Urine Macrophage Migration Inhibitory Factor Reflects the Severity of Renal Injury in Human Glomerulonephritis

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Abstract. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that plays a pathogenic role in experimental crescentic glomerulonephritis (GN). Renal expression of MIF is also upregulated in human GN and correlates with leukocytic infiltration, histologic damage, and renal dysfunction. The study presented here examined whether MIF can be measured in urine and if so, whether the urine MIF concentration reflects the degree of renal injury. Urine and serum MIF was measured by enzyme-linked immunosorbent assay in 10 normal healthy volunteers and in a cohort of 63 patients with GN (2 thin basement membrane disease [TBM], 15 membranous GN, 10 focal segmental glomerular sclerosis, 20 IgA glomerulonephritis, 11 crescentic GN, 10 systemic lupus erythematosus World Health Organization class IV). Renal MIF expression was assessed by immunostaining of biopsy tissue. MIF was detected in urine from normal volunteers (mean ± SD: 191 ± 132 pg MIF/μmol creatinine). The urine MIF concentration was unchanged in patients with nonproliferative nephropathies (343 ± 397 pg MIF/μmol Cr) but was increased 3.4-fold in proliferative nephropathies (645 ± 527 pg MIF/μmol Cr; P < 0.05 versus normal and nonproliferative). Stratified analysis showed the greatest increase in urine MIF in crescentic GN (4.5-fold). In contrast, serum MIF levels were not different between normal patients and any patient group. Immunostaining demonstrated a significant increase in renal MIF expression in proliferative glomerulonephritides that was associated with macrophage and T cell infiltration. There was a significant correlation between the urine MIF concentration and renal MIF expression, but not with serum MIF, indicating a renal origin for the excreted urine MIF. The urine MIF concentration also correlated with the degree of renal dysfunction, histologic damage, and leukocytic infiltration, but not with the amount of proteinuria. In conclusion, this study shows that the urine MIF concentration is significantly increased in proliferative forms of GN and correlates with the degree of renal injury. Urine MIF levels reflect MIF expression within the kidney and may be a useful noninvasive tool for monitoring patients with crescentic GN, particularly in disease exacerbation.

Originally described in 1966 (1,2), macrophage migration inhibitory factor (MIF) is a 12.5-kD protein that has a wide range of proinflammatory and immunomodulatory functions (3). An essential role for MIF has been established in the tuberculin delayed-type hypersensitivity reaction (4) and in T cell activation (5). MIF potentiates lethal endotoxemia in mice and can overcome glucocorticoid-mediated suppression of lethal endotoxia (6–8). A pathologic role for MIF has also been established in experimental models of arthritis and uveoretinitis (9–11).

MIF is constitutively expressed by a variety of cell types and in many tissues (5,12–19). In the kidney, MIF is weakly expressed by some glomerular epithelial cells and by approximatively half of the cortical tubules (20,21). Renal MIF mRNA and protein expression is upregulated in different types of experimental kidney disease, including crescentic anti–glomerular basement membrane glomerulonephritis (GN) (20,22–25). Administration of a neutralizing anti-MIF antibody inhibited macrophage and T cell accumulation and histologic damage, reduced proteinuria, and prevented renal dysfunction in rat crescentic anti–glomerular basement membrane disease (26). Furthermore, delayed administration of the anti-MIF antibody was shown to partially reverse the progressive phase of established crescentic disease in this model (27).

Analysis of renal MIF expression in human biopsy tissue revealed that renal MIF expression is upregulated in proliferative forms of GN (28). Renal MIF expression significantly correlates with renal dysfunction, histologic damage, and leukocytic infiltration (28). Taken together with data from functional blocking studies in the rat, these data suggest that MIF plays an important role in the pathogenesis of human proliferative GN.

The aim of this study was to measure urinary MIF excretion in human GN. This was based on the hypothesis that urinary MIF excretion may reflect the level of MIF production within the kidney; thus, increased MIF production within the injured kidney may be reflected by an increase in the urine MIF concentration.

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Materials and Methods

Patients

Sixty-three renal biopsies from the Department of Nephrology at Monash Medical Center, Clayton, Australia, were examined and classified as part of the routine diagnostic procedure (Table 1). This is a different cohort of patients than those reported in a previous study of MIF immunostaining in human GN (28). In addition, five normal human kidney specimens were analyzed (three from the unaffected poles of nephrectomy specimens performed for renal cell carcinoma and two from unmatched donor kidneys). Blood and urine samples were taken at the time of the biopsy and analyzed for serum creatinine, creatinine clearance, and proteinuria. Ten normal, healthy volunteers also provided blood and urine specimens for comparison.

