see commentary on page 405

The role of capillary density, macrophage infiltration and interstitial scarring in the pathogenesis of human chronic kidney disease

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To assess the relationship between interstitial capillary density and interstitial macrophages we prospectively measured these factors *in situ* in 110 patients with chronic kidney disease. Macrophage numbers and urinary MCP-1/CCL2 levels significantly correlated inversely with capillary density which itself significantly correlated inversely with chronic damage and predicted disease progression. In 54 patients with less than 20% chronic damage, there was a significant correlation between the urinary albumin to creatinine ratio and MCP-1/CCL2, and MCP-1/CCL2 and macrophages but not between MCP-1/CCL2 and capillary density. Conversely, in 56 patients with over 20% chronic damage there was no correlation between MCP-1/CCL2 and macrophages but there were significant inverse correlations between capillary density and both macrophages and chronic damage. The expression of VEGF mRNA significantly correlated with macrophage infiltration, capillary density and chronic scarring. In an ischemic-hypertensive subgroup there was upregulation of the hypoxia marker carbonic anhydrase IX and with over 20% chronic damage an increased macrophage to CCR2 ratio. Our study shows that proteinuria and MCP-1/CCL2 are important for macrophage recruitment in early disease. As renal scarring evolves, alternative pathways relating to progressive tissue ischemia secondary to obliteration of the interstitial capillary bed predominate.

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The evolution of chronic kidney disease is characterized by interstitial fibrosis and tubular atrophy. These changes are promoted by interactions between resident tissue components and infiltrating cells. Animal models and observational studies in humans indicate that infiltrating macrophages have a central role in the initiation and progression of renal scarring.[1] Irrespective of the initial insult, these processes represent a common pathway for the development of injury and progression to end-stage kidney disease.

There are a number of molecules involved in the recruitment of macrophages in chronic kidney disease. We have recently shown a role for the macrophage-directed chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) in a large prospectively recruited cohort of patients with a heterogeneous group of chronic kidney disease: the dominant role for this pathway was in patients with lower levels of renal scarring.[2] A role for MCP-1/CCL2 in renal disease is further supported by smaller human studies and animal models where disease is abrogated by blocking MCP-1/CCL2 or CCR2.[3–10] Interestingly these studies focused on analysis and intervention in early disease.

Progressive tubulointerstitial disease is associated with the loss of interstitial capillaries and a reduced blood flow within those that remain.[11–14] These observations indicate that the renal cortex becomes more ischemic as chronic kidney disease evolves and this may have a central role in disease progression.[15] A number of molecules may be differentially expressed *in situ* in response to hypoxia in chronic kidney disease.[16] For example, vascular endothelial growth factor (VEGF), a hypoxia-induced angiogenic factor,[17] was expressed by renal tubules in human chronic kidney disease with increased expression related to peritubular capillary loss.[12] Similar patterns were seen in a recent study on human immunoglobulin (Ig)-A nephropathy.[18] Other studies have demonstrated increased tubular and interstitial cell

expression of other proinflammatory and profibrotic molecules in response to hypoxia.[19,20] To date, however, the relationship between renal hypoxia and macrophage recruitment to the interstitium has not been studied in human chronic kidney disease.

In these studies, we have analyzed a chronic kidney disease cohort for interstitial capillary density as a surrogate marker for tissue hypoxia. We have assessed the relationship between interstitial capillary density, MCP-1/CCL2, macrophage recruitment, and chronic damage, and analyzed the differential relationship between macrophage recruitment, albuminuria, and capillary density in respect of the stage of chronic damage. Further, we have related these *in situ* changes to VEGF mRNA levels, carbonic anhydrase IX (CA IX) expression (a marker for *in situ* hypoxia), and the differential expression of the chemokine receptors, CCR2 and CX3CR1. These data indicate that the factors that promote macrophage recruitment and retention at interstitial sites may change as kidney disease progresses.

RESULTS

Patients

One hundred and ten patients were studied. This group represented a cohort derived from a population of 215 patients who have previously been reported for index of chronic damage, urinary MCP-1/CCL2, albumin-to-creatinine ratio (ACR), and interstitial macrophages.[2] For this study, we excluded all patients with (i) thin glomerular basement membrane disease ($n=44$); (ii) minimal change nephropathy ($n=5$); (iii) diabetic nephropathy ($n=18$); (iv) extensive background staining that prevented analysis of tissue ($n=28$); and (v) no cortex in the biopsy specimen ($n=10$). Table 1 summarizes the *in situ* data obtained for the cohort.

Quantification of peritubular capillary density

Quantification of *in situ* determinants for potential bias was assessed. There was agreement between measurements of interstitial numbers by the same observer (mean 1.00; 95% CI: 0.98–1.06) or measurements by two observers (mean 0.97; 95% CI: 0.96–1.12). The limits of agreement of measurements by the same observer were 0.85 (CI: 0.79–0.91) and 1.19 (CI: 1.11–1.28), and the limits of agreement of measurements by two observers were 0.69 (CI: 0.60–0.78) and 1.35 (CI: 1.18–1.55).

Peritubular capillary density and macrophage colocalization

There was a uniform distribution of capillaries between tubules in renal tissue without evidence of tubulointerstitial damage (Figure 1a). This was associated with low numbers of interstitial macrophages that were evenly dispersed throughout the interstitium (Figure 1b). Loss of interstitial capillaries was associated with increased interstitial macrophage numbers. Where tubulointerstitial disease was patchy, reduced density of capillaries (Figure 1c) colocalized with increased interstitial macrophage numbers (Figure 1d). In biopsies with an advanced stage of scarring, there was generalized interstitial capillary loss and increased interstitial macrophage numbers.

