The relationship between albuminuria, MCP-1/CCL2, and interstitial macrophages in chronic kidney disease

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Glomerular-derived proteins may activate tubular cells to express the macrophage-directed chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2). Macrophages at interstitial sites have a central role in directing renal scarring. We have prospectively assessed the relationship between albuminuria, urinary MCP-1/CCL2, interstitial macrophage infiltration, *in situ* damage, and clinical outcomes in a large group of patients with chronic kidney disease. We studied 215 patients and quantified albumin–creatinine ratio (ACR), urinary MCP-1/CCL2, interstitial macrophage numbers, and *in situ* damage. ACR correlated with urinary MCP-1/CCL2 (correlation 0.499; $P<0.001$), interstitial macrophage numbers (correlation 0.481; $P<0.001$), and index of chronic damage (correlation 0.363; $P<0.001$). Macrophage numbers closely correlated with *in situ* damage (correlation 0.755; $P<0.001$). By multivariate analysis ACR, urinary MCP-1/CCL2, and interstitial macrophage numbers were interdependent. By Kaplan–Meier survival analysis albuminuria, urinary MCP-1/CCL2, interstitial macrophages, and chronic damage predict the outcome. ACR, macrophage numbers, chronic damage, and creatinine independently predicted renal survival. The association of ACR with other variables was strongest in patients with less advanced disease states. There is a close association between albuminuria, urinary MCP-1/CCL2, and interstitial macrophage infiltration with *in situ* damage and clinical outcomes. These findings support the hypothesis that albuminuria triggers tubular MCP-1/CCL2 expression with subsequent macrophage infiltration. These processes may represent the dominant pathway for the progression of renal injury before the establishment of advanced renal scarring

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In chronic kidney disease, a progressive decline in renal function is associated with worsening tubulointerstitial disease that is characterized by tubular atrophy and interstitial scar formation.[1,2] There is also an interstitial inflammatory infiltrate in which the macrophage is prominent.[3] Animal models indicate that this cell type has a central role in the initiation and progression of renal injury.[4]

The chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) is expressed at sites of injury and inflammation to direct macrophage recruitment; it ligates CC chemokine receptor 2 (CCR2) to promote macrophage adhesion and chemotaxis to disease sites. Increased tubular expression of MCP-1/CCL2 is present in human progressive renal disease,[5,6] and interstitial inflammatory infiltrates express CCR2.[7,8] In animal models, blocking MCP-1/CCL2 or CCR2 is associated with reduced interstitial macrophage infiltration and tubulointerstitial damage.[9–12]

In acute proliferative diseases such as lupus nephritis, mesangiocapillary and crescentic glomerulonephritis, increased urinary MCP-1/CCL2 represents increased glomerular expression of the chemokine.[5] In chronic non-proliferative disease, however, increased urinary levels probably reflect tubular MCP-1/CCL2 expression,[5,13] which itself correlates with urinary albumin levels[14] and interstitial macrophage infiltration.[6,13,15] Recent *in vitro* evidence has demonstrated that tubular epithelial cells express MCP-1/CCL2 in response to proteins including albumin.[16,17] These data support the hypothesis that albuminuria may accelerate disease progression through induction of tubular chemokine expression. However, a direct link between these factors, macrophage infiltration, and *in situ* damage in human disease has not been demonstrated to date.

To address this, we have performed a large prospective study of patients undergoing renal biopsy for investigation of chronic kidney disease. We have used established and validated methodologies to assess the association between albuminuria, MCP-1/CCL2 expression, macrophage infiltration, and renal scarring. These data provide evidence in a

heterogeneous group of diseases of an association between albuminuria and progressive renal scarring secondary to induction of MCP-1/CCL2 and subsequent macrophage recruitment.

RESULTS

Patients

Two hundred and fifteen patients (130 males and 85 females) with chronic renal disease were studied. Two hundred and four patients had urine taken for albumin–creatinine ratio (ACR) measurement prior to their biopsy. Table 1 shows the histological diagnoses of the patients included in this study and the results of the parameters analysed.

Albuminuria correlates with urinary MCP-1/CCL2 and interstitial macrophage numbers

By univariate analysis ACR correlates with urinary MCP-1/CCL2 (correlation 0.499; $P<0.001$), interstitial macrophage numbers (correlation 0.481; $P<0.001$), index of chronic damage (correlation 0.363; $P<0.001$), and serum creatinine (correlation −0.211; $P<0.01$) (Table 2; Figure 1). Categorizing urinary ACR into ranges of albuminuria recognized to characterize very low (ACR < 3 mg/mmol), low (ACR 3–99 mg/mmol), intermediate (ACR 100–299 mg/mmol), and high (ACR > 300 mg/mmol) risk of disease progression showed a direct relationship between an incremental increase in ACR, urinary MCP-1/CCL2 levels, and macrophage numbers, with the highest levels of chemokine and infiltrating cells seen in patients with nephrotic range albuminuria (ACR > 300 mg/mmol) (Figure 2).