Histopathology

Sections (4 μm) of formalin-fixed, paraffin-embedded biopsy tissue were stained with hematoxylin and eosin. The percentage of glomeruli exhibiting crescent formation was scored in 10 to 30 glomerular cross sections (gcs) per biopsy. Glomerular hypercellularity was assessed as follows: 0 = normal (less than 60 cells/gcs); 1 = mild (60 to 90 cells/gcs); 2 = moderate (90 to 120 cells/gcs); and 3 = severe (more than 120 cells/gcs). The percentage of the cortical tubulointerstitial area exhibiting tubulitis, tubular dilation, atrophy, or fibrosis was assessed on hematoxylin and eosin–stained sections as follows: 0 = no lesions; 1 = less than 25%; 2 = 25 to 50%; and 3 = more than 50%. All scoring was performed on sections in a blinded manner.

Urine and Serum Samples

Sterile midstream urine samples were collected from patients and then were stored at 4°C for a maximum of 12 h before processing. A 1-ml aliquot was analyzed for urine creatinine. The urine was centrifuged at 1500 × g for 10 min to separate debris and a protease inhibitor cocktail (Sigma, Castle Hill, New South Wales, Australia) was added; then urine was formed into aliquots and stored at −80°C. Blood samples were collected by venipuncture in plain tubes and left at room temperature for 1 h to clot before being stored at 4°C for up to 4 h. The blood then was centrifuged at 1500 × g for 10 min. The serum was formed into aliquots and stored at −80°C. All samples underwent only one freeze-thaw cycle.

MIF Enzyme-Linked Immunosorbent Assay

Serum and urine MIF concentrations were quantitated by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). In brief, 96-well ELISA plates were coated overnight with 2 μg/ml mouse anti-human MIF capture antibody. Wells were washed with 0.05% Tween-20 PBS (PBST) and then blocked with 5% sucrose, 1% bovine serum albumin (BSA), and 0.05% NaN3 in PBS for 2 h. Test samples (human serum, human urine, or recombinant MIF standards) diluted in 0.1% BSA, 0.05% Tween-20 in 20 mM Tris-HCl, 150 mM NaCl, pH 7.3, were added in triplicate and then incubated at room temperature for 2 h. After washing with PBST, bound MIF was detected by a 2-h incubation with 200 ng/ml biotinylated anti-human MIF antibody diluted in 0.1% BSA, 0.05% Tween-20 in 20 mM Tris-HCl, and 150 mM NaCl, pH 7.3. After washing, samples were incubated with 1.25 ng/ml peroxidase-conjugated streptavidin (Zymed, South San Francisco, CA) for 30 min, washed in PBST, and then incubated for 30 min with 100 μl/well ready-to-use 3,3′,5,5′-tetramethylbenzidine (Zymed) and the colorimetric reaction stopped by the addition of 0.5 M H2SO4. The adsorption at 450/570 nm was measured with a microplate reader.

MIF is stable in human urine; the ELISA measurements are constant for up to 24 h when stored at 4°C or room temperature. The MIF ELISA assay is highly reproducible, and when we analyzed samples (in triplicate) up to 12 times, the SD was 6.6% of the mean value.

Antibodies

Mouse monoclonal antibodies (MoAb) used for immunostaining were as follows: IIID9, mouse MoAb raised against recombinant mouse MIF that cross-reacts with human MIF; UCHL1, mouse anti-CD45RO, which recognizes mature, activated T cells and a subset of monocytes and macrophages (30). Peroxidase and alkaline phosphatase–conjugated goat anti-mouse IgG, mouse peroxidase-conjugated anti-peroxidase complexes, and mouse alkaline phosphatase–conjugated anti-alkaline phosphatase complexes were purchased from Dakopatts (Glostrup, Denmark).

