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Diabetic nephropathy, inflammation, hyaluronan and interstitial fibrosis

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Summary. Hyaluronan (HA) is a ubiquitous connective tissue glycosaminoglycan component of most extracellular matrices and alterations in its synthesis have been suggested to be involved in the glomerular changes of diabetic nephropathy. Similarly it has been suggested that macrophages are involved in the initiation of diabetic glomerular injury. Much less is known regarding the role of the prognostic value of changes in interstitial HA and interstitial inflammatory infiltrate. The aim of this study was to examine the potential association of inflammatory infiltrate, deposition of the matrix component hyaluronan and inter-alpha inhibitor (which is involved in HA assembly) and clinical outcome in diabetic nephropathy.

Histological specimens of 40 patients with biopsy proven diabetic nephropathy were examined. Based on the rate of change in estimated GFR (eGFR, abbreviated MDRD formula), patients were defined as late presenters, progressors or non-progressors.

The degree of interstitial fibrosis was associated with progression of disease and late presentation. There was a significant greater number of CD68-positive cells in the interstitium of patients who subsequently developed progressive renal disease, or those who presented with advanced disease compared to non-progressors. In contrast, there was significant staining for interstitial HA in all the patient groups. Furthermore there was no correlation between the accumulation of HA and CD68-positive macrophages. In addition all patients with biopsy-proven diabetic nephropathy had significantly greater interstitial IαI compared to the normal controls and there was a significant correlation between interstitial HA and IαI.

Increased HA is seen at all stages of diabetic change in the kidney but is not predictive of progression. Macrophage influx, however, is directly related to the progression of diabetic nephropathy and is not associated with HA accumulation.

Key words: Diabetic nephropathy, Hyaluronan, Macrophages, Interstitial fibrosis

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Introduction

Diabetic nephropathy, involving patients with either type I or type II diabetes mellitus, is now the commonest cause of end stage renal disease in the UK, accounting for 20% of all patients requiring renal replacement therapy (UK Renal Registry, 2005). Although the prognosis of patients with diabetic nephropathy has recently improved (Ghavamian et al., 1972; Marcelli et al., 1995), there remains an excess mortality of 70-100 times that of an otherwise matched normal population (Chantrel et al., 1999). Furthermore for patients who require renal replacement therapy, morbidity as assessed by hospitalisation is 2-3 times greater than for non-diabetic patients with end stage renal disease (Marcelli et al., 1995). The overall aim of our work is to establish the mechanisms, which underlie the development of progressive nephropathy in diabetes. More specifically, as it is now evident that progressive decline in renal function in diabetes is closely correlated with the degree of renal interstitial fibrosis (Bohle et al., 1991; Lane et al., 1993), our work has focused on the cellular mechanisms, which drive this.

Diabetes is primarily a metabolic disease therefore the vast majority of papers in this field have focused on mechanisms by which elevated concentrations of glucose alter cell function. Recent studies in experimental models have implicated macrophage infiltration in the pathogenesis of diabetic nephropathy (Young et al., 1995; Lavaud et al., 1996; Sassy-Pringent, et al., 2000). Studies of renal biopsies taken from patients with non-insulin dependent diabetes mellitus have also suggested that macrophages are involved in

the initiation of the glomerular changes (Furuta et al., 1993). To date, however, the prognostic value of an interstitial inflammatory infiltrate, in renal biopsies of patients with diabetic nephropathy has not been defined, although a recent study has demonstrated a correlation between peritubular myofibroblastic cells and interstitial macrophage infiltration (Yonemoto et al., 2006).

It is now clear from many models of inflammation that cell function can be modified by the composition of the extracellular matrix. Hyaluronan (HA) is an ubiquitous connective tissue polysaccharide which *in vivo* is present as a high molecular mass component of the extracellular matrix. It is now known that HA is a major regulator of cell-cell adhesion, migration, proliferation and differentiation, (reviewed in (Laurent, and Fraser, 1992)). As a result it is an important contributor to and a regulator of tissue remodelling, such as that seen at sites of wound healing or fibrosis.

Although HA is not a major constituent of the normal renal corticointerstitium (Hansell et al., 2000), it is highly expressed around proximal tubular cells (PTC) in diverse renal diseases (Wells et al., 1990, 1993; Sibalic et al., 1997; Lewington, et al., 2000). We have previously demonstrated that HA synthesis by PTC *in vitro* was initiated when cells were exposed to high concentrations of glucose (Jones et al., 2001). The alteration in HA generation in the interstitium *in vivo* in diabetic nephropathy however, has not been addressed, and furthermore the functional significance of alterations in HA in the renal tubulo-interstitium is unclear. We have previously demonstrated that it is not simply the presence of HA, but that it assembly into pericellular structures may be critical in determining its effect on cell function (Zhang et al., 2005; Selbi et al., 2006a,b). The inter-alpha trypsin inhibitor (IαI) family includes four plasma proteins, these are free bikunin, IαI, are synthesised predominantly in hepatocytes, we have previously demonstrated that PTC generate PαI variant of the family (Janssen et al., 2001). Heavy chains associated with plasma proteins of the IαI family are known to stabilize pericellular HA (Zhuo et al., 2004). Furthermore we have demonstrated that the heavy chains of PαI are involved in the organisation of pericellular HA-based structures (Selbi et al., 2006a,b).

