Local macrophage proliferation correlates with increased renal M-CSF expression in human glomerulonephritis

Nicole M. Isbel¹, David J. Nikolic-Paterson¹,³, Prudence A. Hill⁴, John Dowling² and Robert C. Atkins¹,³

¹Department of Nephrology, ²Department of Pathology and ³Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia and ⁴Department of Anatomy and Cell Biology, University of Melbourne, Victoria, Australia

Abstract

Background. Macrophage accumulation is a prominent feature in many forms of glomerulonephritis. Local proliferation of macrophages within the kidney has been described in human and experimental glomerulonephritis and may have an important role in augmenting the inflammatory response. The current study examined the relationship between local macrophage proliferation and renal expression of macrophage colony-stimulating factor (M-CSF).

Methods. A total of 118 renal biopsies of patients with a wide range of glomerulonephritides were examined for M-CSF protein and macrophage proliferation (KP1+PCNA+ cells) by single and double immunohistochemistry staining, respectively.

Results. Biopsies of thin membrane disease (TMD) with histologically normal kidney showed M-CSF protein expression by 33% of cortical tubules, while glomerular M-CSF expression was limited to resident macrophages and some podocytes. Glomerular M-CSF expression increased significantly in proliferative forms of glomerulonephritis, with M-CSF staining of infiltrating macrophages, podocytes and some mesangial cells. Segmental areas of strong M-CSF expression, particularly in crescents, co-localized with KP1+PCNA+ proliferating macrophages. There was also an increase in tubular M-CSF expression in most types of glomerulonephritis. Tubular M-CSF staining was strongest in areas of tubular damage and co-localized with KP1+ macrophages, including KP1+PCNA+ proliferating macrophages. Many interstitial macrophages and α-smooth muscle actin-positive myofibroblasts showed strong M-CSF staining. Statistical analysis showed a highly significant correlation between M-CSF expression and local macrophage proliferation in both the glomerulus and tubulointerstitium. Glomerular and tubular M-CSF expression gave a significant correlation with renal dysfunction.

Conclusions. Glomerular and tubulointerstitial M-CSF expression is up-regulated in human glomerulonephritis, being most prominent in proliferative forms of disease. This correlated with local macrophage proliferation, suggesting that increased renal M-CSF production plays an important role in regulating local macrophage proliferation in human glomerulonephritis.

Keywords: macrophage colony-stimulating factor; macrophage, proliferation, human; glomerulonephritis; immunohistochemistry

Introduction

Glomerular and interstitial macrophage accumulation has been described in most types of human glomerulonephritis [reviewed in 1]. Glomerular macrophages and, in particular interstitial macrophages, correlate with renal dysfunction and histologic damage, suggesting that they play an active role in causing renal injury [1]. As such, there has been much interest in the mechanisms by which macrophages accumulate within the injured kidney. Local proliferation has recently been identified as an important mechanism promoting macrophage accumulation in kidney disease [2].

Studies in animal disease models have identified local macrophage proliferation within the injured kidney. Incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), into DNA during the S-phase of the cell cycle, in combination with immunostaining of cell-type specific markers, has been used to identify local macrophage proliferation in experimental glomerulonephritis [2]. Mitotic figures in ED1+ macrophages have also been identified [2]. Phenotypic
analysis of BrdU-labelled cells has shown that it is ED1+ED2− blood monocytes, rather than ED1+ED2+ resident kidney macrophages, which undergo local proliferation in experimental kidney disease [1]. An important observation is that the degree of local macrophage proliferation is related to the nature and severity of renal injury. Occasional proliferating macrophages have been identified in experimental immune complex disease and mesangial proliferative nephritis, while no macrophage proliferation was observed in experimental lipid-induced glomerular injury [reviewed in 1]. In contrast, high levels of macrophage proliferation have been identified in severe renal injury, such as acute renal allograft rejection, crescentic anti-GBM glomerulonephritis and following 5/6 subtotal nephrectomy [2–4].