Immunohistochemistry

Two-color immunohistochemistry staining was performed as described previously (28,31). Paraffin sections (4 μm) were treated with 10-min microwave oven heating in 10 mM sodium citrate, pH 6.0, at 2450 MHz and 800 W. Sections then were preincubated with 10%...
fetal calf serum and 10% normal goat serum in PBS for 20 min, drained, and incubated with KP1 or UCHL1 MoAb overnight at 4°C. Sections then were washed in PBS, endogenous peroxidase inactivated in 0.3% H2O2 in methanol, incubated with peroxidase-conjugated goat anti-mouse IgG, washed in PBS, incubated with mouse peroxidase-conjugated anti-peroxidase complexes, and developed with 3,3-diaminobenzidine to produce a brown color. Slides then underwent a second microwave treatment to denature the bound Ig and prevent antibody cross reactivity (31). Sections then were preincubated with 10% fetal calf serum and 10% normal goat serum in PBS for 20 min, followed by 10% bovine serum albumin in PBS for 20 min, washed, and labeled with the anti-MIF MoAb overnight at 4°C. After washing in PBS, sections then were incubated sequentially with alkaline phosphatase-conjugated goat anti-mouse IgG and mouse alkaline phosphatase-conjugated anti-alkaline phosphatase complexes and then developed with Fast Blue BB Salt (Ajax Chemicals, Melbourne, Australia). Sections were counterstained with periodic acid–Schiff (minus hematoxylin) and mounted in an aqueous medium.

Quantitation of Immunohistochemistry Staining

The number of immunostained cells were counted under high-power microscope fields (×400) in all glomeruli (10 to 30) for each biopsy and expressed as cell per gcs. The number of KP1-positive and UCHL1-positive interstitial cells was counted in high-power fields of the cortex with a 0.02-mm2 graticule fitted in the eyepiece of the microscope for the entire biopsy and expressed as cells per square millimeter. No adjustment of the interstitial cell count was made for tubules or the luminal space. Cortical tubular MIF staining was scored from the entire cortex of the entire biopsy and expressed as cells per square millimeter. No adjustment of the interstitial cell count was made for tubules or the luminal space. Cortical tubular MIF staining was scored from the entire cortex of the biopsy and expressed as the percentage of positive tubules. Data are expressed as means ± SD. All counting was performed on blinded slides.

Statistical Analyses

One-way ANOVA and the Pearson and Spearman single-correlation coefficients were performed by GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Results

Urine MIF Concentration in Human GN

MIF was detected in urine of normal healthy volunteers (mean ± SD: 191 ± 132 pg MIF/μmol creatinine; range, 49 to 433; Figure 1a). The urine MIF concentration in nonproliferative nephropathies (thin basement membrane disease, membranous GN, focal segmental GN) was not different to normal, but there was a significant 3.4-fold increase in urine MIF concentration in proliferative nephropathies (IgA glomerulonephritis, crescentic GN, systemic lupus erythematosus [SLE] World Health Organization class IV) (Figure 1a). A stratified analysis according to disease type found a significant increase (4.5-fold) in the urine MIF concentration in patients with crescentic GN compared with normal volunteers (Figure 1b).

Serum MIF Concentration in Human GN

MIF was readily detected in serum from normal healthy volunteers (mean ± SD: 520 ± 567 pg/ml; range, 99 to 1832; Figure 1c). There was no significant difference in the serum MIF concentration between samples taken from normal subjects and from subjects with nonproliferative and proliferative nephropathies, even when stratified according to disease type (Figure 1, c and d). The wide range of serum MIF levels within these groups was not due to variation in the assay because repeated measurements of individual samples had a SD of 6.6%.

MIF Expression in Renal Biopsies

Immunohistochemistry staining identified weak constitutive MIF protein expression in some visceral and parietal epithelial cells and in approximately 50% of cortical tubules (Figures 2A and 3). A similar pattern of MIF immunostaining was apparent in membranous GN (Figure 2B), and overall, there was no significant change in MIF protein expression in proliferative nephropathies (Figure 3). In contrast, there was a significant increase in glomerular and tubular MIF protein staining in proliferative nephropathies (Figure 3). Areas with strong MIF expression, such as damaged tubules and glomerular crescents, had prominent macrophage and T cell infiltration; double immunostaining showed that infiltrating macrophages and T cells also expressed MIF (Figure 2C).

Quantitation of immunostaining showed significant glomerular macrophage and T cell infiltration in crescentic GN and SLE class IV, whereas a significant interstitial macrophage and T cell infiltrate was apparent in all diseases except thin membrane disease (Figure 4).

Correlation of Urine MIF Concentration with Renal Function, Histologic Damage, MIF Expression, and Mononuclear Cell Infiltration

Analyzing all 63 patients as a single group found a significant correlation between urine MIF concentration and the degree of renal dysfunction, but there was no correlation between urine MIF concentration and the amount of proteinuria or serum MIF level (Table 2). A significant correlation was...
Figure 2. Double immunohistochemistry staining of macrophage migration inhibitory factor (MIF) and leukocytic infiltration in human glomerulonephritis (GN). (A) Normal human kidney from the unaffected pole of a carcinoma showing constitutive MIF expression (blue) by some glomerular cells and approximately half of the tubules. (B) Mild membranous GN showing MIF staining (blue) in some glomerular cells and cortical tubules.
found between the urine MIF concentration and MIF expression within the kidney (Table 2). There was also a significant correlation between the urine MIF concentration and histologic damage, and between urine MIF and renal leukocytic infiltration (except for glomerular T cells) (Table 3).