Capillary density correlates with MCP-1/CCL2, interstitial macrophages, and chronic damage

By univariate analysis, capillary density inversely correlated with urinary MCP-1/CCL2 levels (correlation $=-0.28$, $P=0.005$), interstitial macrophage numbers (correlation $=-0.621$, $P<0.001$), and index of chronic damage (correlation $=-0.82$, $P<0.001$) (Figure 2). There was no correlation with ACR (correlation $=-0.068$, NS).

Capillary density and renal outcome

Kaplan–Meier survival analysis found that those patients with least interstitial capillary density had the worst renal outcome (Figure 3). Univariate Cox regression analysis found interstitial capillary density to significantly impact on renal outcome ($\exp B=0.674$, $P=0.001$). The impact of the other clinical and pathological variables on renal survival as assessed by Kaplan–Meier analysis has been previously described.[2]

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

Histological diagnosis (*n*)	Age (years)	Interstitial capillaries (%)	Index of chronic damage (%)	ACR (mg/mmol)	Urinary MCP-1/CCL2 (pg/mg)	Interstitial macrophages (%)
Ischemic/hypertensive nephropathy (37)	56 (28–81)	6.0 (2.4–11.3)	40 (0–97)	23.8 (0.0–436.6) (*n*=36)	181.5 (13–845.3) (*n*=33)	2.0 (0.9–4.5) (*n*=36)
IgA nephropathy (29)	37 (23–87)	10.3 (2.7–12.6)	9 (0–94)	19.8 (0.4–1097.3)	114.9 (10.6–981.7) (*n*=27)	1.1 (0.8–4.2) (*n*=26)
Focal segmental glomerulosclerosis (20)	49 (30–73)	8.1 (4–11.8)	19.5 (0–75)	345.9 (49.8–1053.4) (*n*=19)	394.9 (41.5–1118.8) (*n*=17)	1.8 (1.1–4.5) (*n*=18)
Other GN (12)	43 (20–71)	10.6 (5–13.3)	8 (0–79)	201.3 (1.8–890.9) (*n*=11)	161.8 (16.9–652.7) (*n*=11)	1.7 (0.9–6.1)
Amyloid/light chain disease (6)	79 (52–85)	7.2 (3.9–9.4)	37.5 (19–93)	91.7 (16–848.4)	510.8 (172.9–737.5) (*n*=5)	3.3 (1.8–7.5)
Others (6)	51 (29–80)	8.8 (5.8–11.6)	27.5 (0–59)	14.3 (0.5–379.1)	155.2 (11.7–604.8) (*n*=5)	1.0 (0.9–5.3) (*n*=5)

ACR, albumin-to-creatinine ratio; GN, glomerulonephritis; IgA, immunoglobulin-A; MCP-1/CCL2, monocyte chemoattractant protein-1. Results are shown as median (range).

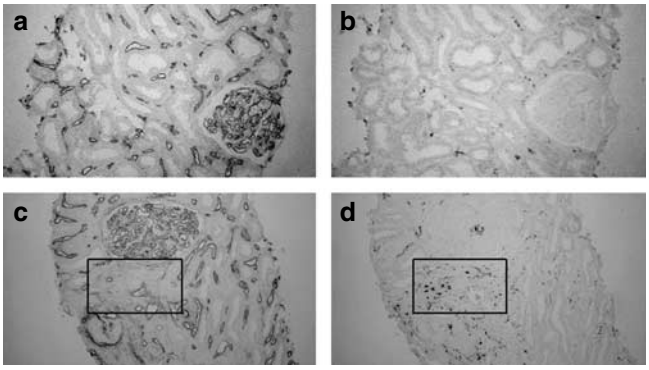

Figure 1 | Capillary loss and macrophage colocalisation. Capillaries (**a**, **c**) by anti-CD34 and macrophages (**b**, **d**) by anti CD68 in renal biopsies, paired serial sections, no counterstaining. No damage, Mild IgA nephropathy (**a**, **b**); (uniform distribution of capillaries and macrophages). Moderate damage, ischemic renal disease (**c**, **d**); patchy (inset box) decreased capillary density and increased macrophage numbers.

Linear regression analysis

Multivariate linear regression analysis was used to assess the relationship between capillary density, interstitial macrophage numbers, urinary MCP-1/CCL2 levels, and ACR, with chronic damage as the dependent variable. There was a strong independent negative correlation between capillary density and chronic damage (-0.574; $P<0.001$) and an independent positive correlation between macrophage numbers and chronic damage (0.363; $P=0.001$). There was no correlation between urinary MCP-1/CCL2 levels and chronic damage (0.035; NS), and no correlation between ACR and chronic damage (-0.063; NS).

The relationship between albuminuria, capillary density, and chronic damage

We have previously shown that over a mean follow-up period of 832 days, the index of chronic damage was a major determinant of renal survival.[2] In patients with an index of chronic damage of less than 20%, 2% of patients reached a renal end point, as defined by doubling of serum creatinine or reaching end-stage renal failure. This is comparable to 26% of patients with an index of chronic damage of 20% or more reaching the renal end point.

To assess the differential relationship of albuminuria and capillary density with chronic damage, macrophage infiltration, and urinary MCP-1/CCL2, subset analysis was performed for patient groups based on the index of chronic damage. Group 1 ($n=54$) comprised patients with biopsies with chronic damage of less than 20%; Group 2 ($n=56$) comprised patients with biopsies with chronic damage of 20% or more.