The relevance of macrophage proliferation in these animal models has been confirmed in a study of renal biopsies from a wide range of glomerulonephritides [5]. Local macrophage proliferation in human glomerulonephritis was demonstrated by double staining for CD68+ macrophages and co-expression of the cell-cycle associated proteins, proliferating cell nuclear antigen (PCNA) and Ki67 [5]. Few, if any, proliferating macrophages were seen in non-proliferative forms of glomerulonephritis, but there was marked local macrophage proliferation in proliferative diseases. The presence of significant numbers of proliferating macrophages has also been reported in ANCA-positive renal vasculitis [6].

These studies indicate that a population of recently recruited blood monocytes can undergo cell division within the kidney. This local proliferation is postulated to amplify the inflammatory response and injury within the kidney. The local nature of the proliferative response suggests that it is regulated by the surrounding microenvironment, although the factor(s) responsible for this response have not been identified. Macrophage colony-stimulating factor (M-CSF, also known as CSF-1) is an important regulator of monocyte/macrophage survival and proliferation [7], and is therefore a logical candidate for the regulation of local macrophage proliferation in kidney disease. Renal M-CSF mRNA is up-regulated in association with macrophage recruitment and the development of kidney disease in lupus-prone mice [8,9]. However, little is known of M-CSF expression in human glomerulonephritis. Therefore, the aim of the current study was to examine the relationship between M-CSF expression and local macrophage proliferation in human glomerulonephritis.

**Patients and methods**

**Patients**

One hundred and eighteen patients undergoing renal biopsy at Monash Medical Centre from 1996 to 1999 were examined (Table 1). Patients gave informed consent and this study was approved by the Monash Medical Centre Human Ethics and Research Committee. This is a different group of patients to that used in a previous study of macrophage proliferation [5]. Disease category was based on histological examination of biopsy specimens. Twenty-one of 32 patients in the IgA nephropathy group had greater than 1 g/day proteinuria and renal dysfunction. Patients with thin membrane disease (TMD) were used as a control group since tissue from kidneys with renal carcinoma were unsuitable because M-CSF expression is increased in other epithelial tumours [10]. Patients with TMD had histologically normal biopsies apart from a thin glomerular basement membrane as measured by electron microscopy (< 200 nM). Serum creatinine, creatinine clearance and 24-h protein excretion were determined at the time of biopsy and assays performed by the Department of Biochemistry, Monash Medical Centre. Full blood count and assessment of circulating blood monocytes was measured using the CELL-DYN 3500 SL system (Abbott Laboratories, Abbott Park, IL, USA). Serum samples were also collected at the time of biopsy and stored at −80 °C. In addition, serum samples were collected from a group of 20 normal volunteers.

**Antibodies**

A polyclonal antibody to rat M-CSF was prepared as follows. A 720 base pair cDNA fragment encoding the N-terminal extracellular region (bases 244–923) of the mature rat M-CSF protein [11], was amplified by PCR, subcloned into pRSET A, and expressed as a His-tag fusion protein in *E. coli* using the Xpress System (Invitrogen, San Diego, CA, USA). Recombinant M-CSF was purified by Ni-column affinity chromatography. A polyclonal antibody was raised by immunization of outbred English shorthaired guinea-pigs with recombinant M-CSF. Affinity chromatography (Avid-AL gel, Haem, Melbourne, Australia), was used to purify the IgG fraction of guinea-pig anti-M-CSF antisera. The anti-M-CSF antibody detects recombinant and native forms of M-CSF from rat, mouse and human by Western blotting (data not shown).

Mouse monoclonal antibodies (mAb) used in this study were: KP1, anti-CD68 present in lysosomes of monocytes and macrophages; PC 10, anti-PCNA which recognizes cells in G1, S and G2 phases of cell cycle; 1A4, anti-z-smooth muscle actin. Peroxidase-conjugated goat anti-mouse IgG, alkaline phosphatase-conjugated goat anti-mouse IgG, mouse peroxidase anti-peroxidase complexes (PAP), and mouse alkaline phosphatase anti-alkaline phosphatase complexes (APAAP) were purchased from Dakopatts (Glostrup, Denmark).

