The sialoadhesin (CD169) expressing a macrophage subset in human proliferative glomerulonephritis

Yohei Ikezumi¹, Toshiaki Suzuki¹, Shinichi Hayafuji¹, Soichiro Okubo¹, David J Nikolic-Paterson³,⁴, Hiroshi Kawachi², Fujio Shimizu² and Makoto Uchiyama¹

¹Division of Pediatrics and ²Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Japan, ³Department of Nephrology and ⁴Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia

Abstract

Background. Sialoadhesin (Sn; CD169) is a lectin-like receptor whose expression is restricted to subsets of tissue and inflammatory macrophages. We have previously identified accumulation of Sn+ macrophages as an important marker of disease progression versus remission in rat mesangial proliferative nephritis. The current study examined the significance of Sn+ macrophages in human proliferative glomerulonephritis.

Methods. Frozen kidney sections from normal adult human kidney (n = 4) and pediatric nephropathy (n = 40) were stained for total macrophages (CD68+ cells), Sn+ macrophages, CD3+ T-cells and collagen type I by immunofluorescence. Leukocyte infiltration and the severity of glomerular lesions and interstitial damage were scored. A second protocol biopsy was performed in 27 cases and clinical and biopsy-based data obtained.

Results. Sn+ macrophages were absent from glomeruli in normal adult human kidney and in thin basement membrane disease (n = 4), but were detected in 4 of 9 cases of purpura nephritis; 7 of 17 IgA nephropathy; 5 of 5 membranoproliferative glomerulonephritis, and 5 of 5 lupus nephritis. Sn+ macrophages were localized in areas of focal glomerular and interstitial damage. Two-colour immunostaining confirmed that Sn+ cells are a subset of total CD68+ macrophages. The number of glomerular Sn+ macrophages correlated with the degree of proteinuria and glomerular lesions (r = 0.44, \( P = 0.0045 \) and \( r = 0.82, P < 0.0001 \); respectively), while interstitial Sn+ macrophages correlated with the degree of proteinuria and interstitial damage (r = 0.59, \( P < 0.0001 \) and \( r = 0.75, P < 0.0001 \); respectively). Combined immunostaining revealed that interstitial Sn+ macrophages and CD3+ T-cells co-localized in areas of tubulointerstitial damage with increased type I collagen deposition. There was significant correlation between the number of interstitial Sn+ macrophages and CD3+ T-cells (r = 0.74, \( P < 0.0001 \)). Most patients responded to a 2 year period of glucocorticoid therapy with a reduction in proteinuria and glomerular lesions and this correlated with the reduction in the number of glomerular Sn+ macrophages.

Conclusion. This study has identified Sn+ cells as a macrophage subset whose accumulation in the kidney correlates with proteinuria and histologic damage. These results, together with recent findings from animal studies, suggest that Sn+ macrophages may play an important role in progressive renal disease.

Keywords: ED3; fibrosis; macrophage activation; proteinuria; T-cells

Introduction

Macrophages have been implicated in the initiation and/or progression of most types of primary and secondary human glomerulonephritis [1]. In addition, many temporal studies in animal models of kidney disease have shown a clear association between macrophage accumulation and the development of renal injury [1]. Furthermore, the degree of macrophage accumulation is predictive of disease progression in IgA nephropathy and lupus nephritis [2,3]. These studies suggest, but do not prove, a role of macrophages as mediators of renal injury. Indeed, macrophages can perform many different functions according to the local microenvironment in which they are...
Table 1. Patient data

<table>
<thead>
<tr>
<th></th>
<th>At the first biopsy</th>
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</tr>
<tr>
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<td>9</td>
<td>5:4</td>
</tr>
<tr>
<td>IgAN</td>
<td>17</td>
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<tr>
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<td>5</td>
<td>2:3</td>
</tr>
<tr>
<td>SLE</td>
<td>5</td>
<td>2:3</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>Normal kidney portion from adult renal carcinoma nephrectomy</td>
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</tbody>
</table>

sCr, serum creatinine; CCr, creatinine clearance standardized to the surface area (1.73 m²); UTP, proteinuria; TBMD, Thin basement membrane disease; HSPN, Henoch-Schönlein purpura nephritis; IgAN, Immunoglobulin A nephropathy; MPGN, Membranoproliferative glomerulonephritis; SLE, Lupus nephritis.