Albuminuria and chronic damage

Albuminuria was correlated with chronic damage in Group 1 (correlation $=0.337$; $P=0.015$), but there was no correlation with worsening advanced chronic damage (Group 2) (Table 2; Figure 4). Similarly, there was a correlation between albuminuria and macrophage numbers in Group 1 (correlation $=0.619$; $P<0.001$), but no correlation in Group 2. There was no correlation in Group 1 or 2 between albuminuria and capillary density.

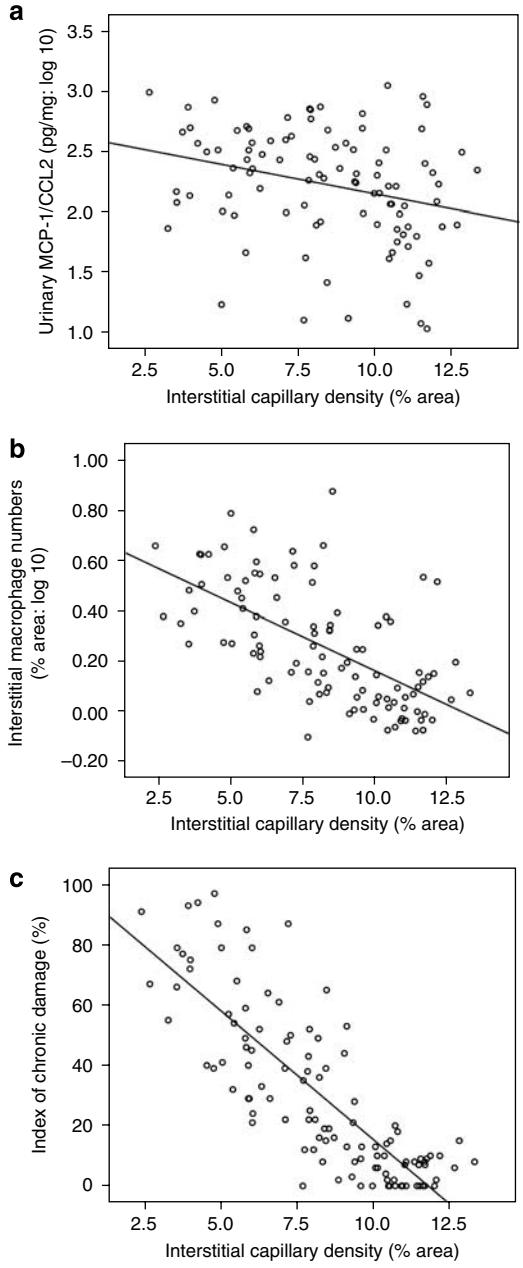

Figure 2 | Capillary density correlations. Plots of correlations (correlation; *P*-value) between (**a**) interstitial capillary density and urinary MCP-1/CCL2 (-0.28; <0.005), (**b**) Macrophage numbers and interstitial capillary density (-0.64; $P<0.001$), and (**c**) interstitial capillary density and index of chronic damage (-0.82; $P<0.001$).

Albuminuria correlated with urinary MCP-1/CCL2 levels in Group 1 (correlation $=0.529$; $P<0.001$) and Group 2 (correlation $=0.483$; $P<0.001$).

Capillary density and chronic damage

Capillary density was negatively correlated with chronic damage in Group 1 (correlation $=-0.281$; $P=0.04$) and

strongly negatively correlated in Group 2 (correlation = −0.562; $P<0.001$) (Figure 4). There was also a strong negative correlation between capillary density and macrophage numbers in Group 2 (correlation = −0.443; $P=0.001$). There was no correlation between capillary density and macrophage numbers when chronic damage was less than 20% (Group 1).

When chronic damage was less than 20% (Group 1), there was a significant correlation between urinary MCP-1/CCL2 and interstitial macrophages (correlation = 0.445; $P=0.001$). There was no significant correlation between urinary MCP-1/CCL2 and interstitial macrophages with more advanced chronic damage (Group 2).

Linear regression analysis for macrophage infiltration by chronic damage group

By multivariate linear regression analysis, for Group 1, there was a significant correlation between ACR and macrophage numbers (correlation = 0.428; $P=0.005$). MCP-1/CCL2 was not significantly correlated indicating a direct relationship with ACR. For Group 2, these patterns of associations changed. Interstitial capillary density independently correlated with macrophage numbers (correlation = 0.479; $P<0.001$). There was no significant correlation between macrophages and ACR or MCP-1/CCL2.

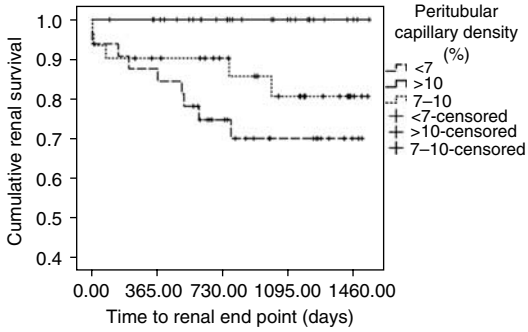

Figure 3 | Kaplan–Meier analysis of interstitial capillary density on renal outcome. Interstitial capillary density categorized into tertiles ($P<0.03$).

