

TNF- α Up-regulates Renal MIF Expression in Rat Crescentic Glomerulonephritis

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ABSTRACT

Background: Macrophage migration inhibitory factor (MIF) is a potent proinflammatory mediator that participates in the pathogenesis of endotoxemia and experimental crescentic glomerulonephritis. However, very little is known about how MIF production is regulated in disease. We therefore examined whether tumor necrosis factor α (TNF- α), a known inducer of MIF expression by macrophages *in vitro*, up-regulates local and systemic MIF expression in a macrophage-mediated rat model of crescentic glomerulonephritis.

Materials and Methods: Anti-glomerular basement membrane (GBM) glomerulonephritis was induced in groups of six primed rats. Animals were treated with 1 mg/kg soluble TNF- α receptor (TNFbp) or saline from the time of disease induction until they were killed on Days 1, 7, or 14. Renal MIF expression was assessed by *in situ* hybridization, immunohistochemistry, and ELISA, and compared with macrophage accumulation and indices of renal damage.

Results: Although TNFbp treatment on Day 1 of the disease had only a partial effect upon the up-regulation

of glomerular MIF expression, on Days 7 to 14 it almost completely abrogated the increase in glomerular and interstitial MIF mRNA and protein expression. In addition, TNFbp treatment significantly inhibited MIF secretion by cultured glomeruli and reduced serum MIF levels. The inhibition of renal MIF expression was paralleled by a significant inhibition of glomerular and interstitial macrophage infiltration ($p < 0.001$ versus saline treated), a significant suppression of renal injury (proteinuria and serum creatinine), and a marked reduction in histologic damage (glomerular hypercellularity, crescent formation, and interstitial fibrosis; all $p < 0.01$ versus saline treated).

Conclusions: This study demonstrates for the first time that TNF- α up-regulates local MIF expression by both infiltrating macrophages and resident kidney cells in rat crescentic glomerulonephritis. In addition, TNF- α regulates systemic MIF production. Thus, TNF- α , together with MIF, may play a pathological role in immunologically induced renal disease.

INTRODUCTION

Macrophage migration inhibitory factor (MIF) was originally described as a product of activated T cells that inhibits the migration of macrophages *in vitro* (1,2). Now, however, it is evident that MIF is not exclusively produced by T cells. Recent studies demonstrate that MIF is constitu-

tively expressed by a variety of cell types, including monocytes/macrophages, anterior pituitary cells, and renal tubular epithelial cells (3–5). Antibody-blocking studies have shown a key pathogenic role for increased MIF production in experimental endotoxemia (6). Glucocorticoids at low levels up-regulate MIF expression via a counter-regulatory mechanism, whereby MIF can then act to override the glucocorticoid effects (7,8). In addition, *in vitro* studies have shown that the proinflammatory cytokines tumor necrosis factor

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α (TNF- α) and interferon γ (IFN- γ) up-regulate macrophage MIF production, and that conversely, MIF induces production of TNF- α and IFN- γ (4,9), which suggests the presence of an amplifying proinflammatory loop response. However, there is little *in vivo* evidence for the functional significance of this amplification pathway.

We have recently shown that MIF production is markedly up-regulated in a rat model of crescentic glomerulonephritis. Increased MIF expression correlated significantly with macrophage infiltration, loss of renal function, and development of renal lesions, including glomerular crescent formation (5). Since TNF- α has been implicated in the pathogenesis of this disease (10,11), we sought to determine whether TNF- α is a physiological inducer of MIF production during the disease process. This was examined by blocking the action of TNF- α in rat crescentic glomerulonephritis by treatment with TNF binding protein (TNFbp), a soluble form of recombinant human TNF- α receptor type 1, and by assessing the effect upon MIF expression and renal injury.

MATERIALS AND METHODS

Nephrotoxic Serum

Rabbit anti-rat glomerular basement membrane (GBM) nephrotoxic serum was raised by repeated immunization of New Zealand white rabbits with purified, particulate rat GBM. The sera was pooled, decomplexed at 56°C for 30 min, absorbed against rat red blood cells at 4°C overnight, and stored at -20°C.

TNF Binding Protein

A dimeric form of recombinant human TNF- α receptor type I, termed the TNF binding protein (TNFbp), was generously supplied by Amgen, Colorado.

Experimental Design

Accelerated anti-GBM glomerulonephritis was induced in inbred male Sprague-Dawley rats (150–200 g) as previously described (12,13). Briefly, animals were immunized *s.c.* with 5 mg normal rabbit IgG in Freund's complete adjuvant and injected *i.v.* with 10 ml/kg rabbit anti-rat GBM serum (12.5 mg IgG/ml) 5 days later

(termed Day 0). Groups of six rats were injected intraperitoneally with 1 mg/kg body weight of TNFbp every second day from 2 hr before administration of anti-GBM serum until being killed on Days 1, 7, or 14. Groups of six control animals had the same schedule, but received saline (vehicle) in place of TNFbp. In addition, one group of six normal rats was also studied. The study was approved by the Animal Experimentation Ethics Committee of Monash Medical Centre.