*p < 0.001, †p < 0.05 compared with the same group at the time of the first biopsy.

present, such as a pro-inflammatory response to microbial infection, removal of apoptotic cells, down-regulation of the innate/immune response, and tissue repair [4]. Indirect evidence that macrophages can induce kidney disease comes from animal studies in which different methods of macrophage depletion (with variable specificity) have resulted in reduced renal injury [1]. Direct evidence that macrophages can mediate renal injury has come from adoptive transfer studies in acute rat anti-GBM glomerulonephritis [5]. The importance of macrophage activation in this process has been demonstrated by the ability of interferon-gamma to augment macrophage-mediated renal injury [6]. Thus, while macrophage activation is clearly a critical aspect of renal injury in experimental kidney disease models, little is known of macrophage activation in human glomerulonephritis.

Although the precise mechanisms of macrophage activation within specific microenvironments is poorly understood, these cells can express a discrete set of surface receptors which are specific for their particular anatomical/functional location. Sialoadhesin (Sn; CD169) is one such receptor. Originally identified as an erythrocyte receptor expressed by subsets of “activated” macrophages, Sn has been characterized as a member of the immunoglobulin superfamily uniquely expressed by macrophages and is a prototypical member of the family of sialic acid binding proteins [7]. Sialoadhesin expression can be induced in macrophages by a variety of stimuli [8,9]. Sialoadhesin expression is normally restricted to macrophages in lymphoid tissue [10,11]; however, accumulation of Sn+ macrophages is observed in chronic inflammatory lesions such as in rheumatoid arthritis, multiple sclerosis and tumors [12,13].

We have previously demonstrated that both CD4+ T-lymphocytes and Sn+ macrophages accumulate in glomeruli and contribute to the development of irreversible glomerular and interstitial damage in a rat model of chronic mesangioproliferative nephritis [13,14]. These findings suggest that Sn+ macrophages may contribute to disease progression in human chronic kidney diseases. Because of the highly restricted pattern of expression of Sn in phenotypically and functionally distinct subpopulations of macrophages, the Sn+ macrophage subset may be an important player, or marker, in the progression of human chronic glomerulonephritis.

The aim of the present study was to determine whether Sn+ macrophages are present in human proliferative glomerulonephritis, and if Sn+ macrophages may play a role in disease progression.

Materials and methods

Patients

Thirty-six patients undergoing diagnostic renal biopsy at the Department of Pediatrics, Niigata University Medical and Dental Hospital, were examined. Patients gave informed consent for the use of renal biopsy tissue, in excess of that required for diagnostic purposes, to be used for research purposes. Disease categories were based on histologic examination of biopsy specimens with clinical findings used to help distinguish between Henoch-Schönlein purpura nephritis (HSPN) and immunoglobulin A nephropathy (IgAN). Details of the patients are summarized in Table 1. All patients underwent the first renal biopsy before commencing steroid treatment and most of the patients in the HSPN, IgAN and membranoproliferative glomerulonephritis (MPGN) groups underwent a second protocol biopsy 2 years (2.2±0.4 years) later, before the completion of the treatment, and clinical data were collected at this time (Table 1). Renal tissue from the uninvolved portion of adult renal carcinoma nephrectomies was used as normal tissue controls. Serum creatinine (sCr), creatinine clearance (CCr), and 24 h protein excretion were determined at the time of biopsy with assays performed by the Department of Biochemistry, Niigata University Medical and Dental Hospital.

Quantification of histologic damage in renal biopsies

For light microscopy, a part of the kidney was fixed with Carnoy's solution, embedded in paraffin and a section was stained with periodic acid-Schiff and periodic acid-methenamine silver. The specimens were reviewed and analysed by an independent anatomic pathologist who was blinded to the
Table 2. Quantification of glomerular alteration

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<td>25–50%</td>
<td>50–75%</td>
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<td>~25%</td>
<td>25–50%</td>
<td>50–75%</td>
<td>global</td>
</tr>
<tr>
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<td>25–50%</td>
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<td>global</td>
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<tr>
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<td>Moderate</td>
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<td>Double contours</td>
<td>(–)</td>
<td>(+)</td>
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<td>Extra capillary change</td>
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<td>(+)</td>
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<tr>
<td>Crescent formation</td>
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<td>cellular</td>
<td>fibrocellular/fibrous</td>
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</table>