Analysis by disease group

We assessed the relationship between the three largest disease groups, focal segmental glomerulosclerosis, ischemic/hypertensive nephropathy, and IgA nephropathy (Table 3). In chronic damage Group 1, there was a significant difference between these disease groups for macrophages ($P=0.001$) and ACR ($P=0.001$). In chronic damage Group 2, there was a significant difference for ACR ($P=0.001$). There were no significant differences for any other studied parameter. However, by multivariate linear regression analysis with chronic damage as the dependent variable, there was a highly significant association with interstitial capillary density in all disease states (Table 3), and a significant association with macrophages in the IgA nephropathy group.

VEGF mRNA

cDNA was available for analysis for 33 patients in the cohort (Figure 5). There were strong correlations with VEGF mRNA ΔC_t and capillary density (0.518; $P=0.002$), macrophages (−0.654; $P<0.001$), and chronic damage (−0.695; $P<0.001$). The lower the ΔC_t the lower the cycle number at which VEGF mRNA is present in relationship to the housekeeping gene, therefore, the higher the expression of VEGF. So, the positive correlation seen with interstitial capillary density means that higher levels of VEGF mRNA are associated with lower capillary density; the inverse correlations seen with macrophages and chronic damage means that higher levels of VEGF mRNA are associated with increasing macrophage infiltration and chronic damage.

When multivariate regression analysis was performed incorporating VEGF mRNA with ACR, MCP-1/CCL2, macrophage numbers, interstitial capillary density, and chronic damage as variables, capillary density (−0.647;

Table 2 | Univariate analysis of correlation between ACR, interstitial capillary density, urinary MCP-1/CCL2, interstitial macrophage numbers, and index of chronic damage by chronic damage groups

	Urinary ACR		Capillary density		Urinary MCP-1/CCL2		Interstitial macrophages	
Chronic damage group	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Urinary ACR	NA	NA						
Capillary density	−0.101 NS (52)	0.161 NS (54)	NA	NA				
Urinary MCP-1/CCL2	0.529; <0.001 (50)	0.483 <0.001 (46)	−0.008 NS (52)	0.004 NS (47)	NA	NA		
Interstitial macrophages	0.619 <0.001 (49)	0.045 NS (50)	−0.188 NS (51)	−0.443 0.001 (52)	0.445 0.001 (48)	0.167 NS (44)	NA	NA
Chronic damage	0.337 0.015 (52)	−0.116 NS (54)	−0.281 0.040 (54)	−0.562 <0.001 (56)	0.076 NS (51)	0.147 NS (47)	0.524 <0.001 (51)	0.710 <0.001 (52)

ACR, albumin-to-creatinine ratio; MCP-1/CCL2, monocyte chemoattractant protein-1; NA, not applicable.
Group 1=chronic damage <20%; Group 2=chronic damage of 20%+.

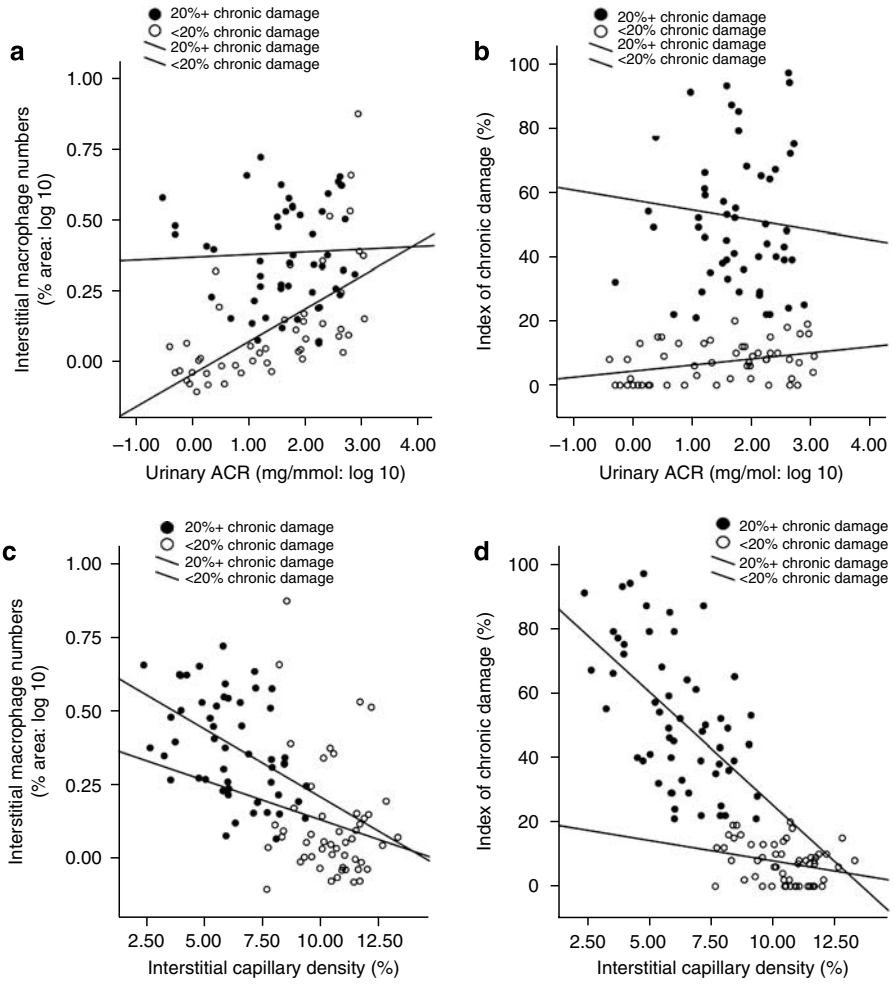

Figure 4 | Subgroup correlations: <20% chronic damage = Group 1; >20% chronic damage = Group 2 (correlation; *P*-value). (**a**) ACR and macrophage infiltration: Group 1 (0.619; <0.001); Group 2 (NS); (**b**) ACR and chronic damage: Group 1 (0.337; 0.015); Group 2 (NS); (**c**) capillary density and macrophage infiltration: Group 1 (NS); Group 2 (−0.443; 0.001); and (**d**) capillary density and chronic damage: Group 1 (−0.281; 0.04); Group 2 (−0.562; <0.001).