The aim of the current study was to quantify both inflammatory infiltrate and the expression and organisation of hyaluronan in renal biopsies of patients with diabetic nephropathy. Furthermore, the correlation of these parameters with the degree of interstitial fibrosis and clinical outcome was also assessed.

Materials and methods

Patients

A computer search was compiled of the pathology archive of the Pathology department, University Hospital of Wales, searching for medical renal biopsies with a diagnosis of diabetic nephropathy. Only patients with a histological diagnosis of diabetic nephropathy were included in the study and any patients with co-existing pathology were excluded. Using these selection criteria 41 biopsies performed between 1992 and 2002 were included. For normal controls, 8 biopsies were obtained from live related kidney donors at the time of donor nephrectomy. The study was approved by the local ethics committee. Renal function was defined as the estimated Glomerular Filtration Rate (eGFR) calculated using the four variable Modification of Diet in Renal Disease Study (MDRD) equation and the stage of renal disease derived from a serum creatinine value measured at the time of renal biopsy (National Kidney Foundation, 2002). Patients who had chronic kidney disease (CKD) stage 5 or requiring renal replacement therapy within 6 months of their renal biopsy were considered as late presenters. Patients who had deterioration of ≥ 5ml/min/year in their eGFR (which also equated to a >10% change in the starting GFR), were termed progressors, and those who did not satisfy these criteria were termed non-progressors. All clinical data was collected at the time of presentation/renal biopsy.

Interstitial fibrosis index

Four-micrometer sections cut from paraffin embedded renal biopsies, were de-waxed, stained with periodic acid Schiff (PAS) reagent and counterstained with hematoxylin. For the evaluation of the extent of interstitial fibrosis, each tubulo-interstitial grid field (renal cortical tubulo-interstitium, 10 grid fields each measuring 0.36 mm^2) was graded semi-quantitatively, and the mean score per biopsy was calculated. Injury was graded according to Shih, Hines, and Neilson on a scale of 0 to 4 (0, normal; 0.5, small focal areas of damage; 1, involvement of less than 10% of the cortex; 2, involvement of 10 to 25% of the cortex; 3, involvement of 25 to 75% of the cortex; 4, extensive damage involving more than 75% of the cortex), with expansion of the intersitial space used as a surrogate marker of interstitial fibrosis (Shih et al., 1988).

Immunohistochemistry

Prior to imaging monocytes in the tissue sections endogenous peroxidase activity was quenched by placing the slides in 0.2% Hydrogen Peroxide containing 0.1% Sodium Azide. Sections were incubated with monoclonal mouse anti-human CD68 (Clone PG-M1) primary antibody (1:100 dilution DakoCytomation, Denmark), and incubated overnight at 4°C. The sections were washed prior to visualisation using the Vectastain Elite ABC kit (Vector Laboratories, CA, USA). Briefly, biotinylated secondary antibody at a dilution of 1:200, was added to each section and incubated for 60 minutes at room temperature. Following further washing steps, the Avidin-Biotin complex was applied for 60 minutes at room temperature, and the reaction visualised by placing in diaminobenzidine tetrahydrochloride (DAB) for 10 minutes. The reaction was quenched by washing in

distilled water. A 25-point graticule (G55, Pyser-SGI LTD, Kent, UK) was used with a Leicia DMLA light microscope, and point counting carried out on five random fields for each biopsy sample.

For HA staining, sections were incubated with a biotinylated HA-binding protein (b-HABP), at a concentration of 5 µg/ml (Seikagaku Corporation, Tokyo, Japan) at 4°C overnight. The slides were washed with PBS prior to incubation with fluorescent avidin-D (20 mg/ml) (Vector laboratories, CA, USA), at room temperature for 1 hour. Following a final washing step, specimens were mounted in Vectasheild (Vector Laboratories). IαI was visualised by incubation of sections with rabbit anti human IαI antibody (DakoCytomation, Denmark) was (final dilution 1:200 dilution), overnight at 4°C. The slides were subsequently washed prior to incubation with Alexa Fluor 568nm goat anti-rabbit IgG (1:1000 dilution) (Molecular Probes Inc, OR, USA) at room temperature for 30 minutes. Seven random fields of each sample were visualised on an Olympus BX51 fluorescent light microscope, photographed using a microscope mounted Zeiss Axiocam HRC, and saved on a computer using Axiovision softwear. Each random field photograph was viewed using Adobe Photoshop 7.0 software and positive staining quantified by point counting using a 144-point graticule was superimposed over each photograph. Points overlying glomeruli and blood vessels were subtracted from the total count, to ensure that only cortical points were included in the final analysis.

