayed by ELISA due to shortage of clinical material. A longitudinal study with protocol kidney biopsies would be the ideal approach but would be expensive and difficult to ensure patient compliance with the biopsy arm of the study. This type of study could also assess the long-term impact of covariates such as blood pressure control and cold

ischemia time. As proteomic techniques develop, they will yield more reproducible data with a greater dynamic range and become less expensive enabling this type of study in the future.

In conclusion, urinary proteomic data correlate well with the more severe CAN Banff grades. Urinary endorepellin, a potential biomarker of advanced CAN, may be indicative of perlecan cleavage and antiangiogenic actions of tissue endorepellin and is a suitable candidate for inclusion in prospective protocol biopsy studies seeking clinically useful biomarkers in the renal transplant population.

Acknowledgments

E. O' R. was in receipt of the Kevin]. and Gloria B. Kiely National Kidney Foundation Fellowship. These studies were supported in part by National Institutes of Health grants DK45462, DK54602, and DK52783 (to M. S. G.) CA39481 and CA47282 (to R. V. I.), and Westchester Artificial Kidney Foundation. We wish to acknowledge the work of Suseela Gogineni in preparation of the histology specimens.

Abbreviation

CAN chronic allograft nephropathy

5 References

- [1]. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, et al. Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N. Engl. J. Med.* 2000; 342:605–612. [PubMed: 10699159]
- [2]. Racusen LC, Solez K, Colvin RB, Bonsib SM, et al. The Banff 97 working classification of renal allograft pathology. *Kidney Int.* 1999; 55:713–723. [PubMed: 9987096]
- [3]. Schwarz A, Mengel M, Gwinner W, Radermacher J, et al. Risk factors for chronic allograft nephropathy after renal transplantation: A protocol biopsy study. *Kidney Int.* 2005; 67:341–348. [PubMed: 15610260]
- [4]. Legendre C, Thervet E, Skhiri H, Mamzer-Bruneel MF, et al. Histologic features of chronic allograft nephropathy revealed by protocol biopsies in kidney transplant recipients. *Transplantation.* 1998; 65:1506–1509. [PubMed: 9645814]
- [5]. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, et al. The natural history of chronic allograft nephropathy. *N. Engl. J. Med.* 2003; 349:2326–2333. [PubMed: 14668458]
- [6]. Hayry P, Paavonen T, Taskinen E, Tomlanovich E, et al. Protocol core needle biopsy and histological chronic allograft damage index as surrogate endpoint for long-term graft survival. *Transplant Proc.* 2004; 36:89–91. [PubMed: 15013309]
- [7]. Seron D, Moreso F. Protocol biopsies and risk factors associated with chronic allograft nephropathy. *Transplant Proc.* 2002; 34:331–332. [PubMed: 11959311]
- [8]. Schaub S, Wilkins J, Weiler T, Sangster K, et al. Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. *Kidney Int.* 2004; 65:323–332. [PubMed: 14675066]
- [9]. Tencer J, Thysell H, Andersson K, Grubb A. Stability of albumin, protein HC, immunoglobulin G, kappa- and lambda-chain immunoreactivity, orosomucoid and alpha 1-antitrypsin in urine stored at various conditions. *Scand. J. Clin. Lab. Invest.* 1994; 54:199–206. [PubMed: 7518610]
- [10]. O'Riordan E, Orlova TN, Mei JJ, Butt K, et al. Bioinformatic analysis of the urine proteome of acute allograft rejection. *J. Am. Soc. Nephrol.* 2004; 15:3240–3248. [PubMed: 15579528]
- [11]. Coombes KR, Fritsche HA Jr, Clarke C, Chen JN, et al. Quality control and peak finding for proteomics data collected from nipple aspirate fluid by surface-enhanced laser desorption and ionization. *Clin. Chem.* 2003; 49:1615–1623. [PubMed: 14500586]