**Immunohistochemistry**

Sections (4 μm) of formalin-fixed, paraffin-embedded tissue were dewaxed, rehydrated and incubated for 20 min in 10% fetal calf serum (FCS) and 10% normal rabbit serum, followed by incubation with 10% BSA for 10 min. The guinea-pig anti-M-CSF antibody was preincubated in 10% normal human serum and 1% BSA before being added to the tissue sections and left overnight at 4 °C. Sections were then washed three times with PBS/0.05% Tween 20 and endogenous peroxidase blocked by incubation in methanol containing 0.3% H2O2 for 20 min. Sections were then incubated with peroxidase-conjugated rabbit anti-guinea-pig IgG,
washed three times in PBS 0.05% Tween 20, and then the signal was amplified using the Biotinyl Tyramide kit with streptavidin-conjugated peroxidase (TSA–Indirect kit, NEN Life Science Products, Boston, MA, USA). The bound peroxidase was developed with diaminobenzidine to produce a brown color followed by a blue nuclear haematoxylin counterstain.

The presence of α-smooth muscle actin was assessed in 4 μm sections of formalin-fixed, paraffin embedded tissue, by a standard three layer PAP method [2]. Sections were incubated with the 1A4 anti-α-smooth muscle actin mAb followed by peroxidase-conjugated goat anti-mouse IgG, and mouse PAP. Sections were developed with diaminobenzidine to produce a brown color and then counterstained using periodic acid-Schiff reagent minus haematoxylin.

Local macrophage proliferation was detected by two-colour immunohistochemistry staining as previously described [5]. Briefly, 4 μm sections of formalin-fixed, paraffin-embedded tissue, were stained for macrophages using a standard three layer PAP method employing the KP1 mAb, peroxidase-conjugated goat anti-mouse IgG, and mouse PAP. Sections were developed with diaminobenzidine to produce a brown color. Sections were then microwaved for 10 min in 0.01 M sodium citrate pH 6.0 to block antigen cross-reactivity and to enhance detection of PCNA [14,26]. Sections were then stained using a standard three layer APAAP method employing anti-PCNA mAb, alkaline phosphatase-conjugated goat anti-mouse IgG, and mouse APAAP. Sections were developed using Fast Blue BB base (Sigma Chemical Co., St. Louis, MO, USA) and then counterstained using periodic acid-Schiff reagent minus haematoxylin.

The following negative controls were used: (i) normal guinea-pig IgG was used in place of the anti-M-CSF antibody; (ii) the primary or secondary antibodies were omitted; and (iii) specificity of M-CSF staining was demonstrated by pre-incubating the anti-M-CSF antibody with a 10-fold molar excess of recombinant human M-CSF for 1 h which largely abrogated the staining reaction.

Quantification of immunohistochemical staining

Glomerular M-CSF staining was assessed in all glomeruli (>5 glomeruli examined in all biopsies) under high power (>400) using a semi-quantitative method as follows: 0 (no labelling); 1 (1–15 cells positive); 2 (15–30 cells positive); and 3 (>30 cells positive). Tubular M-CSF staining was assessed in 500 cortical tubules per biopsy and expressed as the percentage of positively stained tubular cross-sections. A positive tubular cross-section was defined as having two or more stained cells.

The number of KP1+ macrophages and KP1 + PCNA + proliferating macrophages was assessed on double-stained tissue sections. The number of glomerular KP1+ and KP1 + PCNA + cells were counted under high-power (>400). The number of interstitial KP1+ and KP1 + PCNA + cells was determined by counting single and double stained cells in 100–150 consecutive high-power (>400) cortical fields (avoiding glomeruli and large vessels) using a graticule. The number of interstitial KP1+ or KP1 + PCNA + cells was expressed as cells per mm² with no correction made for the tubular area. Interstitial staining of α-smooth muscle actin was assessed by point counting of 50 consecutive high-power (>400) cortical fields, avoiding glomeruli and vessels. All immunohistochemical scoring was performed in a blind fashion using coded slides.
Serum M-CSF levels

Serum M-CSF levels were measured using the Quantikine human M-CSF ELISA kit according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). Comparison of results for immunostaining between the different patient groups used one-way analysis of variance (ANOVA); non-parametric data used the Kruskal-Wallis test with Dunn’s multiple comparison post-test analysis (semi-quantitativeglomerul ar M-CSF scoring), and parametric data (all quantitative staining) used Tukey post-test analysis. Correlation analyses used the Spearman rank correlation coefficient for non-parametric data and the Pearson single correlation coefficient for parametric data.