Interstitial macrophage numbers correlate with urinary MCP-1/CCL2

Interstitial macrophage numbers were successfully quantified in 174 (81%) patients. Extensive background staining prevented analysis of tissue from 31 patients and tissue from 10 patients did not contain cortex. There was no significant bias in macrophage quantification by the same observer (mean 0.99; 95% confidence interval (CI) 0.98–1.07) or between two observers (mean 0.95; 95% CI 0.96–1.11). The limits of agreement of measurements by the same observer were 0.81 (95% CI 0.75–0.88) and 1.21 (95% CI 1.11–1.31), and between two observers were 0.70 (95% CI 0.62–0.79) and 1.29 (95% CI 1.14–1.46).

Macrophage numbers assessed by image analysis (mean % area, 1.76 ± 1.14) correlated with urinary MCP-1/CCL2 levels (correlation 0.451; $P<0.001$) (Table 2; Figure 3).

Interstitial macrophage numbers correlate with chronic damage and creatinine

Interstitial macrophage numbers correlated with the stage of the disease process as assessed by index of chronic damage

Table 1 | Study population: clinical, pathological, and experimental characteristics

Histological diagnosis (*n*)	Age (years)	Creatinine (μmol/l)	Index of chronic damage (%)	ACR (mg/mmol)	Urinary MCP-1/CCL2 (pg/mg)	Interstitial macrophages (%)
Thin GBM disease (*n*=44)	42 ± 14 (*n*=44)	86 ± 12 (44)	3 ± 4 (44)	38 ± 7 (38)	114 ± 86 (38)	0.94 ± 0.11 (38)
IgA nephropathy (43)	44 ± 15 (*n*=43)	150 ± 147 (43)	23 ± 26 (43)	97 ± 183 (42)	204 ± 212 (40)	1.44 ± 0.83 (33)
Ischaemic/hypertensive nephropathy (42)	58 ± 13 (*n*=42)	236 ± 114 (42)	42 ± 48 (42)	54 ± 95 (41)	219 ± 185 (38)	2.22 ± 1.06 (35)
Focal segmental glomerulosclerosis (30)	50 ± 15 (*n*=30)	140 ± 94 (30)	20 ± 16 (30)	352 ± 395 (29)	390 ± 327 (24)	1.81 ± 0.83 (22)
Membranous nephropathy (20)	47 ± 16 (*n*=20)	112 ± 54 (20)	19 ± 24 (20)	373 ± 380 (19)	264 ± 212 (18)	2.32 ± 1.5 (14)
Diabetic nephropathy (18)	57 ± 16 (*n*=18)	153 ± 81 (18)	30 ± 24 (18)	274 ± 323 (17)	354 ± 357 (17)	1.86 ± 0.96 (17)
Minimal change nephropathy (5)	51 ± 23 (*n*=5)	108 ± 54 (5)	10 ± 12 (5)	889 ± 441 (5)	169 ± 10 (2)	1.42 ± 0.25 (4)
Primary amyloidosis (4)	79 ± 7 (*n*=4)	304 ± 301 (4)	43 ± 37 (4)	388 ± 408 (4)	720 ± 24 (2)	5.15 ± 2.03 (3)
Light-chain nephropathy (4)	68 ± 14 (*n*=4)	132 ± 41 (4)	30 ± 12 (4)	156 ± 136 (4)	447 ± 228 (4)	2.20 ± 0.55 (3)
Other (5)	56 ± 21 (*n*=5)	203 ± 122 (5)	27 ± 27 (5)	110 ± 156 (5)	298 ± 208 (4)	2.59 ± 2.03 (5)
All (215)	50 ± 17 (*n*=215)	153 ± 116 (215)	22 ± 25 (215)	175 ± 300 (204)	245 ± 243 (187)	1.76 ± 1.14 (174)

ACR=albumin–creatinine ratio; MCP-1/CCL2=chemokine monocyte chemoattractant protein-1; GBM=glomerular basement membrane; IgA=immunoglobulin A.

Table 2 | Univariate analysis of correlations between ACR, urinary MCP-1/CCL2, interstitial macrophage numbers, index of chronic damage, and serum creatinine

	Urinary ACR Correlation; *P*-value (*n*)	Urinary MCP-1/CCL2 Correlation; *P*-value (*n*)	Interstitial macrophages Correlation; *P*-value (*n*)	Index of chronic damage Correlation; *P*-value (*n*)	Serum creatinine (*n*)
Urinary ACR	1.000 (204)				
Urinary MCP-1/CCL2	0.499; <0.001 (183)	1.000 (187)			
Interstitial macrophages	0.481; <0.001 (165)	0.451; <0.001 (154)	1.000 (174)		
Index of chronic damage	0.363; <0.001 (204)	0.333; <0.001 (187)	0.755; <0.001 (174)	1.000 (215)	
Serum creatinine	−0.211; <0.01 (204)	−0.257; <0.001 (187)	−0.698; <0.001 (174)	−0.690; <0.001 (215)	1.000 (215)

ACR=albumin–creatinine ratio; MCP-1/CCL2=chemokine monocyte chemoattractant protein-1.

(correlation 0.755; $P<0.001$) and serum creatinine (correlation −0.690; $P<0.001$) (Table 2; Figure 4).

Multivariate analyses of correlation

Multivariate linear regression analysis of correlations between the variables found that urinary ACR, urinary MCP-1/CCL2, and interstitial macrophage numbers were interdependent. Serum creatinine and index of chronic renal damage were independent predictors of each other and of interstitial macrophage numbers, but not of the other variables analysed (Table 3).