Quantification of macrophage accumulation. Sections were analysed for: the total number of glomeruli; the number of globally sclerosed glomeruli; glomeruli with crescents, proliferative lesions, and segmental sclerotic lesions; and, changes in glomerular basement membrane (GBM) including thickening and the formation of double contours. The degree of glomerular mesangial proliferation and matrix expansion was scored according to the percentage of glomerular involvement as shown in Table 2. At least 12 (range from 12 to 43, mean 19.5) glomeruli were examined in each patient and the average glomerular damage score calculated. The degree of interstitial inflammation and the degree of interstitial fibrosis/tubular atrophy was scored between 0 and 4 according to the biopsy area demonstrating fibrosis and tubular atrophy as following: 0 (none), 1 (0–5%), 2 (6–25%), 3 (25–50%) and 4 (>50%). At least nine high power fields were evaluated for each patient and expressed as mean ± SD.

Antibodies

Monoclonal antibodies used in this study were: HSn7D2, anti-human sialoadhesin (mouse IgG1; Serotec, Oxford, UK); Y1/82A, anti-CD68 labels human monocyte and macrophages (mouse IgG2b; BD Biosciences Pharmingen, San Diego, CA, USA); and Cris-7, anti-human CD3 labels human T lymphocytes (mouse IgG2a; Cymbus Biotechnology, Chandlers Ford, UK). Other antibodies used were: rabbit anti-human collagen type I (Cosmo Bio, Tokyo, Japan); fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG1 (for HSn7D2 (Southern Biotechnology Associates, Birmingham, AL, USA); tetramethyl-rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG2b (Southern Biotechnology Associates) (for Y1/82A); and aminomethylcoumarin (AMCA)-conjugated goat anti-rabbit IgGs (Chemicon, Temecula, CA, USA).

Immunofluorescence

Tissue samples for immunofluorescence studies were snap-frozen in pre-cooled n-hexane and stored at ~70°C. Macrophages, T-lymphocytes and collagen type I were detected in 3 μm frozen sections by indirect immunofluorescence. The number of cells stained for Sn, CD68 or CD3 antigens were counted at least in 12 glomeruli per patient. Interstitial cells stained for Sn, CD68 or CD3 antigens were counted at least in nine high-power fields (×400).

To examine whether Sn+ cells also expressed the CD68 antigen (a pan macrophage marker), two-colour immunofluorescence studies were performed on tissue sections using HSn7D2 (anti-Sn; mouse IgG1) and Y1/82A (anti-CD68; mouse IgG2b) as primary antibodies followed by incubation of sections with FITC-conjugated anti-mouse IgG1 and TRITC-conjugated goat anti-mouse IgG2b. Interstitial fibrosis was detected by indirect immunofluorescence staining of collagen type I using AMCA-conjugated goat anti-rabbit IgGs as secondary antibody. Three-colour immunofluorescence staining using anti-Sn (HSn7D2), anti-CD3 (cris 7) and anti-collagen type I antibodies.

Statistical analyses

Comparisons were made between two groups by Mann–Whitney test (GraphPad 4.0, San Diego, CA). The Pearson single correlation analysis was used to compare quantification of glomerular, tubular and interstitial Sn, CD68 or CD3 staining with proteinuria. The Spearman correlation analysis was used to compare the number of glomerular or interstitial Sn, CD68 or CD3 cells with the degree of glomerular or interstitial damage, respectively. Data are shown as the mean ± SD.

Results

Patients

All patients underwent renal biopsy before commencing treatments such as steroids and other immunosuppressants (Table 1). No patients presented with renal dysfunction at the time of biopsy as assessed by serum creatinine levels and CCr except for one patient with lupus nephritis who presented with a slight reduction in CCr (73 ml/min/1.73). Most of the patients, except those with thin basement membrane disease (TBMD), presented with proteinuria, while 1 of 9 HSPN, 5 of 17 IgAN, 1 of 5 MPGN, and 1 of 5 lupus nephritis patients presented with nephrotic syndrome with proteinuria greater than 3.5 g/day.