Renal MIF expression correlated with the degree of renal dysfunction, histologic damage and renal leukocytic infiltration (Tables 2 and 3); however, renal MIF expression did not correlate with proteinuria. Serum MIF levels failed to correlate with any parameter.

**Discussion**

This study demonstrates that MIF is readily detected in the urine of normal, healthy volunteers. Urinary MIF was present at normal levels in nonproliferative forms of GN but was increased more than threefold over normal levels in the proliferative nephropathies. The urine MIF concentration correlated with the degree of renal dysfunction, histologic damage, leukocytic infiltration, and renal MIF expression. The relationship of urinary MIF excretion to MIF expression within the kidney and the potential clinical utility of the urine MIF concentration are considered below.

The increase in urinary MIF concentration seen in proliferative nephropathies is probably the result of increased local production and secretion of MIF within the injured kidney. This postulate is supported by two findings. First, there was a significant correlation between renal MIF expression assessed by immunohistochemistry staining and the urine MIF concentration. MIF expression was increased in the glomerulus (resident and infiltrating mononuclear cells) and in tubular epithelial cells—both potential sites of MIF secretion into the urinary space. In support of this concept, we have reported that interferon gamma can induce rapid secretion of MIF by mesangial cells and tubular epithelial cells in vitro (32). The increase in MIF immunostaining in biopsy tissues in proliferative forms of GN is consistent with a previous study in which the upregulation of MIF mRNA and protein expression was described in a different cohort of GN patients (28). Second, no relationship was seen between the urine MIF concentration and serum MIF levels or proteinuria, indicating that increased urinary MIF excretion is not simply a reflection of increased clearance of serum MIF or increased protein excretion generally.

This study has shown that in an individual patient, the urine and renal MIF correlated with the severity and activity of GN (glomerular hypercellularity and crescent formation, the degree

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**Figure 3.** Quantitation of renal migration inhibitory factor (MIF) expression in human glomerulonephritis (GN). (a) Glomerular MIF protein expressed as MIF-positive cells per glomerular cross section (gcs) in human kidney samples from normal patients and from patients with nonproliferative and proliferative diseases. (b) Glomerular MIF protein in individual disease types. (c) Tubular MIF protein expressed as the percentage of MIF-positive cortical tubules in human kidney samples from normal patients and from patients with nonproliferative and proliferative diseases. (d) Tubular MIF protein in individual disease types. Memb, membranous GN; FSGS, focal segmental GN; Cresc, crescentic GN; Thin BM, thin basement membrane disease; SLE, systemic lupus erythematosus. Data are means ± SD. *, P < 0.05, ***, P < 0.001 versus normal; #<sup>+</sup>, P < 0.05, ###<sup>+</sup>, P < 0.001 versus nonproliferative diseases, by ANOVA.

**Figure 4.** Quantitation of macrophages and T cells in human glomerulonephritis (GN). (a) Glomerular macrophages (CD68<sup>+</sup> cells) expressed per glomerular cross section (gcs). (b) Glomerular T cells (UCHL<sub>1</sub> cells). (c) Interstitial macrophages (CD68<sup>+</sup> cells). (d) Interstitial T cells. Memb, membranous GN; FSGS, focal segmental GN; Cresc, crescentic GN; Thin BM, thin basement membrane disease; SLE, systemic lupus erythematosus. Data are means ± SD. *, P < 0.05, **, P < 0.01, ***, P < 0.001 versus normal by ANOVA.

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A mild infiltrate of UCHL<sub>1</sub> T cells (brown) is also apparent. (C) Crescentic GN showing strong MIF staining (blue) in a crescent, together with many CD68<sup>+</sup> macrophages (brown). MIF staining is also seen in cells in the glomerular tuft, interstitial cells, and most tubules. Many macrophages with MIF staining can be seen and note the presence of with strong MIF staining in areas of tubulitis. (D) Negative control staining of the same biopsy as in (C) using isotype-matched irrelevant antibodies. Sections were counterstained with period acid–Schiff minishematoxylin. Original magnifications: ×100 in A and B, ×200 in C and D.
of interstitial damage, mononuclear cell infiltrate, and loss of renal function). Therefore, the urine MIF concentration may be useful in monitoring patients for the degree of disease activity. However, the urine MIF concentration is not useful in identifying a specific type of GN in an individual patient, although a high urine MIF level does suggest a more severe proliferative form of GN. Further studies are needed to assess how urine MIF excretion changes with time in individual patients. In particular, it is important to determine whether urine MIF could be an early indicator of a flare of disease activity.