Renal outcome

Data on renal outcome were available on 165 (77%) of the 215 patients at a mean of 832 days (±431) following their renal biopsy. Twenty-seven patients reached the renal end point (nine patients doubled their serum creatinine; 18 patients reached end-stage renal failure) after a mean period of 439 days (range 1–1283 days; one patient with end-stage focal segmental sclerosing glomerular disease commenced dialysis at 1 day). Those who progressed were significantly older (60±16 years) than those who did not (49±16 years) ($P=0.002$). Kaplan–Meier survival analyses found that high levels of albuminuria, particularly ACR > 300 mg/mmol, were predictive of a poorer renal outcome. Similarly, those with high levels of urinary MCP-1/CCL2, interstitial macrophages,

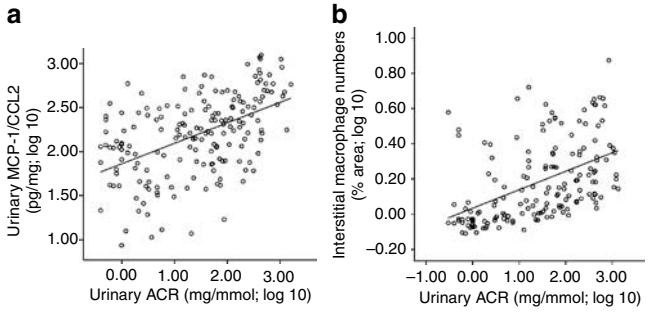

Figure 1 | Plots of correlations between ACR and urinary MCP-1/CCL2, and between ACR and interstitial macrophage numbers (correlation; *P*-value). (**a**) Urinary ACR and urinary MCP-1/CCL2 (0.499; $P<0.001$). (**b**) Urinary ACR and interstitial macrophage numbers (0.481; $P<0.001$).

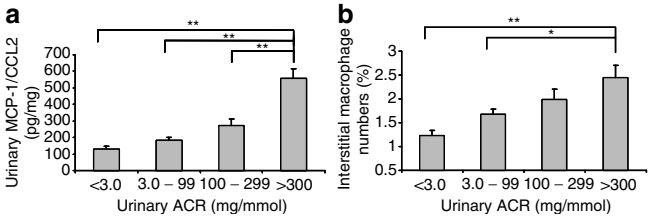

Figure 2 | Albuminuria range and associated urinary MCP-1/CCL2 and interstitial macrophage numbers. (**a**) Urinary ACR and urinary MCP-1/CCL2 (ANOVA, $P<0.001$). (**b**) Urinary ACR and interstitial macrophage numbers (ANOVA, $P<0.001$) (** and *, Bonferroni *post-hoc* test significance $P<0.001$ and 0.01, respectively).

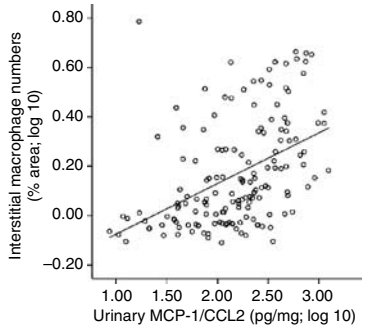

Figure 3 | Plot of correlation between urinary MCP-1/CCL2 and interstitial macrophage numbers (0.451; $P<0.001$).

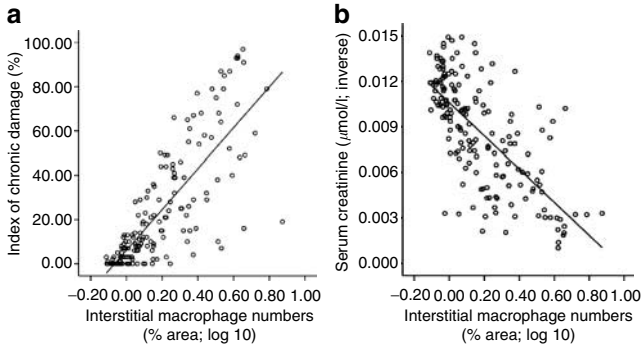

Figure 4 | Plots of correlation between interstitial macrophage numbers and chronic damage and between interstitial macrophage numbers and serum creatinine. (**a**) Interstitial macrophage numbers and index of chronic damage (0.755; $P<0.001$). (**b**) Interstitial macrophage numbers and serum creatinine (−0.698; $P<0.001$).

Table 3 | Multivariate linear regression analysis of correlations between ACR, urinary MCP-1/CCL2, interstitial macrophage numbers, index of chronic damage, and serum creatinine

	Dependent variable				
	Urinary ACR Correlation; *P*-value	Urinary MCP-1/CCL2 Correlation; *P*-value	Interstitial macrophages Correlation; *P*-value	Index of chronic damage Correlation; *P*-value	Serum creatinine Correlation; *P*-value
Urinary ACR	NA	0.343; <0.001	0.162; <0.01	NS	NS
Urinary MCP-1/CCL2	0.336; <0.001	NA	0.175; <0.01	NS	NS
Interstitial macrophages	0.352; <0.001	0.329; <0.001	NA	0.520; <0.001	−0.339; <0.001
Index of chronic damage	NS	NS	−0.263; <0.001	NA	−0.457; <0.001
Serum creatinine	NS	NS	0.445; <0.001	−0.359; <0.001	NA

ACR=albumin-creatinine ratio; MCP-1/CCL2=chemokine monocyte chemoattractant protein-1; NA=not applicable; NS=not statistically significant.