Table 1. Patient characteristics.

Age	Sex	eGFR presentation	Δ eGFR ml/min/yr
Non progressors			
39	M	48	4.55
66	M	70	4.38
56	M	59	0.58
76	F	17	1.02
72	F	13	4.01
49	M	57	0.3
52	M	59	4.75
62	M	49	2.37
58	M	43	2.6
51	M	36	0.71
40	F	67	1.1
52	M	32	0.5
25	M	45	3.8
60	M	25	1.8
56	M	60	4.25
59	M	31	0.9
47	M	60	0.75
Progressors			
42	F	56	5.7
69	M	14	9
27	F	12	7
55	F	12	7.1
74	M	20	10.1
59	F	15	10.3
52	M	92	11.0
55	M	93	9.53
62	M	46	9.24
58	M	61	6.2
48	M	27	16
44	M	24	7
57	M	30	7
29	M	28	14.1
77	F	17	10
Late presenters			
73	M	5	n/a
75	M	7	n/a
60	F	6	n/a
40	M	7	n/a
35	F	5	n/a
50	M	8	n/a
53	M	6	n/a
45	M	9	n/a
77	M	10	n/a

Statistical analysis

Statistical analyses were performed using SPSS version 11.0.2 software (SPSS Inc, Chicago, IL). Differences between groups were tested using analysis of variants (ANOVA) with the Bonferroni post hoc test used for multiple comparisons. A value of $P<0.05$ was considered to represent a significant difference.

Results

Patient characteristics

The patient characteristics are shown in Table 1. There was no significant difference between the patient age and sex distribution in the patients categorised into the non-progressor, progressor and late presenter groups. Patients categories as non-progressors had a significantly longer duration of follow up to progressors

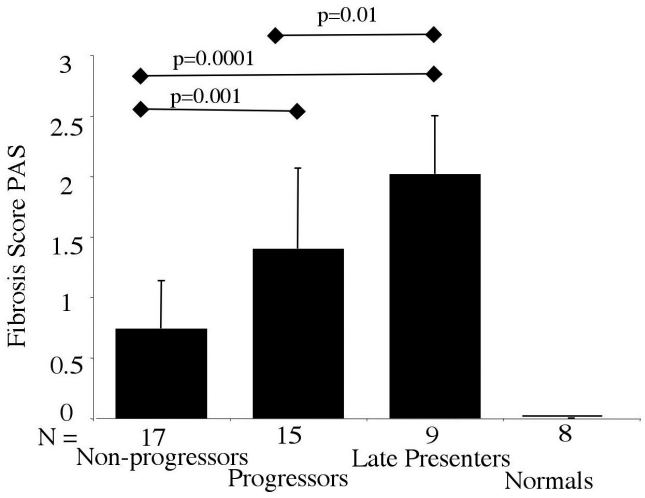

Fig. 1. Interstitial fibrosis score for normal renal biopsies and patients with diabetic nephropathy according to renal outcome. Data represents median and inter-quartile range, with open circles representing outliers (>2sd from the population mean).

(6.11±2.75years vs 3.67±2.85years, p=0.01), thus suggesting that differences between groups were not a reflection of an inadequate period of follow up.

For each patient, the mean of all blood pressure, cholesterol and blood glucose measurements for the six-month period following the renal biopsy was taken a reflection of these risk factors at the time of presentation/biopsy. There were no significant differences in any of these clinical parameters between the three patient groups (Table 2).

Furthermore there was no significant difference in the percentage of patients on an ACE inhibitor at the time of biopsy between each of the patient groups (29% non progressors, 30% progressors, 37% late presenters).