- [12]. Izmirlan G. Application of the random forest classification algorithm to a SELDI-TOF proteomics study in the setting of a cancer prevention trial. *Ann. N. Y. Acad. Sci.* 2004; 1020:154–174. [PubMed: 15208191]
- [13]. Qu Y, Adam BL, Yasui Y, Ward MD, et al. Boosted decision tree analysis of surface-enhanced laser desorption/ ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. *Clin. Chem.* 2002; 48:1835–1843. [PubMed: 12324514]
- [14]. Bix G, Fu J, Gonzalez EM, Macro L, et al. Endorepellin causes endothelial cell disassembly of actin cytoskeleton and focal adhesions through $\alpha 2\beta 1$ integrin. *J. Cell Biol.* 2004; 166:97–109. [PubMed: 15240572]
- [15]. Antus B, Yao Y, Song E, Liu S, et al. Opposite effects of testosterone and estrogens on chronic allograft nephropathy. *Transpl. Int.* 2002; 15:494–501. [PubMed: 12389082]
- [16]. Freese P, Svalander CT, Molne J, Norden G, et al. Chronic allograft nephropathy-biopsy findings and outcome. *Nephrol. Dial. Transplant.* 2001; 16:2401–2406. [PubMed: 11733633]
- [17]. Isoniemi H, Taskinen E, Hayry P. Histological chronic allograft damage index accurately predicts chronic renal allograft rejection. *Transplantation.* 1994; 58:1195–1198. [PubMed: 7992362]
- [18]. Iozzo RV. Matrix proteoglycans: From molecular design to cellular function. *Annu. Rev. Biochem.* 1998; 67:609–652. [PubMed: 9759499]
- [19]. Mongiat M, Sweeney SM, San Antonio JD, Fu J, et al. Endorepellin, a novel inhibitor of angiogenesis derived from the C terminus of perlecan. *J. Biol. Chem.* 2003; 278:4238–4249. [PubMed: 12435733]
- [20]. Cornell LD, Colvin RB. Chronic allograft nephropathy. *Curr. Opin. Nephrol. Hypertens.* 2005; 14:229–234. [PubMed: 15821415]
- [21]. Mauiyyedi S, Pelle PD, Saidman S, Collins AB, et al. Chronic humoral rejection: Identification of antibody-mediated chronic renal allograft rejection by C4d deposits in peritubular capillaries. *J. Am. Soc. Nephrol.* 2001; 12:574–582. [PubMed: 11181806]
- [22]. Randhawa PS, Finkelstein S, Scantlebury V, Shapiro R, et al. Human polyoma virus-associated interstitial nephritis in the allograft kidney. *Transplantation.* 1999; 67:103–109. [PubMed: 9921805]
- [23]. Hueso M, Alia P, Moreso F, Beltran-Sastre V, et al. Angiotensin converting enzyme genotype and chronic allograft nephropathy in protocol biopsies. *J. Am. Soc. Nephrol.* 2004; 15:2229–2236. [PubMed: 15284309]
- [24]. Dimeny E, Tufveson G, Lithell H, Larsson E, et al. The influence of pretransplant lipoprotein abnormalities on the early results of renal transplantation. *Eur. J. Clin. Invest.* 1993; 23:572–579. [PubMed: 8243529]
- [25]. Nankivell BJ, Fenton-Lee CA, Kuypers DR, Cheung E, et al. Effect of histological damage on long-term kidney transplant outcome. *Transplantation.* 2001; 71:515–523. [PubMed: 11258430]
- [26]. Seron D, Moreso F, Fulladosa X, Hueso M, et al. Reliability of chronic allograft nephropathy diagnosis in sequential protocol biopsies. *Kidney Int.* 2002; 61:727–733. [PubMed: 11849416]
- [27]. Sousa MM, Norden AGW, Jacobsen C, Willnow TE, et al. Evidence for the role of Megalin in renal uptake of transthyretin. *J. Biol. Chem.* 2000; 275:38176–38181. [PubMed: 10982792]
- [28]. Tormey WP, O'Brien PA. Clinical associations of an increased transthyretin band in routine serum and urine protein electrophoresis. *Ann. Clin. Biochem.* 1993; 30:550–554. [PubMed: 8304723]
- [29]. Saraiva MJ, Costa PP, Goodman DS. Biochemical marker in familial amyloidotic polyneuropathy, Portuguese type. Family studies on the transthyretin (prealbumin)-methionine-30 variant. *J. Clin. Invest.* 1985; 76:2171–2177. [PubMed: 3908483]
- [30]. Sousa MM, Norden AG, Jacobsen C, Willnow TE, et al. Evidence for the role of megalin in renal uptake of transthyretin. *J. Biol. Chem.* 2000; 275:38176–38181. [PubMed: 10982792]
- [31]. Xu S, Venge P. Lipocalins as biochemical markers of disease. *Biochim. Biophys. Acta.* 2000; 1482:298–307. [PubMed: 11058770]
- [32]. Steinhoff J, Feddersen A, Wood WG, Hoyer J, et al. Glomerular proteinuria as an early sign of renal-transplant rejection. *Clin. Nephrol.* 1991; 35:255–262. [PubMed: 1873939]