Table 3 | Multivariate linear regression analysis with chronic damage as the dependent variable.

	Urinary ACR	Capillary density	Urinary MCP-1/CCL2	Interstitial macrophages
Focal segmental glomerulosclerosis	β: 0.523 *P*=0.058	β: 1.139 *P*=0.001	β; 0.000 NS	β; 0.279 NS
Ischemia/hypertensive	β: 0.20 NS	β: 0.646 *P*<0.001	β; 0.160 NS	β; 0.252 *P*=0.058
IgA	β: 0.05 NS	β: 0.252 *P*=0.005	β; 0.068 NS	β; 0.831 *P*<0.001

ACR, albumin-to-creatinine ratio; IgA, immunoglobulin-A; MCP-1/CCL2, monocyte chemoattractant protein-1.
Note a highly significant association with interstitial capillary density in all disease states, and a significant association with macrophages in the IgA nephropathy subgroup. (β=standardized β-coefficient).

$P<0.001$) and VEGF mRNA ΔC_t (−0.336; $P=0.009$) showed significant associations.

CA IX expression

To further assess the relationship between hypoxia and *in situ* determinants of chronic damage, we analyzed tissue from patients with ischemic/hypertensive nephropathy ($n=20$) for CA IX, a transmembrane glycoprotein that is hypoxia inducible and may therefore be an endogenous marker of hypoxia. There was a significant difference between expression of CA IX in tissue with less than 20% chronic damage and 20% + chronic damage (Figure 6a–c). The large majority of expression of CA IX was restricted to tubular epithelium.

CCR2 and CX3CR1 expression

The differential expression of two chemokine receptors important for the recruitment and retention of macrophage trafficking was assessed. Receptor expression appeared

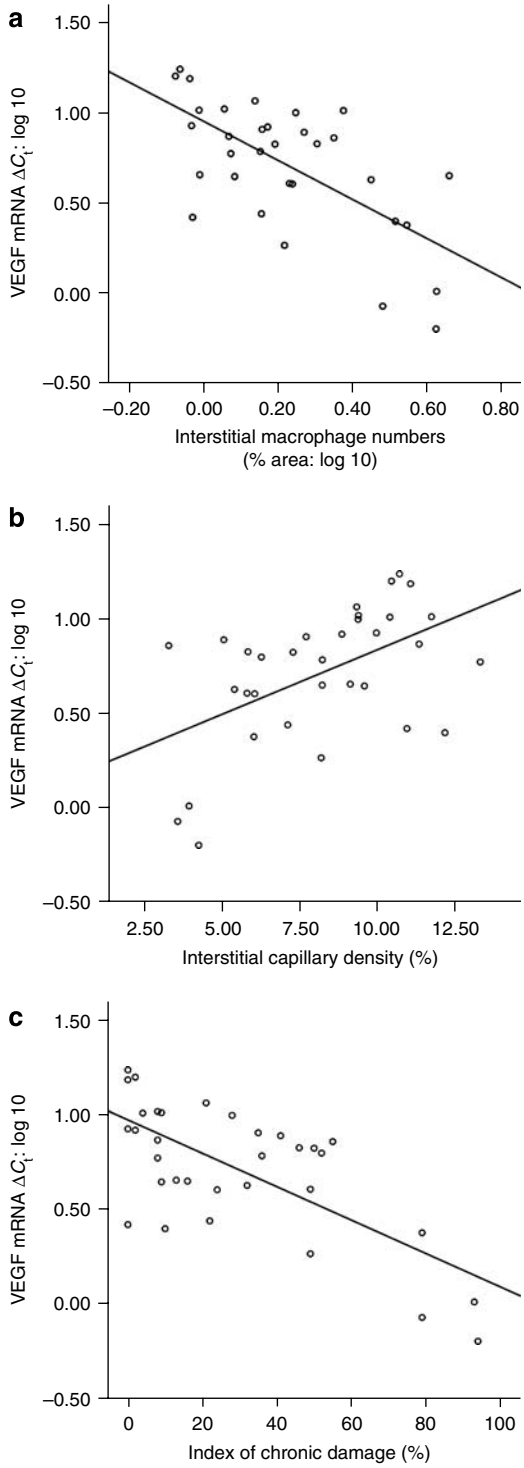

Figure 5 | VEGF mRNA and *in situ* parameters. Correlations between VEGF mRNA expression (ΔC_t) and (**a**) macrophage infiltration (−0.654; $P<0.001$), (**b**) capillary density (0.518; $P=0.002$), and (**c**) chronic damage (−0.695; $P<0.001$).

localized to cell infiltrates. The ratio between macrophage infiltration and CCR2 expression was increased in biopsies with 20%+ chronic damage compared with biopsies with less than 20% chronic damage (Figure 6d–f), while macrophage/CX3CR1 ratio was maintained (Figure 6g–i). This indicates that CCR2 may have a more important role for the recruitment of macrophages in early chronic damage. As chronic damage progresses, the role for CX3CR1 for the recruitment and retention of cells is maintained.