Results

Immunostaining of M-CSF in renal biopsies

Assessment of M-CSF expression in normal human kidney is difficult since the usual source of such tissue is the ‘uninvolved’ pole from renal carcinoma nephrectomies. Such tissue is unsuitable since M-CSF expression is increased in various epithelial tumours [10]. Therefore, acknowledging the limitations, we used biopsies from patients with thin membrane disease (TMD) who had histologically normal kidney as the control group. An overview of M-CSF staining in TMD is shown in Figure 1a. Glomerular M-CSF staining of TMD was limited to some podocytes and some parietal epithelial cells (Figure 1b). Double staining found that resident glomerular macrophages express M-CSF (not shown). Immunostaining of M-CSF was evident in approximately 33% of cortical tubules (Figures 1a and c). In addition, most medullary tubules showed strong M-CSF staining (Figure 1d). There was little change in glomerular M-CSF expression in non-proliferative forms of glomerulonephritis, although there was variation within individual cases. However, there was a significant increase in glomerular M-CSF immunostaining in proliferative forms of disease, particularly in SLE class IV (Figures 2a and 3a). In proliferative diseases, glomerular M-CSF staining was evident in podocytes, infiltrating macrophages and some mesangial cells. There was also a marked increase in the percentage of cortical tubules stained for M-CSF in both non-proliferative and proliferative forms of disease (Figures 1e, 1f, and 3b). Many M-CSF-positive interstitial cells were also evident in severe disease (Figures 1f and 2c).

Co-localization of M-CSF and local macrophage proliferation

Glomerular and interstitial KPI + macrophages and the presence of KPI + PCNA + proliferating macrophages were assessed by double immunostaining. Small numbers of KPI + macrophages and occasional KPI + PCNA + proliferating macrophages were seen in non-proliferative forms of glomerulonephritis (Figure 3c and d). In contrast, macrophage infiltration and local proliferation were prominent in proliferative forms of disease, particularly SLE and crescentic glomerulonephritis (Figure 3c and d).

Glomerular M-CSF staining was most prominent within segmental proliferative lesions and crescents (Figure 2a). This co-localized with focal accumulation of KPI + macrophages, including the presence of proliferating macrophages (KPI + PCNA + cells) (Figure 2b). Strong M-CSF expression by damaged tubules was also evident in areas of macrophage accumulation and local proliferation (Figure 2c and d).

Analysis of all patients as one group identified a highly significant correlation between M-CSF expression and macrophage accumulation and local macrophage proliferation in both glomerular and tubulointerstitial compartments (Figure 4). Furthermore, glomerular and tubular M-CSF expression correlated with renal dysfunction, but not proteinuria (Figure 4). Blood monocyte numbers did not correlate with glomerular or tubular M-CSF expression.

Co-localization of M-CSF and interstitial myofibroblasts

M-CSF immunostaining of tubules and interstitial cells was evident in areas of interstitial fibrosis. Staining of serial sections showed intense interstitial M-CSF staining co-localized with abundant β-smooth muscle actin-positive myofibroblasts (Figure 2e and f). There was also co-localization of β-smooth muscle actin and M-CSF staining in areas of periglomerular fibrosis and within fibrocellular crescents. Interstitial β-smooth muscle actin staining was quantified by a point counting method and, analysing all patients as one group, found a positive correlation between interstitial β-smooth muscle actin staining and tubular M-CSF expression \( (r = 0.52; P = 0.0007) \).

Serum M-CSF levels in glomerulonephritis

Normal volunteers and patients with TMD had similar levels of serum M-CSF (615 ± 66 and 824 ± 138 ng/ml, respectively). There was a 4- to 5-fold increase in serum M-CSF levels in both non-proliferative and proliferative glomerulonephritis (Figure 5). Analysing all patients as one group, serum M-CSF levels correlated with tubular M-CSF expression \( (r = 0.27, P = 0.01) \) and renal function \( (r = –0.34; P = 0.001) \). There was no correlation with proteinuria or blood monocyte numbers.