Accumulation of Sn+ macrophages in glomerulonephritis

Immunofluorescence staining revealed small numbers of Sn+ cells in the interstitium of normal adult human kidney, but Sn+ cells were absent from glomeruli...
Patients with proliferative glomerulonephritis showed many glomerular and interstitial Sn$^+$ cells (Fig. 1C–E). Glomerular accumulation of CD3$^+$ T lymphocytes was also observed in some patients with proliferative glomerulonephritis (Fig. 1F).

Glomerular accumulation of Sn$^+$ cells was not seen in adult normal kidney or in TBMD, but was observed in 4 of 9 cases of purpura nephritis (44%); 8 of 17 cases of IgA nephropathy (47%); 5 of 5 cases of MPGN (100%), and 5 of 5 cases of lupus nephritis (SLE) (100%). In contrast, significant glomerular accumulation of CD68$^+$ macrophages was seen in all patients (Figure 2A). In IgAN, glomerular Sn$^+$ cells were detected in only those patients with severe proteinuria (mean 2.7 g/day), and in 5 of 8 cases presenting with nephrotic syndrome (mean proteinuria of 3.8 g/day). In lupus nephritis, despite the variety of histological change (two with WHO class II, two with class III and one with class IV), Sn$^+$ cells were detected in all patients.

Dual immunofluorescence staining revealed that Sn$^+$ cells also express CD68, a pan macrophage marker, confirming that Sn$^+$ cells are a subpopulation of macrophages (Figure 3A–C). Most of the glomerular macrophages in patients with MPGN or SLE expressed Sn, while only 30–40% of glomerular macrophages in HSPN or IgAN expressed Sn (Figure 2A). In contrast, most interstitial CD68$^+$ macrophages expressed Sn in all disease groups (Figure 2B).

Taking all patients as a single cohort, there was a significant correlation between the number of glomerular Sn$^+$ cells and the degree of proteinuria and glomerular lesions. There was also a significant correlation between the number of interstitial Sn$^+$ cells and the degree of proteinuria and interstitial damage.
Fig. 1. A comparison of renal accumulation of Sn+ cells and CD68+ macrophages. (A) The number of glomerular Sn+ cells (closed bars) and CD68+ macrophages (open bars) per glomerular cross-section (gcs). (B) The number of interstitial Sn+ cells (closed bars) and CD68+ macrophages (open bars) per high power field (HPF). Statistical analysis used Mann–Whitney test.

Fig. 2. Combined immunofluorescence studies. Immunofluorescence staining of a case of lupus nephritis showing glomerular (A) Sn+ cells (green), and (B) CD68+ macrophages (red). Overlay of the stains reveals yellow staining indicating that most glomerular CD68+ macrophages also express Sn, demonstrating that Sn+ cells are a subset of CD68+ macrophages (C). Combined immunofluorescence of a case of lupus nephritis shows the presence of interstitial (D) Sn+ cells (green), (E) T-lymphocytes (red), and (F) deposition of collagen type I (blue). Overlay of the stains reveals co-localisation of Sn+ cells and T-cells in an area of tubulointerstitial damage with increased type I collagen deposition (G).
Fig. 4A–D. In addition, the total number of CD68+ macrophages in the glomerular and interstitial compartments correlated significantly with proteinuria and tissue damage (Fig. 4E–H). In contrast, the calculated CD68+Sn− macrophage subset did not correlate with proteinuria or with glomerular or tubulointerstitial damage.

CD68+ macrophages were identified within glomerular crescents, but only rare Sn+ cells were seen in crescents. Whether this reflects that macrophages
within crescents are recently recruited blood monocytes that have yet to express Sn, or if crescent macrophages have a different phenotype compared to the macrophages in the glomerular tuft and interstitium is not clear. However, analysing all patients as a single cohort, the percentage of glomerular crescents correlated with glomerular \((r = 0.42; P = 0.038)\) and interstitial \((r = 0.51; P = 0.0003)\) Sn+ macrophages and with glomerular \((r = 0.44; P = 0.025)\) and interstitial \((r = 0.39; P = 0.0066)\) CD68+ macrophages.

Combined immunofluorescence staining revealed that interstitial Sn+ cells and CD3+ T-cells often co-localized in areas of tubulointerstitial damage with increased type I collagen deposition (Figure 3D–G). Furthermore, there was a significant correlation between the number of glomerular Sn+ cells and CD3+ T-cells \((r = 0.76, P < 0.0001)\) and between interstitial Sn+ cells and CD3+ T-cells \((r = 0.74, P < 0.0001)\). In contrast, there was no correlation between the CD68+Sn− macrophage subset and CD3+ T cells in the glomerulus or interstitium.