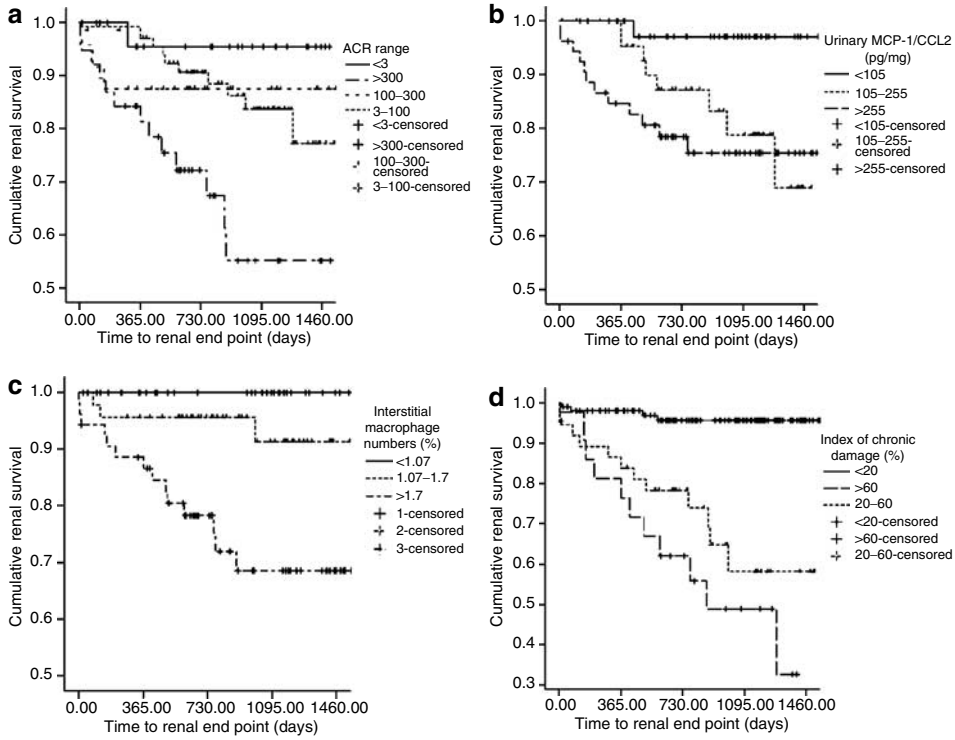

Figure 5 | Kaplan–Meier analysis of variables on renal outcome. (**a**) Urinary ACR (log rank statistic 14.01; $P<0.005$). (**b**) Urinary MCP-1/CCL2 (log rank statistic 7.26; $P<0.026$). (**c**) Interstitial macrophage numbers (log rank statistic 14.93; $P<0.001$). (**d**) Index of chronic damage (log rank statistic 37.16; $P<0.001$).

Table 4 | Univariate and multivariate analysis of the impact of studied variables on renal survival

		Interstitial macrophages Correlation; *P*-value	Urinary MCP-1/CCL2 Correlation; *P*-value	Urinary ACR Correlation; *P*-value	Serum creatinine Correlation; *P*-value	Index of chronic damage Correlation; *P*-value	Age Correlation; *P*-value	Sex
Univariate	Exp(B); *P*-value	107.2; <0.001	4.247; 0.012	2.149; <0.001	0.0002; <0.001	26.8; <0.001	1.039; 0.003	NS
Multivariate		6.02; 0.02	NS	9.154; 0.002	0.0001; 0.002	477; 0.003	NS	NS

MCP-1/CCL2=chemokine monocyte chemoattractant protein-1; NS=not statistically significant.

and chronic damage had the poorest renal outcome (Figure 5). Univariate Cox regression analysis found that ACR, urinary MCP-1/CCL2, interstitial macrophage numbers, index of chronic damage, serum creatinine, and patient age had a significant impact on renal outcome (Table 4). Multivariate analysis found urinary ACR, interstitial macrophage numbers, index of chronic damage, and serum creatinine to be independent variables that significantly predicted renal survival (Table 4). Urinary MCP-1/CCL2 and patients' age and sex were not found to be significant.

Subset analyses

Histological diagnosis. To test further the unifying role of albuminuria and MCP-1/CCL2 in interstitial inflammation and damage, we analysed data from patients with immunoglobulin A (IgA) nephropathy, focal segmental glomerulosclerosis (FSGS), and ischaemic/hypertensive nephropathy independently (Table 5). In each group, univariate analysis demonstrated the correlation between urinary albumin and MCP-1/CCL2 levels and urinary MCP-1/CCL2 and interstitial macrophage numbers. In IgA nephropathy and focal segmental glomerulosclerosis urinary ACR correlated with interstitial macrophage numbers, but this association was not present in ischaemic/hypertensive nephropathy. Multivariate analyses of correlation and renal outcome analyses were not performed as the patient numbers in individual disease groups were relatively small.