Renal Function

Blood samples and 24-hr urine collections were taken on Days 0, 1, 7, and 14. Urinary protein excretion was determined with the Manual Poncau Red method. Serum creatinine was measured with the standard Jaffe rate reaction (Alkaline picrate). All analyses were performed at the Department of Biochemistry, Monash Medical Centre.

Histopathology

Kidney tissues for histological examination were fixed in 10% formalin and 4- μ m paraffin sections were stained with hematoxylin and eosin or periodic acid-Schiff (PAS). Coded slides were assessed as previously described (12,13). Twenty glomerular cross sections per animal were examined under high power to determine glomerular hypercellularity (0–3) and the percentage of glomeruli with crescent formation, while the degree of cortical tubulointerstitial fibrosis (0–4) was also determined.

Immunohistochemistry

Single and double immunostaining was performed on 4- μ m paraffin sections of formalin-fixed kidney with a newly developed method of microwave-based, multiple immunoenzymatic staining (14). Briefly, sections were dewaxed, placed in 0.01 M sodium citrate buffer, pH 6.0, and heated for 2 \times 5 min in a microwave oven at 2450 MHz/800 W. This procedure facilitates retrieval of cytoplasmic antigens. Sections were then incubated with mouse ED1 monoclonal antibody (MAb) (anti-CD68, which recognizes monocytes and macrophages) (15) or mouse anti-human MIF MAb, which also recognizes rat MIF (16), using a three-layer peroxidase anti-peroxidase (PAP) staining protocol, and developed with 3,3-diaminobenzidine, thereby giving a brown product. For double immunostaining,

sections were microwave heated for a second time after completion of the three-layer PAP staining with the ED1 MAb to denature bound immunoglobulin molecules and prevent antibody cross-reactivity. Sections were then labeled with the anti-MIF MAb by means of a three-layer alkaline phosphatase-anti-alkaline phosphatase (APAAP) method and developed with Fast Blue BB Base (Sigma Chemical Co., St. Louis, MO), which yielded a blue product. Sections were counterstained with PAS reagent without hematoxylin and coverslipped in an aqueous mounting medium. An isotype-matched mouse antibody 73.5 against human CD45R was used as the negative control.

In Situ Hybridization

A 420 bp *Xba* I/*Bam* HI fragment of mouse MIF cDNA in the Bluescript SK+ vector (Stratagene, La Jolla, CA) (17), which is highly homologous to the rat nucleotide sequence (18), was used to prepare sense and antisense MIF cRNA probes labeled with digoxigenin-11-UTP by in vitro transcription according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany). The incorporation of digoxigenin-labeled nucleotides was assayed by spotting serial dilutions of probes onto Hybond nylon membrane (Amersham, Little Chalfont, Buckinghamshire, U.K.) and detecting the bound probe with alkaline phosphatase-conjugated Fab fragments of anti-digoxigenin IgG with 4-nitroblue tetrazolium chloride and X-phosphate substrates according to the manufacturer's instructions (Boehringer Mannheim). Only probes detectable at a concentration of 0.1 pg/ μ l or lower were used.

In situ hybridization was performed on 4- μ m paraffin sections of formalin-fixed tissue by using a newly developed microwave-based technique (19). After dewaxing, sections were treated with a microwave oven for 2 \times 5 min as outlined above, incubated with 0.2 M HCl for 15 min, followed by 1% Triton X-100 for 15 min, and then digested for 20 min with 10 μ g/ml proteinase-K at 37°C (Boehringer Mannheim). Sections then were washed in 2 \times SSC, prehybridized, and then hybridized with 0.3 ng/ μ l DIG-labeled sense or anti-sense cRNA probe overnight at 42°C in a hybridization buffer containing 50% deionized formamide, 4 \times SSC, 2 \times Denhardt's solution, 1 mg/ml salmon sperm DNA (Sigma), and 1 mg/ml yeast tRNA. Sections were washed in 0.1 \times SSC at 42°C and the hybridized probe

detected by means of alkaline phosphatase-conjugated sheep anti-digoxigenin IgG and color development with NBT/X-phosphate. The sections were counterstained with PAS and mounted in an aqueous medium. Some sections were subsequently microwave oven treated and used for three-layer PAP staining with the anti-MIF or ED1 MAb, as described above.

Quantitation of in Situ Hybridization and Immunohistochemistry Staining

The quantitation of positively stained cells in tissue sections was performed as previously described (5). Briefly, the number of cells labeled by the MIF cRNA probe or anti-MIF MAb were counted in at least 50 consecutive glomerular cross sections (gcs) in each animal. In double-stained sections, the number of ED1⁺, MIF⁺, and ED1⁺MIF⁺ cells was counted in 50 glomerular cross sections. Macrophage accumulation within the tubulointerstitium was assessed by counting ED1⁺ cells in 20 consecutive high-power fields (\times 40) by means of a 0.02 mm² graticule fitted into the eyepiece of the microscope. Counting progressed from the outer cortex to the inner cortex, avoiding only large vessels and glomeruli. In addition, the number of labeled tubules was scored in at least 1000 consecutive cortical tubular cross sections. Data from groups of six animals are expressed as the mean \pm standard error of the mean (SEM) per glomerular cross section or per square millimeter (mm²).