This study found that serum MIF levels were not different between normal volunteers and the various patient groups, including SLE class IV GN. This contradicts a recent report in which a fourfold increase in serum MIF levels was described in patients with SLE (33). Unfortunately, this report gave no patient details, so that the severity and systemic symptoms in these SLE cases are unknown, thus making it difficult to compare with the patient group in this study. One possible explanation for the apparent discrepancy is that increased serum MIF reflects systemic symptoms because the SLE patient group in this study had primarily renal involvement without systemic disease. Further studies are needed to clarify this issue.

In summary, this study found that the urine MIF concentration is significantly increased in proliferative forms of GN and correlated with the degree of renal dysfunction, histologic damage, and leukocytic infiltration. Urine MIF reflects MIF expression within the injured kidney and may be a useful noninvasive tool for monitoring patients with crescentic GN and their exacerbation of disease.

**Acknowledgments**

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**References**


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**Table 2. Correlation of the urine macrophage migration inhibitory factor (MIF) concentration with renal function and serum MIF in human glomerulonephritis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Serum Creatinine (μmol/L)</th>
<th>Creatinine Clearance (ml/min)</th>
<th>Proteinuria (g/d)</th>
<th>Urine MIF (pg/μmol Cr)</th>
<th>Serum MIF (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine MIF (pg/μmol Cr)</td>
<td>0.26^b</td>
<td>0.23^b</td>
<td>−0.02^c</td>
<td>0.30 b</td>
<td>0.28 b</td>
</tr>
<tr>
<td>Glomerular MIF (cells/gcs)</td>
<td>0.70^d</td>
<td>−0.27^d</td>
<td>0.007^e</td>
<td>0.25^b</td>
<td>0.09^e</td>
</tr>
<tr>
<td>Tubular MIF (%)</td>
<td>0.79^d</td>
<td>−0.36^e</td>
<td>0.09^e</td>
<td>0.24^b</td>
<td>0.09^e</td>
</tr>
</tbody>
</table>

^a Cr, creatinine; gcs, glomerular cross section. Data from all patients were analyzed with the Pearson single correlation coefficient. ^b P < 0.05. ^c NS. ^d P < 0.001. ^e P < 0.01.

**Table 3. Correlation of the urine macrophage migration inhibitory factor concentration with histologic damage and mononuclear cell infiltrate in human glomerulonephritis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glomerular Hypercellularity (1–3)</th>
<th>Glomerular Crescent (%)</th>
<th>Glomerular Macrophage (cells/gcs)</th>
<th>Glomerular T cells (cells/gcs)</th>
<th>Intestinal Damage (0–3)</th>
<th>Intestinal Macrophage (cells/mm²)</th>
<th>Intestinal T Cells (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine MIF (pg/μmol Cr)</td>
<td>0.29^b</td>
<td>0.27^b</td>
<td>0.30^b</td>
<td>0.18^e</td>
<td>0.28^b</td>
<td>0.24^b</td>
<td>0.24^b</td>
</tr>
<tr>
<td>Glomerular MIF (cells/gcs)</td>
<td>0.56^d</td>
<td>0.39^d</td>
<td>0.80^d</td>
<td>0.69^d</td>
<td>0.34^e</td>
<td>0.58^d</td>
<td>0.48^d</td>
</tr>
<tr>
<td>Tubular MIF (%)</td>
<td>0.79^d</td>
<td>−0.36^e</td>
<td>0.09^e</td>
<td>0.24^b</td>
<td>0.09^e</td>
<td>0.24^b</td>
<td>0.09^e</td>
</tr>
</tbody>
</table>

^a MIF, macrophage migration inhibitory factor; Cr, creatinine; gcs, glomerular cross section. Data from all patients were analyzed with the Pearson single correlation coefficient, except for glomerular hypercellularity and interstitial damage; Spearman’s single correlation coefficient correlation coefficient was used to analyze all other data. ^b P < 0.05. ^c NS. ^d P < 0.001. ^e P < 0.01.
for macrophage migration inhibitory factor in T-cell activation. 

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