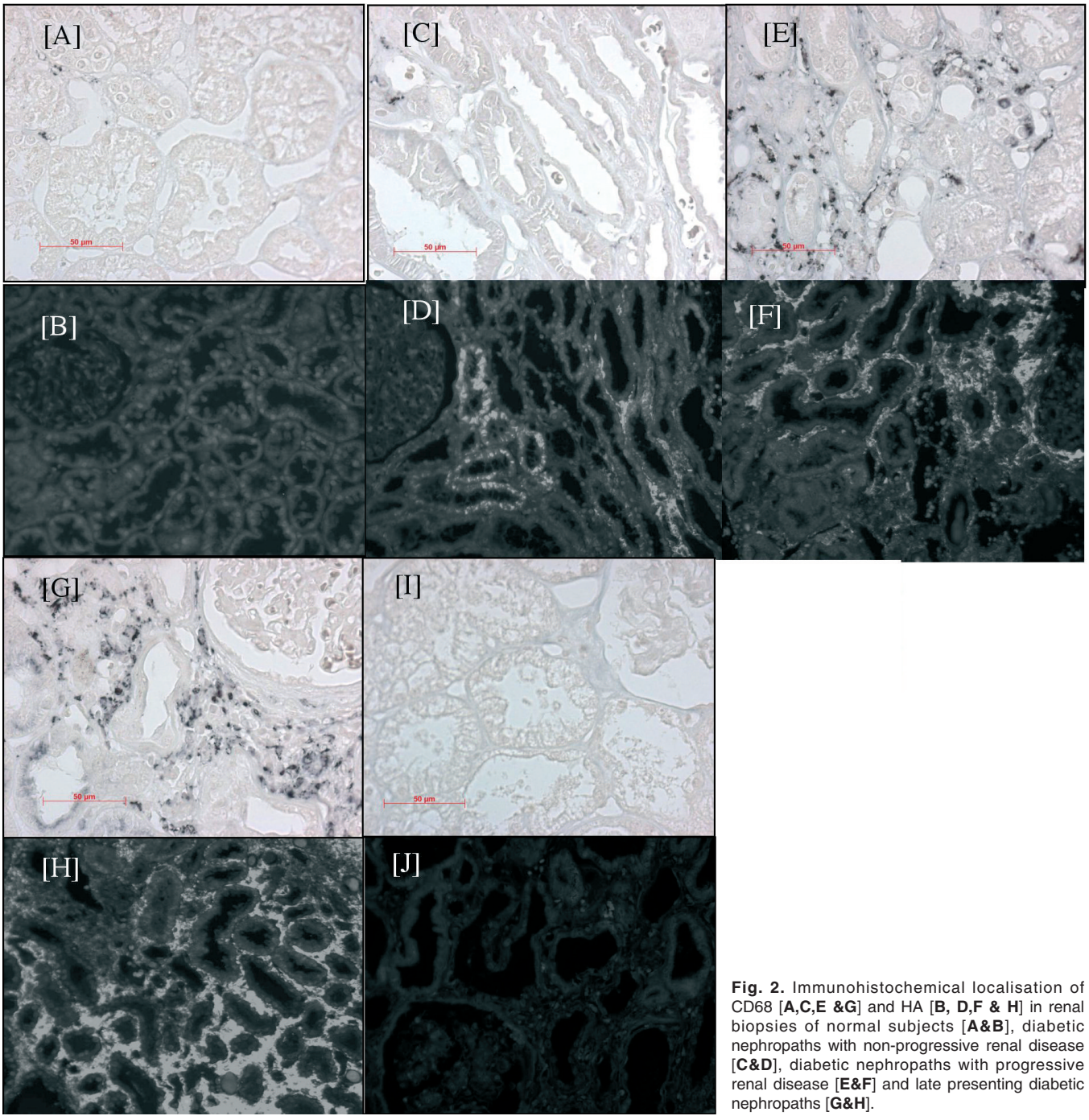

Fig. 2. Immunohistochemical localisation of CD68 [**A,C,E &G**] and HA [**B, D,F & H**] in renal biopsies of normal subjects [**A&B**], diabetic nephropaths with non-progressive renal disease [**C&D**], diabetic nephropaths with progressive renal disease [**E&F**] and late presenting diabetic nephropaths [**G&H**].

Interstitial fibrosis predicts outcome

Compared to normal control biopsies, the fibrosis score was significantly greater for all patients with diabetic nephropathy (Fig. 1). The fibrosis score was significantly higher in patients who subsequent developed progressive renal disease compared to non-progressors. The fibrosis score was highest for those patients with stage 5 CKD at presentation.

Inflammatory cell infiltrate predicts outcome

Representative images of CD68 staining of renal biopsy sections for each of the patient groups are shown in Figure 2. Very few CD68 positive cells were detected in the normal renal interstitium (Fig. 2A). In contrast, there was a significant increase in the number of CD68 positive cells seen in the patients with diabetic nephropathy (Figure 2C,E,G). There was a positive correlation between the interstitial fibrosis index and the number of infiltrating CD68 positive cells (Fig. 3A). All patients with diabetic nephropathy had significantly greater numbers of infiltrating CD68 positive cells compared to the normal controls. In addition within the individual groups there was also a significant greater number of CD68 positive cells in the "progressors" and "late presenters" as compared to the "non-progressors"

Table 2. Clinical parameters.