- [33]. Schaub S, Wilkins JA, Antonovici M, Krokhn O, et al. Proteomic-based identification of cleaved urinary beta2-microglobulin as a potential marker for acute tubular injury in renal allografts. *Am. J. Transplant.* 2005; 5:729–738. [PubMed: 15760396]
- [34]. Hampel DJ, Sansome C, Sha M, Brodsky S, et al. Toward proteomics in uroscopy: Urinary protein profiles after radiocontrast medium administration. *J. Am. Soc. Nephrol.* 2001; 12:1026–1035. [PubMed: 11316862]
- [35]. Câmara NO, Silva MS, Nishida S, Pereira AB, et al. Proximal tubular dysfunction is associated with chronic allograft nephropathy and decreased long-term renal-graft survival. *Transplantation.* 2004; 78:269–275. [PubMed: 15280689]
- [36]. Teppo AM, Honkanen E, Finne P, Törnroth T, et al. Increased urinary excretion of alpha-1 microglobulin, at 6 months after transplantation is associated with urinary excretion of transforming growth factor beta-1 and indicates poor long term renal outcome. *Transplantation.* 2004; 78:719–724. [PubMed: 15371675]
- [37]. Spahr CS, Davis MT, McGinley MD, Robinson JH, et al. Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. *Proteomics.* 2001; 1:93–107. [PubMed: 11680902]
- [38]. Pieper R, Gatlin CL, McGrath AM, Makusky AJ, et al. Characterization of the human urinary proteome: A method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. *Proteomics.* 2004; 4:1159–1174. [PubMed: 15048996]
- [39]. Smith G, Barratt D, Rowlinson R, Nickson J, et al. Development of a high-throughput method for preparing human urine for two-dimensional electrophoresis. *Proteomics.* 2005; 5:2315–2318. [PubMed: 15887186]
- [40]. Arikawa-Hirasawa E, Watanabe H, Takami H, Hassell JR, et al. Perlecan is essential for cartilage and cephalic development. *Nat. Genet.* 1999; 23:354–358. [PubMed: 10545953]
- [41]. Costell M, Carmona R, Gustafsson E, Gonzalez-Iriarte M, et al. Hyperplastic conotruncal endocardial cushions and transposition of great arteries in perlecan-null mice. *Circ. Res.* 2002; 91:158–164. [PubMed: 12142349]
- [42]. Tran PK, Tran-Lundmark K, Soininen R, Tryggvason K, et al. Increased intimal hyperplasia and smooth muscle cell proliferation in transgenic mice with heparan sulfate-deficient perlecan. *Circ. Res.* 2004; 94:550–558. [PubMed: 14739157]
- [43]. Pillarisetti S. Lipoprotein modulation of subendothelial heparan sulfate proteoglycans (perlecan) and atherogenicity. *Trends Cardiovasc. Med.* 2000; 10:60–65. [PubMed: 11150731]
- [44]. Gonzalez EM, Reed CC, Bix G, Fu J, et al. BMP-1/Tolloid-like metalloproteases process endorepellin, the angiostatic C-terminal fragment of perlecan. *J. Biol. Chem.* 2005; 280:7080–7087. [PubMed: 15591058]
- [45]. Bonsib SM, Abul-Ezez SR, Ahmad I, Young SM, et al. Acute rejection-associated tubular basement membrane defects and chronic allograft nephropathy. *Kidney Int.* 2000; 58:2206–2214. [PubMed: 11044243]

Figure 1. Multidimensional scaling plot of distribution of urine samples from healthy volunteers ($\diamond$), patients with a Banff chronic score = 0 ($\triangle$), and patients with a Banff chronic score = 1 ($\blacklozenge$). Note how the healthy volunteers form a relatively tight cluster while the transplant recipients also form distinct groupings dependent on the presence of CAN. (Scales are arbitrary).

Figure 2. Proteins identified by mass/charge ratio using a boosting algorithm distinguishing between (a) patients with CAN Banff grade 0 *versus* CAN Banff grade 2 and (b) patients with CAN Banff grade 0 *versus* CAN Banff grade 3.

Figure 3.

Boxplot representation of significant difference in urinary endorepellin/creatinine ratios between patients with normal histological appearance and early grades of CAN (Banff) and those with more severe grades (Banff 2 and 3). ($p < 0.01$, Mann–Whitney).

Figure 4.

Comparison of endorepellin (red) and perlecan domain III (green) fluorescence for control renal biopsies (see text), patients with CAN and patients with acute rejection (AcR). Intensity and overlap of green and red fluorescence is shown in typical line-scans obtained using Metamorph software. Note the broad spread of endorepellin labeling, exceeding that of perlecan, in CAN. Abbreviations GBM-glomerular basement membrane, TBM-tubular basement membrane. (Magnification 600×) White bar in upper left panel = 200 μm.