Chronic damage. To assess whether the stage of disease progression affected the relationships between ACR, urinary MCP-1/CCL2, and interstitial macrophage numbers, we analysed patients with an index of chronic damage of less than 20% and compared them with those with an index of 20% or greater. Univariate analysis showed that ACR, urinary MCP-1/CCL2, and interstitial macrophage numbers were highly significantly correlated in those patients (128 patients) with an index of chronic damage of <20% (all with

Table 5 | Univariate analysis of correlation between ACR, urinary MCP-1/CCL2, interstitial macrophage numbers, index of chronic damage, and serum creatinine by histological diagnosis: (a) IgA nephropathy; (b) focal segmental glomerulosclerosis and (c) ischaemic/hypertensive nephropathy

	Urinary ACR Correlation; *P*-value (*n*)	Urinary MCP-1/CCL2 Correlation; *P*-value (*n*)	Interstitial macrophages Correlation; *P*-value (*n*)	Index of chronic damage Correlation; *P*-value (*n*)	Serum creatinine Correlation; *P*-value (*n*)
(a) IgA nephropathy					
Urinary ACR	1.000 (42)				
Urinary MCP-1/CCL2	0.633; <0.001 (40)	1.000 (40)			
Interstitial macrophages	0.604; <0.001 (33)	0.521; <0.001 (32)	1.000 (33)		
Index of chronic damage	0.53; <0.001 (42)	0.514; 0.001 (40)	0.825; <0.001 (33)	1.000 (43)	
Serum creatinine	−0.398; <0.01 (42)	−0.438 <0.01 (40)	−0.817; <0.001 (33)	−0.713; <0.001 (43)	1.000 (43)
(b) Focal segmental glomerulosclerosis					
Urinary ACR	1.000 (29)				
Urinary MCP-1/CCL2	0.792; <0.001 (24)	1.000 (24)			
Interstitial macrophages	0.529; <0.05 (21)	0.586; <0.05 (18)	1.000 (22)		
Index of chronic damage	0.347; NS (29)	0.033; NS (24)	0.534; <0.05 (22)	1.000 (30)	
Serum creatinine	−0.202; NS (29)	−0.093; NS (24)	−0.421; 0.05 (22)	−0.375; <0.05 (30)	1.000 (30)
(c) Ischaemic/hypertensive nephropathy					
Urinary MCP-1/CCL2	0.318; 0.05 (37)	1.000 (38)			
Urinary ACR	1.000 (40)				
Interstitial macrophages	−0.129; NS (33)	0.427; <0.05 (31)	1.000 (35)		
Index of chronic damage	0.190; NS (40)	0.287; NS (38)	0.789; <0.001 (35)	1.000 (42)	
Serum creatinine	−0.186; NS (40)	0.268; NS (38)	−0.606; <0.001 (35)	−0.606; <0.001 (42)	1.000 (42)

ACR=albumin–creatinine ratio; IgA=immunoglobulin A; MCP-1/CCL2=chemokine monocyte chemoattractant protein-1; NS, not statistically significant.

$P<0.001$). In patients with more chronic damage ($n=87$), there was close correlation between albuminuria and urinary MCP-1/CCL2 ($\beta=0.463$, $P<0.001$). Interstitial macrophage numbers correlated with urinary MCP-1/CCL2 ($\beta=0.238$, $P=0.05$), but not with the ACR ($\beta=0.011$, $P=$NS).

DISCUSSION

In these studies, we demonstrate an association between albuminuria and a pathway for the development of renal injury that progresses from the generation of MCP-1/CCL2 to macrophage recruitment and *in situ* damage in a heterogeneous group of renal diseases. The strength of this association is directly related to the amount of urinary albumin leak; the strongest correlations exist in nephrotic range albuminuria. Subset analyses of different types of renal disease also show that the association between interstitial macrophage infiltration and damage is strong in all disease states and at different levels of renal scarring. However, the relationship between ACR, urinary MCP-1/CCL2, and interstitial macrophage numbers is strongest in those patients with lower levels of scarring and affected by disorders associated with distinct patterns of glomerular injury, such as IgA nephropathy and focal segmental glomerulosclerosis. In more advanced renal disease, including ischaemic/hypertensive nephropathy, these associations are less strong, indicating that other mechanisms may have an increasingly important role on the recruitment of these cells in this setting.

The role of macrophages in the progression of chronic renal injury is well established; their presence is a consistent feature of the tubulointerstitial changes that occur irrespective of the original disease.[1,2] Experimental models indicate that macrophages mediate damage at this site through a number of mechanisms, which include the generation of radical oxygen species, nitric oxide, complement factors, and proinflammatory cytokines.[4] Macrophages also affect supporting matrix and vasculature through the expression of metalloproteinases and vasoactive peptides and promote the transdifferentiation of tubular epithelial cells into interstitial fibroblasts, which themselves contribute to renal scarring.[18]

The chemokine MCP-1/CCL2 acting through its receptor CCR2 is a potent chemoattractant for macrophages and has been demonstrated to direct macrophage infiltration and injury in a number of renal and non-renal diseases.[9–12,19,20] Recent experiments using novel gene therapy techniques[10,11] in progressive kidney disease have specifically blocked tubular MCP-1/CCL2 expression with a subsequent reduction in interstitial macrophage infiltration and *in situ* injury. In human chronic non-proliferative kidney diseases, MCP-1/CCL2 expression has been demonstrated by immunohistochemistry[5,6] and *in situ* hybridization[5,21] to be primarily restricted to tubular epithelial cells. These cells are local to peritubular capillaries and can therefore direct the infiltration of macrophages from the intravascular compartment.