	Non progressors	Progressors	Late Presenters	
SBP mmHg (mean±sd)	146.8±12.0	153.6±116.6	128.4±26.1	n/s
DBP mmHg (mean±sd)	82.7±8.7	85.3±8.9	71.4±9.9	n/s
Cholesterol mmol/l (mean±sd)	4.7±0.6	6.3±1.8	4.9±1.6	n/s
Glucose mmol/l (mean±sd)	14.3±8.7	12.79±8.9	8.3±2.12	n/s

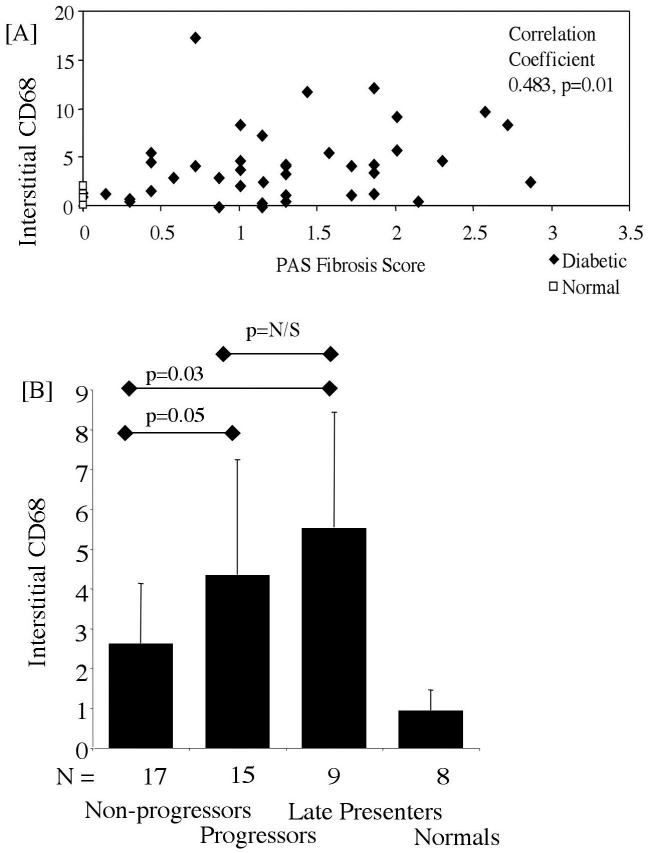

Fig. 3. Correlation between interstitial CD68 count and fibrosis score for both normals (open squares) and all diabetic nephropaths (closed diamonds) [**A**] and interstitial CD68 count for normal renal biopsies and patients with diabetic nephropathy according to renal outcome [**B**]. Data in **B** represents median and inter-quartile range. * p<0.001 as compared to each of the diabetic nephropathy groups. Negative control for CD68 was generated by replacement of primary antibody with mouse IgG and HA by omission of the bHABP.

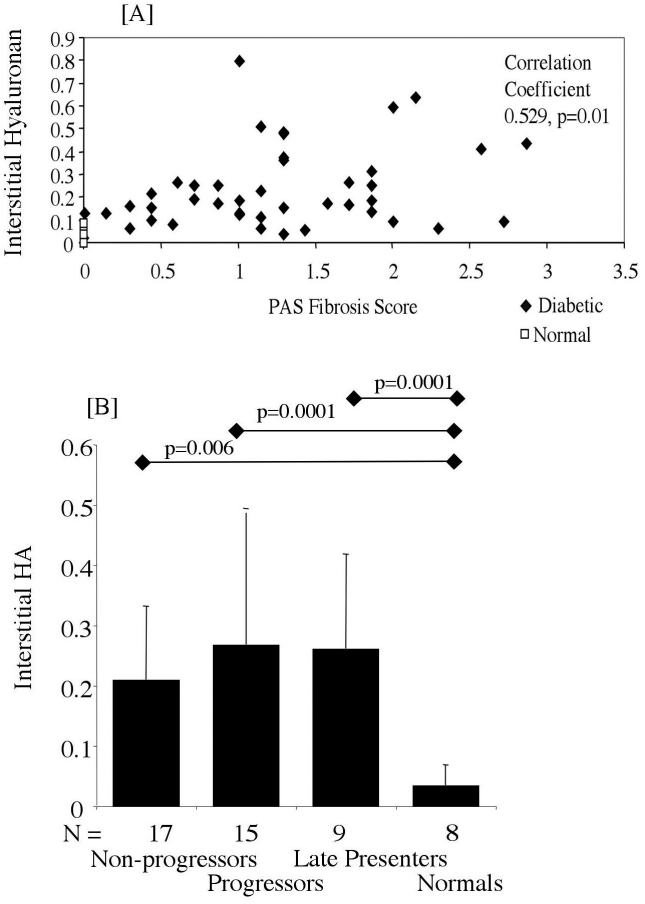

Fig. 4. Correlation between interstitial HA score and fibrosis score for both normals (open squares) and all diabetic nephropaths (closed diamonds) [**A**] and interstitial HA score for normal renal biopsies and patients with diabetic nephropathy according to renal outcome [**B**]. Data in **B** represent median and inter-quartile range. P>0.05 between each of the diabetic nephropathy groups.