DISCUSSION

We have previously shown that albuminuria correlates with MCP-1/CCL2 production in human chronic kidney disease and may represent an important pathway for the recruitment of macrophages to interstitial sites.[2] We found the strongest associations at lower levels of renal scarring; in more advanced renal disease these associations were less strong. To address the hypothesis that other factors may become increasingly important as renal scarring develops, in these studies we have analyzed the role of interstitial capillary density, as a marker for tissue hypoxia. We demonstrate an association between interstitial capillary loss, macrophage recruitment, and chronic damage. Although there is a significant correlation with MCP-1/CCL2 across the study group, this is not an independent association.

In patients with renal scarring of 20% or more, levels that may predict accelerated deterioration of renal function, we found strong associations between capillary density, macrophage infiltration, and chronic damage. In patients with lower levels of scarring (<20%), there was no correlation between capillary density and macrophage numbers and a weak correlation with chronic damage. This pattern was reversed for albuminuria, with a strong association with macrophage numbers (and MCP-1/CCL2 levels) in patients with less than 20% scarring and no significant associations in patients with scarring of 20% or more. These relationships appeared independent of disease and indicate that in early but not late renal damage, albuminuria and MCP-1/CCL2 are important pathways for macrophage recruitment. As human chronic kidney disease evolves, the relationship between capillary density, macrophage recruitment and chronic damage is of increasing importance. The lack of an association between capillary density and MCP-1/CCL2 despite a strong association between capillary density and macrophage numbers in more severe scarring (>20% chronic damage) is intriguing, and suggests that the dominant pathways for macrophage recruitment and proliferation in this setting may be MCP-1/CCL2 independent.

Progressive tubulointerstitial disease is associated with loss of interstitial capillaries and reduced blood flow in those that remain. Recent *in vivo* studies in animal models demonstrated increased cortical tubular hypoxia with disease progression using novel immunohistochemical markers.[21,22] Tubulointerstitial hypoxia has also been demonstrated early in the development of inflammation and injury, even before capillary architecture perturbation.[21] There is also evidence of early tubulointerstitial hypoxia in human disease. *In vivo* studies have demonstrated reduced peritubular capillary flow preceding tubulointerstitial disease in patients with progressive forms of nephrotic syndrome compared with