Relatively small studies of human chronic disease have (i) demonstrated associations between semiquantitative assessment of MCP-1/CCL2 expression *in situ* and macrophage infiltration[5,6] and damage,[5] and (ii) correlated urinary MCP-1/CCL2 with ACR[13] and macrophage infiltration.[14] In a retrospective study of 25 patients, there was more tubular MCP-1/CCL2 expression and macrophage infiltration in patients with progressive disease.[15] The study reported here

is much larger than previous analyses, is prospective, quantitative, and powered for subset analyses, and to explore associations that demonstrate a pathogenic role with an impact on clinical end points.

Several theories have been proposed to explain the link between the initial renal insult and progressive tubulointerstitial inflammatory injury. In acute proliferative renal diseases, glomerular-derived cytokines may have effects on cells downstream in the nephron by, for example, activating tubular epithelial cells to express leukocyte adhesion molecules and proinflammatory cytokines, including MCP-1/CCL2.[22] This theory is most relevant to crescentic membranoproliferative glomerulonephritides, where marked increases in glomerular expression of proinflammatory cytokines have been demonstrated.[5,6,23]

We analysed a group of patients with chronic kidney diseases where previous studies have shown that proteinuria is a rigorous predictor of tubulointerstitial disease progression.[24] As glomerular injury evolves, tubular epithelial cells are exposed to higher concentrations of glomerular filtrated proteins, including albumin; these may promote phenotypic changes necessary for the recruitment of macrophages to interstitial sites.[24] *In vitro* proximal tubular epithelial cells express MCP-1/CCL2 in a dose–response manner to protein.[16,17] In animal models, strategies that decrease albuminuria are also associated with a reduction in tubular MCP-1/CCL2 expression and interstitial inflammation.[25–27] The results of this study indicate that albuminuria-induced tubular MCP-1/CCL2 expression is important in interstitial macrophage recruitment and disease progression in human chronic kidney disease. As well as demonstrating strong correlations between ACR, urinary MCP-1/CCL2, interstitial macrophage numbers and scarring, we found that these factors predict renal outcome. The association between urinary MCP-1/CCL2 levels, macrophage infiltration, and clinical outcomes had not previously been prospectively studied.

Consistent with other studies, nephrotic range albuminuria was associated with the highest risk of disease progression, and these patients had the highest levels of urinary MCP-1/CCL2 and interstitial macrophage numbers. To demonstrate causality however requires the means of reducing albuminuria or blocking its effect on tubular cells, in the absence of other factors that may trigger this pathway. The protein overload proteinuria model is the best animal model currently available to study the effects of proteinuria on the development of tubulointerstitial disease. In this setting, where no glomerular abnormalities are induced, the importance of tubular derived MCP-1/CCL2 in disease progression has been demonstrated.[10]

Albumin is clearly not the only serum protein that is leaked into the urinary space in disease. Experiments with human proximal tubular epithelial cells (PTEC) have demonstrated that serum proteins of molecular weight 40–100 kDa increase proximal tubular epithelial cell MCP-1/CCL2 expression.[16] Albumin has a molecular weight within this range (60 kDa), as does transferrin, which has also has biological effects on proximal tubular epithelial cells, including increasing chemokine expression.[28] Larger-molecular-weight proteins also enter the urinary space in disease states. These include immunoglobulins, which also increase tubular MCP-1/CCL2 expression.[29] The biological effects of these larger-molecular-weight proteins are particularly important in renal prognosis, as their detection in urine is more predictive of disease progression than albumin.[24] Indeed, in minimal change disease there is selective proteinuria, where large-molecular-weight proteins are not leaked. This disease is not associated with the development of tubulointerstitial disease and renal failure. There is evidence that the fatty acids bound to albumin are an important determinant of biological effect, and the relative lack of fatty acid content of albumin in minimal change disease may also be associated with a benign prognosis.[30] *In vitro* however, the use of lipated albumin resulted in MCP-1/CCL2 expression by proximal tubular epithelial cells at levels similar to de-lipidated albumin.[17]