(Fig. 3B).

Interstitial HA is increased in diabetic nephropathy but does not predict outcome

Representative images of HA staining for each of the patient groups are shown in Fig. 2. There was negligible staining for HA in the corticointerstitium of normal renal tissue (Fig. 2B). In contrast, all patient groups with diabetic nephropathy showed strong interstitial staining for HA (Fig. 2D-H) and there was a significant positive correlation between fibrosis score and the expression of

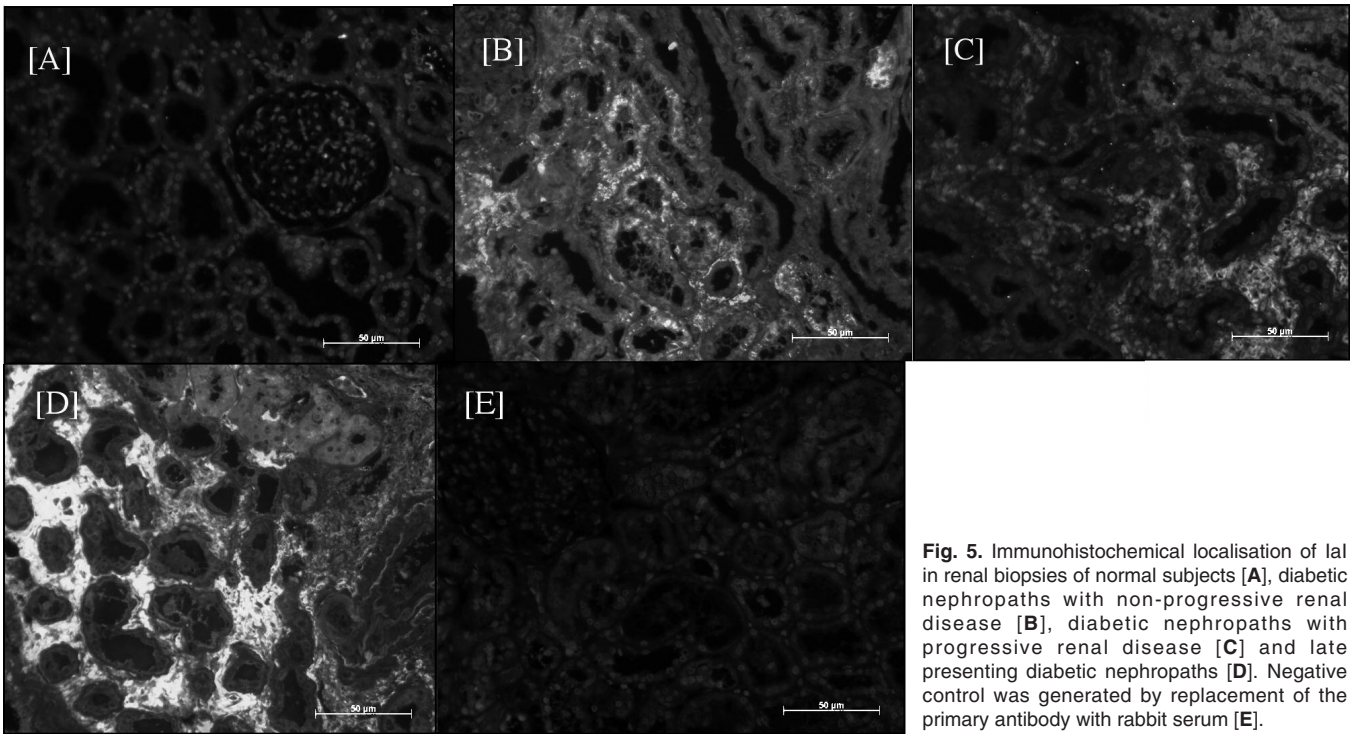

Fig. 5. Immunohistochemical localisation of IaI in renal biopsies of normal subjects [**A**], diabetic nephropaths with non-progressive renal disease [**B**], diabetic nephropaths with progressive renal disease [**C**] and late presenting diabetic nephropaths [**D**]. Negative control was generated by replacement of the primary antibody with rabbit serum [**E**].