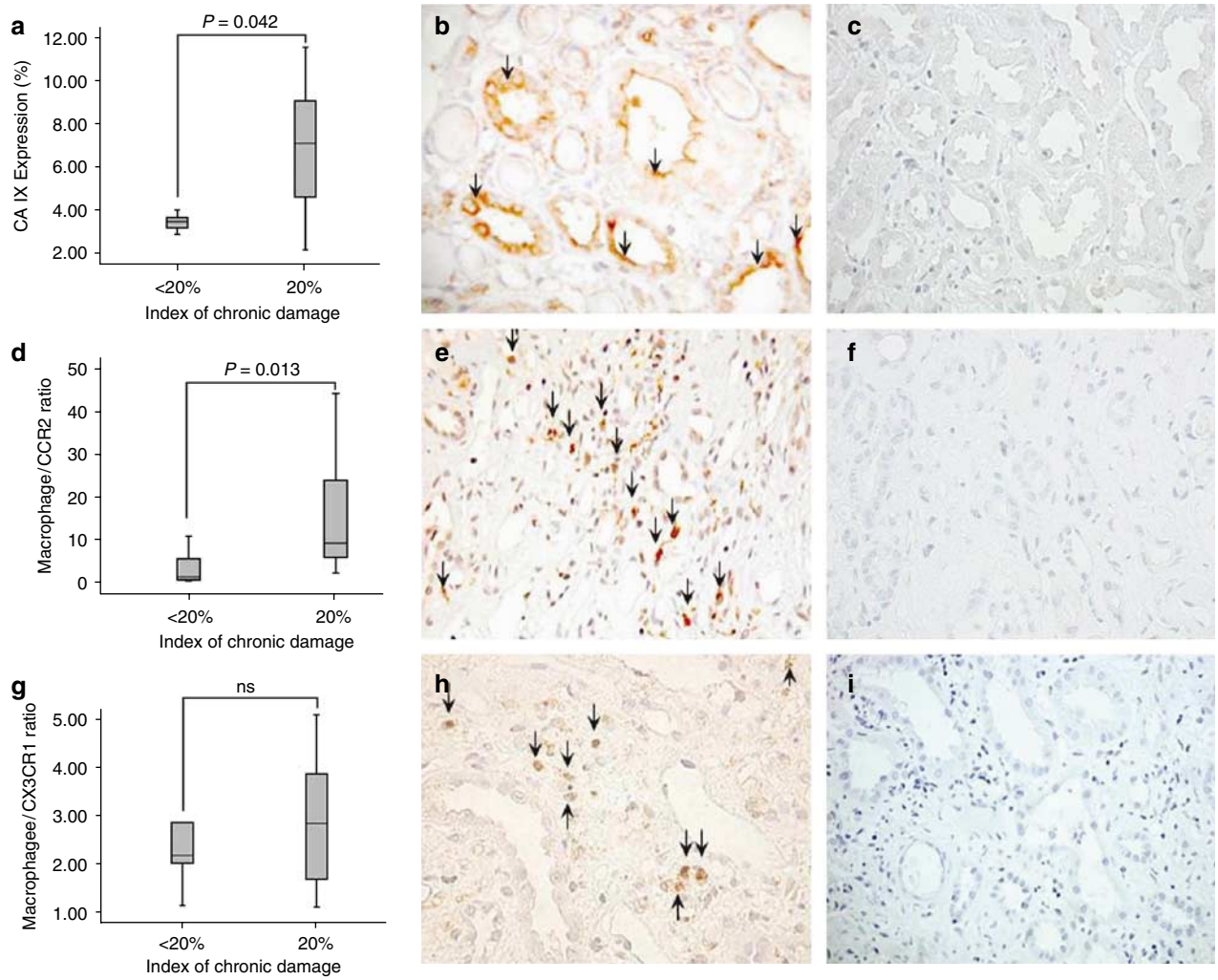

Figure 6 | Immunohistochemical staining of renal biopsies, comparison of antigen expression in <20% chronic damage, and >20% chronic damage (ischemic/hypertensive nephropathy. (**a**) CA IX expression in the two subgroups, (**b**) CA IX expression on tubular epithelial cells (original magnification ×400), (**c**) negative (isotype matched) control for CA IX, (**d**) macrophage/CCR2 ratio in the two subgroups, (**e**) CCR2 chemokine receptor expression (arrowed) on interstitial cells (original magnification ×400), (**f**) negative (isotype matched) control for CCR2; (**g**) macrophage/CX3CR1 ratio, (**h**) chemokine receptor expression on infiltrating interstitial cells (original magnification ×400), (**i**) negative (isotype matched) control for CX3CR1.

nonprogressive diseases where peritubular capillary flow is preserved.[23]

Hypoxia may promote injury and scar formation by pathways that include extracellular matrix production by interstitial fibroblasts and proximal tubular epithelial cells and the promotion of proximal tubular epithelial cells transdifferentiation to fibroblasts.[24,25] It also induces tubular epithelial cell expression of proinflammatory molecules including intercellular adhesion molecule-1 and tumor-necrosis factor-α as well as inducing tubular apoptosis.[19]

Adaptive physiological response to hypoxia include increased expression of VEGF and CA IX.[26,27] VEGF is a gene product of hypoxic pathways and has previously been reported as showing increased expression in human chronic kidney disease in association with loss of interstitial capillaries.[12,18] To further assess the *in situ* microenvironment, we analyzed expression of VEGF mRNA and found a direct relationship between VEGF mRNA production *in situ*, interstitial capillary density, and macrophage infiltration. The increased expression patterns of VEGF are consistent with activation of hypoxic pathways in association with capillary loss. The development of functional consequences of tissue hypoxia with capillary loss was further supported in the ischemic/hypertensive subgroup by upregulated CA IX expression in biopsies with 20% or more chronic damage. CA IX has been widely validated as a marker of hypoxia.[28,29]

Hypoxia may also downregulate inflammatory molecules, indeed a recent *in vitro* study indicates that hypoxia downregulates both constitutive expression and upregulation by tumor-necrosis factor-α of MCP-1/CCL2 by proximal

tubular epithelial cells.[30] As chemokine receptors direct the differential recruitment of cell subsets into the kidney,[31,32] and MCP-1/CCL2 may be less important for macrophage recruitment as chronic kidney disease progresses, we analyzed CCR2 and CX3CR1 expression in ischemic/hypertensive nephropathy. CCR2 is the targeted receptor for MCP-1/CCL2; CX3CR1 is the fractalkine/CX3CL receptor, and may contribute to the recruitment and retention of monocyte/macrophages and other leukocyte subsets in human kidney disease.[32] MCP-1/CCL2 may enhance monocyte CX3CR1 recruitment and adhesion through CCR2[33] and as macrophages differentiate CCR2 expression may be downregulated and CX3CR1 expression upregulated.[34] Consistent with a diminishing role for MCP-1/CCL2 in more advanced CKD, there was a higher ratio of macrophage/CCR2 expression in biopsies with 20%+ chronic damage. Conversely, for CX3CR1, the expression ratio was maintained.

The balance between proteinuria and hypoxia in the development of chronic kidney disease is of great interest. We hypothesize that in early proteinuric disease, macrophages are recruited by MCP-1/CCL2-dependent pathways. This may be a focal process that represents the patchy nature of disease and may localize macrophages to affected nephrons. The majority of MCP-1/CCL2 in early disease will reflect increased production of this chemokine by tubular epithelial cells in response to albumin and other glomerular filtered proteins. As nephron segments become scarred, ischemia may then become a dominant factor in further promoting macrophage infiltration and proliferation at these sites. Hypoxic and angiogenic molecules are produced and modulate the local microenvironment. These local processes will then involve bystander nephrons, with the development of both primary and secondary proteinuria and ischemia. Although proteinuria will remain a surrogate marker for disease progression as scarring progresses, it may be of less pathogenic relevance in late disease. Indeed, it is now clear that drugs that target the renin-angiotensin-aldosterone-system have important direct anti-inflammatory as well as antiproteinuric effects, which may explain their continuing clinical efficacy in advanced renal disease.

The data reported here are observational and associative, but do indicate that hypoxic damage secondary to obliteration of the interstitial capillary bed may represent an important pathway in human chronic kidney disease for the recruitment, and *in situ* retention and proliferation of macrophages, the dominant infiltrating cell in the progression of chronic kidney disease.

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 and fixed as previously indicated.[2] Urine for quantification of MCP-1/CCL2 was collected immediately before the biopsy.