The results from our subset analyses suggest that proteinuria-induced tubular MCP-1/CCL2 expression is particularly important in the recruitment of macrophages early in disease. Correlations between interstitial macrophage numbers, urinary MCP-1/CCL2, and albumnuria were particularly significant in those patients with an index of chronic damage $<20\%$. However, in those at a more advanced stage the ACR did not significantly correlate with interstitial macrophage numbers, although both factors were independently correlated with urinary MCP-1/CCL2 and macrophage numbers. Thus, other factors may become increasingly important in promoting MCP-1/CCL2-dependent interstitial macrophage infiltration with disease progression. These may include tissue hypoxia, which can generate tubular MCP-1/CCL2 through activation of the renin–angiotensin system.[31,32] Hypoxia also increases adhesion molecule expression by endothelial cells and tubular epithelial cells. Recruited macrophages may themselves promote a hypoxic microenvironment. Macrophages colocalize with tubular cells at sites of vascular endothelial growth factor downregulation, and *in vitro* macrophage-derived cytokines downregulate tubular vascular endothelial growth factor expression.[33] Vascular endothelial growth factor is an important proangiogenic cytokine. In patients with hypertensive/ischaemic nephropathy where tissue hypoxia is likely, we found that interstitial macrophage numbers did not directly correlate with albuminuria, although albuminuria and macrophage numbers remained significantly correlated with urinary MCP-1/CCL2.

A reduction in proteinuria to <1 g per day in response to blood pressure reduction and renin–angiotensin system blockade is associated with a better renal prognosis.[24] Patients who do not achieve this response have a significantly poorer prognosis. We did not collect data on renin–angiotensin system blockade therapy at the time of their biopsy, nor prospectively studied the effect of renin–angiotensin system blockade introduction on those patients who subsequently commenced this treatment. We would hypothesize,

however, that renin–angiotensin system blockade would reduce urinary MCP-1/CCL2 expression. In proteinuric renal disease, baseline and urinary MCP-1/CCL2 levels are unlikely to be a more specific indicator of prognosis than measuring proteinuria in those with proteinuric renal disease. However, in non-proteinuric diseases such as ischaemic nephropathy, measuring MCP-1/CCL2 may be valuable. This requires further study.

This study highlights the central role of the interstitial macrophage in chronic kidney disease progression in respect of tubular-derived MCP-1/CCL2. Therefore, this chemokine, the receptor CCR2, or the factors that increase expression of either molecule may represent attractive therapeutic targets. The use of gene therapy and chemokine receptor antagonists that target MCP-1/CCL2-dependent interstitial macrophage recruitment in animal models has yielded encouraging results.[9–12] Animal models studying chemokine receptors in glomerulonephritides highlight a further layer of complexity. Models of anti-glomerular basement membrane disease in mice deficient of the chemokine receptor CCR2 developed more severe disease.[34] These findings may indicate that MCP-1/CCL2 and/or CCR2 may have important homeostatic roles in some settings.

In summary, we show a close association between albuminuria, urinary MCP-1/CCL2, and interstitial macrophage numbers with *in situ* damage and clinical outcomes in human chronic kidney disease. This relationship is strongest in early disease and less pronounced in ischaemic/hypertensive nephropathy. These findings support the theory that increased albuminuria triggers tubular MCP-1/CCL2 expression with subsequent macrophage infiltration. These processes may represent the dominant pathway for the progression of renal injury with limited renal scarring.

MATERIALS AND METHODS

Patients

Following local ethical committee permission and informed consent, we recruited patients who underwent percutaneous renal biopsy in our department for investigation of renal disease between June 1999 and June 2002. Renal biopsy specimens were obtained prospectively from 215 patients with chronic kidney disease under real-time ultrasound guidance using a 16-G semi-automatic biopsy needle. The specimens were immediately fixed in formal saline and glutaraldehyde. Urine for quantification of MCP-1/CCL2 and ACR was collected immediately before the biopsy. A normal ACR is <2.5 mg/mmol for men, and <3.5 mg/mmol for women. An ACR of 100 mg/mmol approximates to a 24-h urinary albumin excretion of 1 g, whereas an ACR of 300 mg/mmol or more approximates to nephrotic range albuminuria. Multiplying by 8.8 converts the ACR from mg/mmol creatinine to mg/mg creatinine.

Patients found to have mesangiocapillary glomerulonephritis, crescentic glomerulonephritis, or lupus nephritis were excluded from further study, as in these diseases the glomeruli is a significant source of any MCP-1/CCL2 detected in the urine.[5] Patients with chronic nonproliferative diseases were included where urinary MCP-1/CCL2 probably reflects tubular expression.

Follow-up data were available on 165 patients (77%) of the total cohort and we analysed clinical outcomes for this group. We defined a doubling in serum creatinine from the time of renal biopsy and/or initiation of renal replacement therapy (calculated in days) as the end point for renal outcome. Data were unavailable on 50 patients who had been referred back to their primary care physician or referring hospital. The majority (35 patients) had been diagnosed as having either thin glomerular basement membrane disease or IgA nephropathy as a cause of their isolated microscopic haematuria.

Renal diagnosis

Histological diagnoses were made on formal saline- and glutaraldehyde-fixed biopsy specimens. Light microscopic sections (2 μm) were examined in orthodox ways and immunostaining carried out with an immunoperoxidase method for IgG, IgA, IgM, and the complement component C9. The glutaraldehyde-fixed specimen was embedded in Araldite and sectioned for electron microscopy when indicated.

Urinary MCP-1/CCL2 assay

Quantification of urinary MCP-1/CCL2 was performed using a commercially available sandwich ELISA kit according to the manufacturers' instructions (R&D Systems, Minneapolis, MN, USA). These levels were corrected for urinary creatinine concentration.