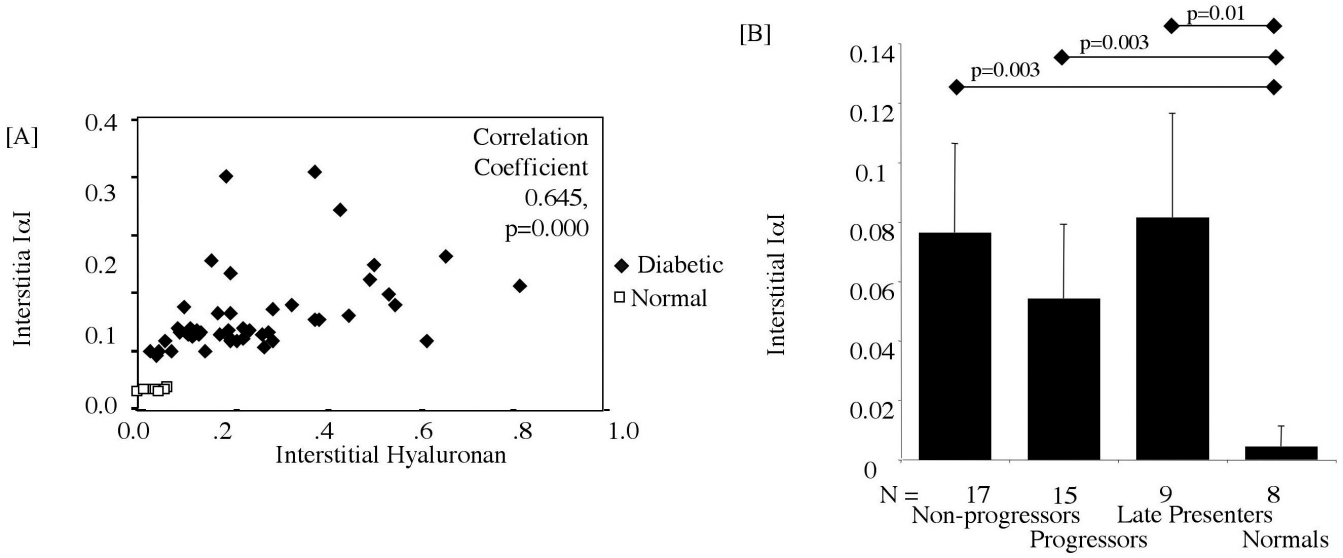

Fig. 6. Correlation between interstitial HA score and interstitial Ial score for both normals (open squares) and all diabetic nephropaths (closed diamonds) [**A**] and interstitial HA score for normal renal biopsies and patients with diabetic nephropathy according to renal outcome [**B**]. Data in **B** represents median and inter-quartile range, with open circles representing outliers (>2sd from the population mean).

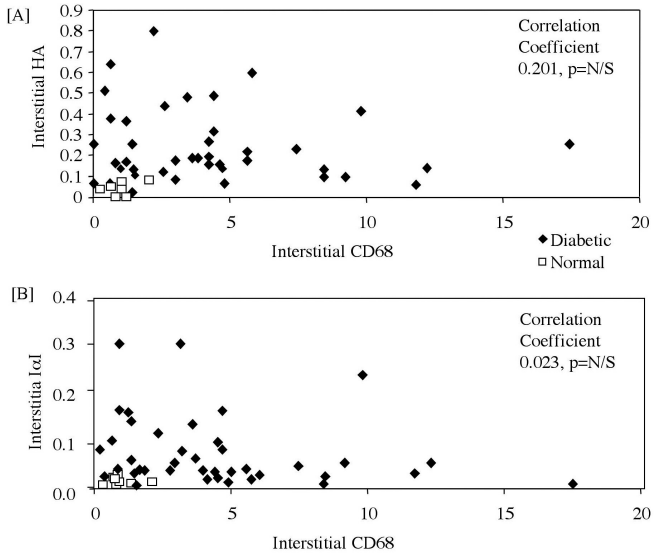

Fig. 7. Lack of correlation between interstitial CD68 count and HA score for both normals (open squares) and all diabetic nephropaths (closed diamonds).

hyaluronan in the corticointerstitium (Fig. 4A). Although all patients with biopsy proven diabetic nephropathy had significantly greater interstitial HA compared to the normal controls, there was no significant difference in the interstitial HA score between patients categorised as "progressors", "non-progressors" or those classified as late presenters (Fig. 4B). As with HA all patients with biopsy-proven diabetic nephropathy had significantly greater interstitial IαI compared to the normal controls (Fig. 5) and there was a significant correlation between interstitial HA and IαI (Fig. 6A). There was, however, no significant difference in the interstitial IαI score between "progressors", "non-progressors" and late presenters (Fig. 6B).

Interstitial HA does not correlate with inflammatory infiltrate

Despite the correlation of both HA and CD68 with the fibrosis score, there was no direct correlation between interstitial HA staining and the CD68 cell counts in diabetic nephropaths (Fig. 7A). Similarly there was no significant correlation between interstitial IaI staining and the CD68 counts (Fig. 7B).