All patients received glucocorticoid-based immunosuppression, which reduced proteinuria in most cases and all patients maintained normal renal function (Table 1). Patients with HSPN, IgAN or MPGN underwent a second, protocol biopsy which was analysed for leukocytes by immunofluorescence. As a single cohort, patients showed a significant reduction in proteinuria and glomerular lesions at the second biopsy, although tubulointerstitial damage was unaltered (Figure 5A–C). There was also a significant reduction in the number of glomerular and interstitial CD68+ total macrophages, Sn+ macrophages and CD3+ T cells in response to glucocorticoid therapy (Figure 5D–I).

The change in the number of glomerular Sn+ macrophages in response to glucocorticoid therapy correlated with the change in proteinuria and glomerular lesions seen in response to therapy (Table 3). In addition, the change in the number of glomerular CD68+ total macrophages and CD3+ T cells correlated with the change in proteinuria and glomerular lesions. Among the changes in the number of interstitial leukocyte populations, a significant correlation with the change in proteinuria was only seen with interstitial Sn+ macrophages. The change in the number of interstitial leukocyte populations failed to correlate with change in tubulointerstitial damage (Table 3). The change in the number of the CD68+Sn− macrophage subset did not correlate with the change in proteinuria or histologic damage. Of note, the number of glomerular Sn+ cells correlated with the severity of proteinuria at the time of the second biopsy \((r = 0.59; P = 0.0012)\), whereas the CD68+, CD3+ and CD68+Sn− cell populations failed to correlate.

**Discussion**

Macrophages have been implicated as mediators of renal injury in human glomerulonephritis based upon correlative studies at the time of biopsy and as predictors of disease progression [1–3]. However, macrophages are highly heterogeneous cells and have the potential to perform many functions, including those involved in tissue repair [4]. Thus, it has been proved to be difficult to attribute specific functions to the macrophage infiltrate seen in individual biopsies. Most studies examining macrophage functional heterogeneity have been performed in vitro using functional bioassays or altered expression of individual gene products in bone marrow-derived macrophages or blood monocytes. A few studies have examined macrophage subsets in human glomerulonephritis. One such study found that the expression of the S100 family members, MRP8 and MRP14, and the formation of MRP8/MRP14 complexes in glomerular macrophages correlated with the severity of the inflammatory process [15]. In a separate study examining the T-cell co-stimulation molecule CD86, glomerular and interstitial CD86+ cells were found to be at least 60% macrophages. Interstitial CD86+ cells, but not glomerular CD86+ cells, were found to correlate with renal dysfunction [16].

There are very few examples of molecules that can be used as specific markers of macrophage ‘activation’ in the setting of the renal biopsy. Sialoadhesin is one candidate marker of macrophage ‘activation’ since: (i) Sn expression is restricted to the macrophage lineage; (ii) Sn is not expressed by blood monocytes, (iii) Sn expression is induced by pro-inflammatory cytokines, and; (iv) Sn+ macrophages can present antigen to T-cells.

In the current study, glomerular and interstitial Sn+ macrophages were present in greatest numbers in the more severe types of glomerulonephritis (MPGN and lupus nephritis), but were only seen in approximately half of the cases of IgAN and purpura nephritis. The number of glomerular and interstitial Sn+ macrophages showed a significant correlation with proteinuria and histologic damage. Glucocorticoid-based therapy of patients with HSPN, IgAN and MPGN was effective in reducing proteinuria and glomerular lesions. This response correlated with the change in the number of glomerular and interstitial Sn+ macrophages. In addition, the degree of proteinuria observed after 2 years of glucocorticoid therapy correlated with the number of glomerular Sn+ macrophages present in the second biopsy. These data suggest that Sn+ macrophages may play a role in mediating renal injury and that this population is amenable to suppression by glucocorticoid therapy in these patients. However, these data do not rule out the possibility that the accumulation of Sn+ macrophages simply reflects the severity of proteinuria, although the lack of correlation between the number of total CD68+ macrophages and the severity of proteinuria at the time of the second biopsy suggests that macrophage accumulation per se is not a simple response to renal injury. In support of a role for Sn+ macrophages in mediating renal injury, we have previously demonstrated that glomerular accumulation of Sn+ (ED3+) macrophages is...
associated with glomerular T-cell infiltration and production of interferon-γ and interleukin-2 in a rat model of progressive mesangioproliferative glomerulonephritis [13]. Furthermore, depletion of CD4+ T-cells prevented glomerular accumulation of Sn+ (ED3+) macrophages and suppressed renal injury [14].