Immunohistochemistry for macrophages

The immunohistochemical detection of tissue macrophages was performed using established methods. Briefly, dewaxed and rehydrated paraffin-embedded sections (2 μm) were processed for antigen retrieval in 0.01 mol/l of sodium citrate buffer (pH 6.0) at 95°C for 30 min. Slides were then incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, followed by sequential treatment with 0.1% avidin and 0.01% biotin (Dako Ltd, Ely, UK) to block endogenous biotin activity. Three-stage indirect immunohistochemistry was performed by sequential incubation with a primary mouse monoclonal antibody directed against the pan-macrophage antigen CD68 (5 mcg/ml; clone PG-M1; Dako Ltd) for 1 h, a biotinylated secondary rabbit anti-mouse antibody (50 mcg/ml; Dako Ltd) for 1 h, and a horse radish peroxidase-conjugated streptavidin–biotin complex (Dako Ltd) for 20 min. Between two incubations, sections were washed in Tris-buffered saline (pH 7.4). All incubations were performed at room temperature. Binding was visualized by the addition of 3′,3-diaminobenzidine (Vector Laboratories Ltd, Peterborough, UK). Sections were then counterstained with Mayer's haematoxylin (Sigma-Aldrich, Poole), except those used for quantitative analysis, which were left unstained (see below). Mouse IgG1 (Dako Ltd) was used as an isotype control antibody and substituted for the primary antibody on serial sections.

Tissue sections were batched and stained in one sitting over a course of 4 days to help maintain consistency in the technique used between samples. The PG-M1 monoclonal antibody was used to identify the macrophage-specific antigen CD68. The protein is present in the cytosol of all macrophages and its expression does not vary with the maturity and activation status of the cell. This is in contrast with CD14, which has also been been used in immunohistochemical studies as a marker for macrophages.[35]

Interstitial macrophage quantification

An interactive image analysis system was used for blinded assessment of interstitial macrophage numbers. This technique has previously been reported as a reliable method for the analysis of human and animal renal sections.[36,37] Coded sections stained for

CD68 were visualized at ×200 magnification and the image captured digitally by an Aequitas image database and image archive management system (Dynamic Data Links, Cambridge). Each image was then converted to a two-colour scale image by Aequitas image analysis software (Dynamic Data Links). By altering the threshold, the image was processed so that positive staining was represented by black pixels and measured as a percentage of the area of the total image analysed. For each patient, the mean measurement of five randomly selected non-confluent microscopic fields of renal cortex was determined. Glomerular staining was excluded from the analysis by the computer software. Sections where background staining made it impossible to digitally differentiate specific staining were excluded from analysis.

Quantification of chronic damage

The extent of chronic damage within each biopsy specimen was assessed by an established and validated method that is a rigorous predictor of renal outcome.[38] Briefly, one routinely prepared section of each specimen was stained by periodic acid-methenamine silver and examined under a microscope. Chronically scarred tissue was identified as glomeruli showing global sclerosis but not segmental sclerosis, areas of interstitial fibrosis, which appeared more solid and deeply stained than normal or oedematous interstitial tissues, and atrophic tubules, defined as tubules smaller than normal, with thickened basement membranes, or tubules larger than normal, with thin epithelium, including those large enough to be considered cysts. Arteries and arterioles were not judged to have chronic damage unless they were completely occluded. Images at a magnification of ×10 were captured digitally by the Aequitas image database and image archive management system (Dynamic Data Links). Using Aequitas image analysis software, the extent of scarring observed within the cortex of the kidney biopsy section was quantified and expressed as a percentage of total tissue analysed.

Statistics

To assess the validity of the methods used to quantify interstitial macrophages, inter- and intra-observer variabilities were tested. For inter-observer variation, two observers measured 20 randomly selected specimens independently; one observer had helped to develop the method (D Zehnder) and the other would measure the entire series (KS Eardley). The 20 specimens were also measured twice at intervals by Eardley to test for intra-observer variability. Agreement was assessed by the method of Bland and Altman after log transformation (necessary because the differences between measurements were proportional to the mean).[39] This method gives the bias, or mean difference between measurements, and limits of agreement, or 2 s.d.'s either side of the mean, with 95% CIs for the bias and limits of agreement, all expressed as ratios when back-transformed.

Linear regression analyses were performed to determine correlations between normally distributed data variables. When required, as determined by normality testing for skewness, data variables were normalized by log transformation. In the case of serum creatinine the reciprocal was used, and for index of chronic damage 1 was added to the value prior to log transformation. Correlations are presented by expressing the β correlation coefficient along with the *P*-value. Linear regression stepwise multivariate analysis of these correlations with a dependent variable was also performed. Comparison of data means was performed using either a two-tailed '*t*' test or analysis of variance (ANOVA) with Bonferroni *post-hoc* tests when comparing multiple groups. Univariate and multivariate analyses of the impact of variables on renal outcome were performed using Cox regression analysis. Renal outcome was also assessed by Kaplan–Meier survival analysis with log rank testing. Kaplan–Meier analyses of urinary MCP-1/CCL2 and interstitial macrophage numbers were performed after their categorization in tertiles. All statistical tests were performed using SPSS for Windows, version 12.0, and the level of significance was set at $P<0.05$.

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