Discussion

Macrophages have been shown to cause renal injury in experimental glomerulonephritis and macrophage
Fig. 1. Immunostaining of M-CSF in human glomerulonephritis. (a) Thin membrane disease (TMD) showing M-CSF staining by some cortical tubules and a small number of glomerular cells. (b) TMD showing glomerular M-CSF expression by some podocytes (arrows). Some parietal epithelial cells are also stained for M-CSF (arrowhead). (c) High power view of the cortex showing several M-CSF stained tubules in TMD. (d) Most medullary tubules show strong M-CSF staining in TMD. (e) A case of IgA nephropathy showing an increase in tubular M-CSF expression. There is also an increase in glomerular M-CSF staining by podocytes, infiltrating leukocytes and mesangial cells. (f) A case of crescentic glomerulonephritis showing a focal increase in glomerular M-CSF and an increase in M-CSF expression by tubules and interstitial cells. Sections counterstained with haematoxylin. Original magnifications: ×160 (a,f), ×250 (d,e), ×400 (b,c).
Fig. 2. Association of M-CSF expression with local macrophage proliferation in human glomerulonephritis. (a) Strong focal M-CSF staining of macrophages and crescent cells within a segmental proliferative lesion with a crescent in a case of SLE class IV. (b) Double immunostaining of a section from the same glomerulus as in (a), showing co-localization of KPI + PCNA + proliferating macrophages (arrows) with M-CSF staining. (c) An area of strong M-CSF staining of tubules and interstitial cells in a case of crescentic glomerulonephritis. (d) Double immunostaining of a section from the same area as in (c), showing the presence of KPI + PCNA + proliferating macrophages (arrows). (e) A case of IgA nephropathy in which an area of focal tubulointerstitial damage with tubular atrophy shows strong M-CSF staining within the interstitium which co-localizes with the presence of a dense infiltrate of α-smooth muscle actin-positive myofibroblasts shown in a serial section (f). Sections were counterstained with PAS (b,d,e,f) or haematoxylin alone. Original magnifications: ×250 (c,f) and ×400 (a–d).
accumulation correlates with renal dysfunction and histologic damage in human glomerulonephritis and is a predictor of renal outcome [1]. This study has identified M-CSF protein within the glomerulus and tubules in TMD and shown that M-CSF expression is up-regulated in glomerulonephritis, particularly in aggressive diseases such as SLE class IV and crescentic glomerulonephritis. There was good co-localization between areas with increased M-CSF expression and local proliferation in both the glomerulus and tubulo-interstitium. Furthermore, there was a highly significant correlation between up-regulation of glomerular and tubular M-CSF expression and local macrophage proliferation. As expected from this close association between M-CSF and macrophages, there was a significant correlation between renal M-CSF expression and renal dysfunction.

The results of this study demonstrate an association between up-regulation of renal M-CSF expression and local macrophage proliferation. Although these data do not prove a cause and effect relationship—a limitation of human-based studies—the findings do support the postulate that M-CSF is the major factor driving local macrophage proliferation in human glomerulonephritis. The results support animal studies which have examined the potential role of M-CSF in promoting macrophage accumulation in experimental models of kidney disease. Elevated levels of M-CSF mRNA in the kidney, spleen and liver in two lupus-prone mouse strains (NZB/W and MRL lpr/lpr) are associated with increased numbers of macrophages in these organs [8].