Renal diagnosis

Renal diagnosis and exclusion criteria for further analysis have been previously described.[2] Histological diagnoses were made on formal saline and glutaraldehyde-fixed biopsy specimens. Light microscopic sections (2 μm) were examined in orthodox ways and immunostaining done 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

Interstitial capillaries. The immunohistochemical detection of tissue macrophages was performed as previously described.[2] Briefly, for interstitial capillaries, studies were performed on serial sections using established methods. Sections were prepared for the application of the primary antibody as previously described. There was then sequential incubation with a primary mouse monoclonal antibody directed against the endothelial cell marker CD34 (1 μg/ml; clone QBEnd 10; Dako UK Ltd, Cambridgeshire, UK) for 1 h, a biotinylated secondary rabbit anti-mouse antibody (50 μg/ml; Dako Ltd) for 1 h and an HRP-conjugated streptavidin ABC complex (Dako Ltd) for 20 min. Between each incubation periods, 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). Mouse IgG1 (Dako Ltd) was used as an isotype control antibody and substituted for the primary antibody on serial sections. Tissue sections were batched over a short period of 4 days to help maintain consistency in the technique used between samples.

CCR2, CX3CR1, and CA IX. Biopsy sections from patients with ischemic/hypertensive nephropathy were analyzed. Sections were heated in an oven at 56°C for 30 min and then placed in one-step dewaxing and epitope retrieval solution W-Cap TEC Buffer pH 8.0AP (Surgipath Europe Ltd, Peterborough, UK) at 96°C for 30 min. Sections were then allowed to cool in W-Cap for 20 min. Sections were then treated with 0.3% solution of hydrogen peroxide in methanol for 10 min. Endogenous biotin was blocked using a sequential 10-min treatment with 0.1% avidin and 0.01% biotin (Dako UK Ltd). For CA IX antigen retrieval, the citrate buffer epitope retrieval method was used instead of W-Cap one-step dewaxing and epitope retrieval technique.

Chemokine receptor expression was studied with a CCR2 rabbit anti-human monoclonal antibody ([ab32144]; Abcam plc, Cambridge, UK) at 1:300 dilution, and CX3CR1 by a rabbit anti-human polyclonal antibody ([AB1891]; Chemicon Europe Ltd, Hampshire UK) at a 1:125 dilution. CA IX was studied by a rabbit anti-human polyclonal antibody ([ab15086]; Abcam plc, dilution 1:500), diluted in Dako REAL Antibody Diluent (S2022; Dako UK Ltd) for 60 min at room temperature. ChemMate DAKO EnVision Detection kit (K5007; Dako UK Ltd) was used for detection of positive staining. In this two-step technique, sections were incubated with the ChemMate DAKO EnVision/HRP, rabbit/mouse reagent for 30 min. Following which, sections were washed with phosphate-

buffered saline and then incubated with 1:40 dilution of ChemMate DAB + Chromogen in ChemMate Substrate Buffer for 10 min. Sections were then mounted in an aqueous medium (Immumount; Life Sciences International, Basingstoke, Hants, UK).

Controls used included omission of the primary antibody and substitution with an irrelevant rabbit IgG (Dako UK Ltd) isotype control at equivalent concentrations in parallel using adjacent sections on the same slide. Sections from renal cell carcinoma tissue were used as positive controls for CA IX staining.

In situ quantification

An interactive image analysis system was used for quantitative assessment of *in situ* antigens as previously described.[2] Coded sections were stained for CD68, CD34, CCR2, CX3CR1, and CA IX, and the image captured digitally, converted to a two-color scale image and processed. For each patient, the mean measurement of five randomly selected nonconfluent microscopic fields of renal cortex was determined. Glomerular staining was excluded from the analysis by the computer software.

Quantification of chronic damage

Chronic damage was assessed as previously described.[2,35]

Real-time PCR

Expression of mRNA for VEGF was assessed using real-time PCR in an ABI 7500 system (Applied Biosystems, Warrington, UK). mRNA had been isolated and reverse transcribed for 33 patients of the original cohort as previously described.[36] The reactions were performed in 25 µl aliquots on a 96-well optical reaction plate (Applied Biosystems). Primers for 18 s were used in conjunction with target primers as an internal reference. Reactions contained TaqMan universal PCR master mix (Applied Biosystems), 500–900 nmol primers, 100–200 nmol TaqMan probe, and 25–50 ng cDNA. Target gene probes were labeled with FAM and 18 s probes were labeled with VIC. Reactions occurred as follows: 50°C for 2 min, 95°C for 10 min, 44 cycles of 95°C for 15 s, and 60°C for 1 min. Data were obtained as C_t values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) according to the manufacture's guidelines, and used to determine ΔC_t values (C_t of target gene$-C_t$ of housekeeping gene) as raw data for gene expression. TaqMan gene expression assays for VEGF (ID: Hs00900054_m1) were purchased from Applied Biosystems.

Statistics

The validity of the methods was assessed as previously outlined and extended to include the other determinants.[2] 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. Correlations are presented by expressing the β-correlation coefficient along with the P-value. Comparison of data, mean or median, was performed using a two-tailed t-test, where data were normally distributed and a Mann–Whitney or Kruskal–Wallis test, where the data were not parametric. Renal outcome was also assessed by Kaplan–Meier survival analysis with logrank testing for interstitial capillary density after categorization in tertiles. All statistical tests were performed using SPSS for Windows, version 14.0.

DISCLOSURE

All the authors declared no competing interests.

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