Figure 5.
Box plot representation of digitally measured fluorescence intensity in control kidney biopsies (see text), patients with CAN and patients with acute rejection.

Table 1

Conditions and binding buffers used for the different chip surfaces

Chip type	Maximum molecular mass (kDa)		Optimized range (kDa)		Laser intensity		Deflector mass (kDa)		Buffer used	
	Low	High	Low	High	Low	High	Low	High	Low	High
CM10	100	200	2–20	20–50	197	200	2	50	100 mM NaAc pH 4.0	
Q10	100	200	2–20	20–50	197	200	2	50	50 mM Tris HCl pH 9.0	
H50	100	200	2–20	20–50	192	200	2	50	30% ACN 1 mM HEPES	
IMAC30	100	200	2–20	20–50	192	200	2	50	0.2 M NaCl in PBS	

Table 2

Patient clinical data according to Banff Classification

Classification	Banff = 0 (n = 23)	Banff = 1 (n = 22)	Banff = 2 (n = 16)	Banff = 3 (n = 14)
Serum Creatinine (mg/dL)				
Nadir post transplant	1.6 ± 1.1	1.8 ± 1.0	1.8 ± 0.7	2.3 ± 1.0
Time of sample	2.7 ± 1.6	2.8 ± 1.5	3.3 ± 2.0	3.8 ± 1.6
Last follow-up	2.3 ± 1.5	2.8 ± 1.4	3.8 ± 2.3	4.8 ± 3.0
Drugs				
Tacrolimus	18	18	13	10
Cya	4	4	3	3
Rapamycin	12	20	14	10
MMF	9	6	2	3
Steroid	19	20	15	10
Other	1	0	0	0
Mean arterial blood pressure	93.6 ± 12.6	102.1 ± 12.3	98.8 ± 10.9	98.7 ± 12.5
Time from transplant to biopsy (days)	349 ± 610	559 ± 878	654 ± 952	10586 ± 1190

Cya, Cyclosporin A; MMF, Mycophenolate Mofetil.

Table 3

Acute and chronic Banff histopathology scores for interstitial (*i*), tubular (*t*), glomerular (*g*), and vascular (*v*) compartments

Classification	Banff = 0 (<i>n</i> = 23) <i>n</i>	Banff = 1 (<i>n</i> = 22) <i>n</i>	Banff = 2 (<i>n</i> = 16) <i>n</i>	Banff = 3 (<i>n</i> = 14) <i>n</i>	
Acute Banff Scores					
<i>i</i>	0	12	8	4	2
	1	7	10	6	7
	2	1	2	5	2
	3	3	2	1	3
<i>t</i>	0	14	13	5	4
	1	3	3	5	6
	2	1	5	5	4
	3	5	1	1	0
<i>g</i>	0	21	21	15	14
	1	2	1	0	0
	2	0	9	1	0
	3	0	9	0	0
<i>v</i>	0	20	22	14	13
	1	0	0	1	1
	2	3	0	1	0
	3	0	0	0	0
Chronic Banff Scores					
<i>ci</i>	0	23	0	0	0
	1	0	20	0	0
	2	0	2	15	1
	3	0	0	1	13
<i>ct</i>	0	23	1	0	0
	1	0	21	1	0
	2	0	0	15	2
	3	0	0	0	12
<i>cg</i>	0	22	21	18	10
	1	0	0	0	1
	2	1	0	0	1
	3	0	1	0	2
<i>cv</i>	0	9	3	4	3
	1	9	8	4	2
	2	3	6	7	4
	3	2	5	1	5

n represents number of patients with that score.

Table 4

Comparison of patients with no chronic histological change with those with a Banff CAN score 2

Adaboost algorithm ↓Actual Predicted→		CAN = 2	CAN = 0	Error rate (%)
CAN = 2		12	1	7.6
CAN = 0		2	12	14.2
Sens. = 85.7%		Spec. = 92.3%	NPV = 85.7%	PPV = 92.3

Table 5

Characterization of proteins identified as important in distinguishing patients with Banff grade 2 or 3 from patients with normal allograft histology

Protein name	Measured mass (kDa)	Actual mass (Da)	Mean spectral intensity	% Amino acid coverage	Number of distinct peptides
β2-Microglobulin	13.9	13 714.6	4.29e8	11	4
Prealbumin	13.9	15 887.0	1.72e8	44	4
Perlecan	20.9	46 8828	4.59e8	2	8
α1-Microglobulin	43.4	38 999.7	8.76e8	21	6