In a more detailed study, Bloom et al. [9] used in situ hybridization to demonstrate increased levels of renal M-CSF mRNA prior to the development of macrophage accumulation and the onset of renal disease in MRL lpr/lpr mice. In addition, isolated glomerular macrophages from the pre-proteinuric phase of disease in MRL lpr/lpr mice were shown to be dependent on M-CSF for survival and proliferation [9]. Direct evidence for M-CSF driving macrophage accumulation was provided by studies in which local secretion of M-CSF under the kidney capsule (via implanted tubular epithelial cells transfected to secrete M-CSF) induced macrophage accumulation and renal damage in susceptible MRL-lpr, but not normal MRL-(++) mice [12,13]. Administration of M-CSF to mice has been shown to enhance endotoxin-induced proteinuria and glomerular macrophage accumulation [14]. Indeed, there is a case report in which a patient given M-CSF as an adjunct to chemotherapy developed nephrotic syndrome with diffuse mesangial proliferation and marked glomerular macrophage infiltration, suggesting that M-CSF treatment may have enhanced an underlying renal disease through promoting glomerular macrophage infiltration and proliferation [15]. However, blocking studies in experimental glomerulonephritis are necessary in order to prove that M-CSF drives local macrophage proliferation.
Fig. 4. Correlation analysis of glomerular (a–d) and tubular (e–h) M-CSF expression with: macrophage accumulation expressed as KP1+ cells per glomerular cross-section (gcs) or per mm² (a,e), macrophage proliferation expressed as KP1+ PCNA+ cells per gcs or per mm² (b,f), renal function (c,g) and proteinuria (d,h). Data from all patients was analysed as one group using the Spearman’s rank correlation coefficient (a–d) or the Pearson single correlation coefficient (e–h).

The results of the present study are consistent with a previous report of glomerular M-CSF expression in human glomerulonephritis. Matsuda et al. [16] reported weak M-CSF immunofluorescence staining in a small number of glomerular and parietal epithelial cells in normal kidney specimens. In contrast, M-CSF mRNA and protein expression was prominent in glomerular mesangial cells and podocytes in IgA nephropathy and SLE, and a significant correlation was seen between glomerular M-CSF expression and glomerular macrophage accumulation [16]. We also found an increase in glomerular M-CSF expression in SLE, although this was not evident in IgA nephropathy. This difference may be due to the use of TMD as the control group in our study, or in the method used to provide a semi-quantitative assessment of
glomerular M-CSF expression (i.e. approximate number of cells vs fluorescence intensity). Our results significantly extend that reported by Matsuda et al. [16], with the examination of tubular M-CSF expression and a comparison of M-CSF expression with local macrophage proliferation. Indeed, the results of this and other studies [1,2,14] demonstrate that interstitial rather than glomerular macrophage accumulation correlates best with renal dysfunction and is predictive of renal outcome. It is apparent that tubules are a major source of M-CSF within the diseased kidney and that this correlates with interstitial macrophage proliferation. This is consistent with in vitro studies demonstrating secretion of biologically active M-CSF by human renal tubular epithelial cells [17].

An interesting finding in the current study was the prominent M-CSF staining of z-smooth muscle actin-positive myofibroblasts in areas of fibrosis. Fibroblast production of M-CSF is well documented [7,18], but the role of this growth factor in fibrosis is not well understood. The application of M-CSF to dermal ulcers in rabbits was found to stimulate wound healing with increased granulation tissue and elevated levels of TGF-b1 mRNA [19]. This suggests a potential role for M-CSF in the fibrotic process, but this remains to be determined.

Serum M-CSF levels were elevated in all forms of glomerulonephritis and were not discriminatory. This may simply reflect accumulation in the face of renal impairment. Elevated serum M-CSF levels have been reported previously in patients with chronic renal failure regardless of the aetiology of the renal disease [20].

In summary, this study has demonstrated that glomerular and tubulointerstitial M-CSF expression is up-regulated in human glomerulonephritis, being most prominent in proliferative forms of disease. This correlated with local macrophage proliferation, suggesting that increased M-CSF production within the kidney plays an important role in regulating local macrophage proliferation in human glomerulonephritis.

Acknowledgements. We thank Paul Kramer for cutting biopsy sections, Rita Foti for help with cDNA cloning and expression of M-CSF, and Drs Steven Chadban and Yannick Le Meur for critical review of this manuscript. This study was funded by the National Health and Medical Research Council of Australia and the Australian Kidney Foundation.

References

Fig. 5. Serum M-CSF levels in normal volunteers and human glomerulonephritis. Serum M-CSF levels (pg/ml) were measured by ELISA. Data are shown as the mean ± SEM. *P < 0.05, ***P < 0.001 vs normal by ANOVA. Abbreviations are explained in the legend to Figure 3.

Received for publication: 6.10.90
Accepted in revised form: 12.3.01