Sialoadhesin is normally restricted to a subset of tissue macrophages, such as those found in lymphoid tissue [7]. Since Sn is absent from blood monocytes, the expression of Sn by monocyte/macrophages within the diseased kidney must be induced by factors within the local glomerular and/or interstitial microenvironment. Combinations of the pro-inflammatory cytokines interferon-γ, tumour necrosis factor-α and interleukin-1 can induce Sn expression in bone marrow-derived macrophages, although when used individually these factors do not modulate Sn expression [9]. The most potent inducer of Sn expression in monocyte/macrophages is glucocorticoids, and this induction is augmented by interferons β and γ and interleukin-4 [8]. Thus, macrophage expression of Sn does not readily fit into one of the four modes of macrophage activation (innate, humoral, classical or alternative) [4]. The role of Sn+ macrophages in disease has not been clearly elucidated. Since Sn is not a phagocytic receptor, it is unlikely to be involved directly in scavenging functions [7]. Recent studies have been suggested that Sn contributes to macrophage cell–cell and cell–matrix interactions.

Fig. 5. Follow-up biopsies were performed in the HSPN, IgAN and MPSN patient groups. Taking all patients as a single cohort, the degree of: (A) proteinuria, (B) glomerular lesions, (C) tubulointerstitial damage, is shown at the time of the first and second biopsies. The number of glomerular (D–F) and interstitial (G–I) CD68+ macrophages, Sn+ macrophages and CD3+ T cells is shown. Data were analysed using the Wilcoxon matched pairs test.
interactions during inflammatory reactions [11]. In particular, Sn has been identified as a lymphocyte adhesion molecule [17], suggesting that Sn+ macrophages may contribute to the recruitment of lymphocytes into inflamed tissues. In addition, a recent study has demonstrated that Sn+ macrophages play an important role in the induction of T-cell immunity [18]. Using a mouse model of T-cell-mediated graft versus leukaemia, the T-cell response to eradicate the tumour was dependent upon Sn+ macrophages as shown by administration of an anti-Sn antibody. In vivo, Sn+ host macrophages in the liver formed clusters with donor CD8+ T cells. In vitro, purified Sn+ macrophages were shown to process exogenous antigens and stimulate MHC class I peptide-restricted CTL responses [18].

Our study showed that Sn+ macrophages and CD3+ T cells co-localized in areas of tubulointerstitial damage with increased type I collagen deposition, and there was a significant correlation between the number of Sn+ macrophages and CD3+ T-cells in both the glomerulus and interstitium. Thus, the leukocyte adhesion and antigen presentation functions of Sn+ macrophages may promote T-cell immunity and chronic disease progression in human glomerulonephritis. Clearly, further studies are needed to examine the functional capacities of Sn+ macrophages for co-stimulation of renal T-cell immunity and inducing kidney damage.

Conclusions

This is the first study to identify the Sn+ subset of ‘activated’ macrophages in human glomerulonephritis. Accumulation of Sn+ macrophages correlated with the degree of proteinuria and histologic damage. In addition, the response to glucocorticoid-based therapy correlated with a reduction in the number of Sn+ macrophages. These results, together with recent findings from animal studies, suggest that Sn+ macrophages may play an important role in progressive renal disease. Further studies are warranted to elucidate the functional capacity of Sn+ macrophages and their potential for mediating renal injury.

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Conflict of interest statement. None declared.

References


Table 3. Correlation of the change in renal leukocyte populations in response to treatment

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Data are from all a single cohort of 27 patients. NS, not significant. The change in the number of leukocytes, proteinuria or of glomerular or tubulointerstitial lesions is defined as the measurement at the time of the first biopsy minus the measurement at the time of the second biopsy. Analysis used the Pearson correlation coefficient for proteinuria data and the Spearman correlation coefficient for the histologic data.


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