Discussion

The importance of interstitial fibrosis in predicting the outcome of patients with diverse renal diseases including diabetic nephropathy has been well established by the numerous studies of Bohle and his colleagues (Bohle et al., 1991). Consistent with these studies we have demonstrated that a higher interstitial fibrosis score clearly predicts a poor renal outcome in that it picks out those patients who are approaching end stage at the time of renal biopsy and those who were likely to progress over the follow-up period following the initial biopsy.

The striking and novel finding in this study is that the presence of an inflammatory infiltrate as a prognostic marker in human diabetic nephropathy. A significant increase in the number of CD68 positive cells was demonstrated in both those patients with stage5 CKD, and also those in whom there was a significant deterioration of renal function during the study follow-up period. Of note however there was no statistical increase in the number of inflammatory cells between normal renal tissue and the biopsies of patients whose renal function was maintained over the study period. These data are consistent with those seen in experimental models of diabetic nephropathy. Studies utilizing the db/db mouse, a model of type II diabetes, have demonstrated macrophage accumulation and activation in the kidneys associated with prolonged hyperglycaemia and progressive renal injury (Chow et al., 2004). A pathogenic role for infiltrating macrophages is supported by studies by the same group demonstrating that ICAM-1 deficiency is protective against nephropathy in this model (Chow et al., 2005). As with the type II db/db model, ICAM-1 deficient mice are resistant to renal injury after streptozotocin induced diabetes (Okada et al., 2003). In contrast to studies in experimental models of diabetic nephropathy, far fewer studies have characterised the involvement of macrophages in human diabetic nephropathy. Studies characterising the role of macrophages in human diabetic nephropathy have focused on glomerulosclerosis in patients with type II diabetes (Furuta et al., 1993). Their association with interstitial fibrosis and the prognostic significance of an interstitial inflammatory infiltrate in patients with diabetic nephropathy however has not been previously described.

Although numerous studies have demonstrated an association between increased staining for HA in the renal cortex following injury, the functional significance of this is not clear. A role for HA in the pathogenesis of diabetic nephropathy has been postulated following the publication of the observations that increased hyaluronan production, is involved in initiation of glomerular hypercellularity in the streptozotocin model of diabetes (Mahadevan et al., 1995). In addition we have previously demonstrated that exposure of proximal tubular cells to elevated D-glucose concentrations stimulates the synthesis of HA (Jones et al., 2001). It is generally assumed that the association between renal injury and increased HA generation suggests that HA may be involved in the pathogenesis of progressive disease. Work performed *in vitro*, however, has suggested that HA may have either disease promoting activity or potentially beneficial disease-limiting effects. Hyaluronan of low molecular weight has been demonstrated to induce monocyte chemoattractant protein-1 expression (Beck-Schimmer et al., 1998) and up-regulate of ICAM-1 and VCAM-1 (Oertli et al., 1998) in renal tubular epithelial cells. In contrast we have demonstrated that PTC form peri-cellular HA

cable-like structures that bind mononuclear leukocytes *via* their cell surface HA receptor CD44 (Selbi et al., 2004). HA based cables modulate the interaction between infiltrating inflammatory cells and resident cells, and decrease monocyte dependent TGF-ß1 generation by the epithelial cells (Selbi et al., 2004; Zhang et al., 2004). Furthermore engagement of CD44 by HA in PTC, attenuates signalling of the pro-fibrotic cytokine TGF-ß1 (Ito et al., 2004a,b).

While HA staining was negligible in our normal patient group, all patients with diabetic nephropathy had elevated corticointerstitial HA staining. This is consistent with our previous *in vitro* work demonstrating that exposure of proximal tubular epithelial cells to 25 mM D-glucose stimulates transcriptional activity of the HAS2 isoform of hyaluronan synthase and a concomitant increase in HA synthesis (Jones et al., 2001). Previous studies have shown that the heavy chains associated with plasma proteins of the IαI family stabilize pericellular HA (Zhuo et al., 2004). The positive correlation in the expression of IαI and HA, supports the concept that HC transfer may be important in stabilization of extracellular HA associated with renal fibrosis. Although there was a correlation between HA staining and the degree of interstitial fibrosis, unlike the presence of an inflammatory infiltrate, a change in HA staining was unable to distinguish between patients who subsequently developed progressive renal failure from those whose renal function was maintained over the study period.

The overall aim of this study was to examine the potential association between HA, interstitial inflammation and interstitial fibrosis. It is clear that the presence of macrophages in the renal interstitium differentiates patients who are likely to develop progressive interstitial fibrosis from those who do not progress to end stage renal failure. In contrast, HA is a marker of interstitial fibrosis and is present in all patients with diabetic nephropathy. The lack of correlation of HA staining with the CD68 count suggests that interstitial HA is not involved in inflammatory cell recruitment and is unlikely that it plays a direct role promoting the fibrotic response. In contrast an inflammatory infiltrate, may be involved in the pathogenesis of progressive renal interstitial fibrosis in diabetic nephropathy.

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