MIF Secretion by Cultured Glomeruli

Glomeruli were purified by differential sieving. Briefly, a half kidney from each animal was placed in RPMI 1640 with 5% fetal calf serum (FCS), diced finely, gently pressed through a 250- μ m wire mesh, and poured through a 106- μ m and then a 75- μ m wire mesh. Glomeruli remaining on the top of a 75- μ m mesh were washed extensively with RPMI/5% FCS and collected. Isolated glomeruli were >95% pure as assessed by phase-contrast microscopy. Glomeruli were cultured at 3000 glomeruli/ml in RPMI 1640/5% FCS for 24 hr and the supernatant was harvested. The concentration of MIF in supernatant samples was quantitated by a sandwich ELISA (7).

Serum MIF Levels

Serum MIF levels were assessed by Western blotting since the presence of rat serum proteins

TABLE 1. Effect of TNFbp treatment on renal injury in anti-GBM disease

	Proteinuria (mg/24 hr)	Serum Creatinine (μ mol/L)	Glomerular Hypercellularity (0–3)	Glomerular Crescents (%)	Interstitial Fibrosis (0–4)
Normal	9 \pm 2	42.7 \pm 1.0	0	0	0
Saline treated					
Day 1	65 \pm 22 ^a	47.8 \pm 6.0	0.82 \pm 0.10	0	0.8 \pm 0.2
Day 7	204 \pm 22 ^c	59.3 \pm 4.4 ^b	1.30 \pm 0.17	10.3 \pm 2.1	2.3 \pm 0.3
Day 14	288 \pm 14 ^c	67.2 \pm 2.4 ^c	1.40 \pm 0.06	17.3 \pm 2.6	3.2 \pm 0.3
TNFbp treated					
Day 1	43 \pm 4 ^a	49.0 \pm 4.4	0.71 \pm 0.07	0.3 \pm 0.3	0.5 \pm 0.2
Day 7	125 \pm 18 ^{c,f}	47.2 \pm 5.1	0.99 \pm 0.12	0.3 \pm 0.2 ^f	1.2 \pm 0.2 ^d
Day 14	190 \pm 16 ^{c,f}	46.0 \pm 1.8 ^e	0.89 \pm 0.07 ^e	0.9 \pm 0.3 ^f	1.2 \pm 0.2 ^e

Data shown as mean \pm SEM. ^a*p* < 0.05, ^b*p* < 0.01; ^c*p* < 0.001 versus normal; ^d*p* < 0.05; ^e*p* < 0.01, ^f*p* < 0.001 compared to time-matched saline-treated animals.

interfered with the MIF ELISA. Five-microliter serum samples from all animals were resolved by SDS polyacrylamide gel electrophoresis, blotted onto nitrocellulose membrane, incubated sequentially with rabbit anti-murine MIF polyclonal antibody and horse radish peroxidase-conjugated goat anti-rabbit antibody, and developed with chloronaphthol/H₂O₂, as previously described (4). Recombinant MIF was used as a control to compare levels of MIF expression.

Statistical Analyses

One-way analysis of variance (ANOVA) from the Complete Statistical Analysis program (CSS, Statsoft, Tulsa, OK, USA) was used to analyze differences in MIF expression, macrophage accumulation, and renal functional in normal animals, TNFbp-treated, and saline-treated anti-GBM disease. Differences in histology were compared by the nonparametric Mann-Whitney U test.

RESULTS

Renal Function and Histopathology

The induction of anti-GBM disease in saline-treated animals resulted in severe proteinuria and a significant increase in serum creatinine levels (Table 1). Saline-treated animals showed significant glomerular hypercellularity and crescent formation plus marked interstitial fibrosis on Day 14 of anti-GBM disease. In contrast, TN-

Fbp treatment caused a significant reduction in proteinuria and maintained normal levels of serum creatinine (Table 1). Consistent with these measurements, TNFbp-treated animals showed only mild glomerular hypercellularity and interstitial fibrosis, whereas crescent formation was almost entirely abolished.

Glomerular MIF Expression and Macrophage Accumulation

In situ hybridization and immunohistochemistry staining showed that a small number of glomerular visceral and parietal epithelial cells constitutively expressed MIF mRNA (3–10 cells/gcs) and protein (0–3 cells/gcs) (Fig. 1a, b). The induction of anti-GBM disease in saline-treated animals caused a rapid increase in the number of glomerular cells expressing MIF mRNA and protein on Day 1, and this was further increased on Days 7 and 14 (Figs. 1 and 2). This up-regulation of glomerular MIF expression was paralleled by macrophage accumulation over the 14-day period (Fig. 1c). TNFbp treatment caused only a partial reduction in glomerular MIF expression on Day 1 of disease compared with expression in saline-treated animals, and this did not affect glomerular macrophage accumulation (Fig. 1a–c). However, TNFbp treatment almost completely prevented any further increase in MIF mRNA or protein expression during the rest of the course of the disease, which was paralleled by inhibition of glomerular macrophage accumulation (Figs. 1 and 2).

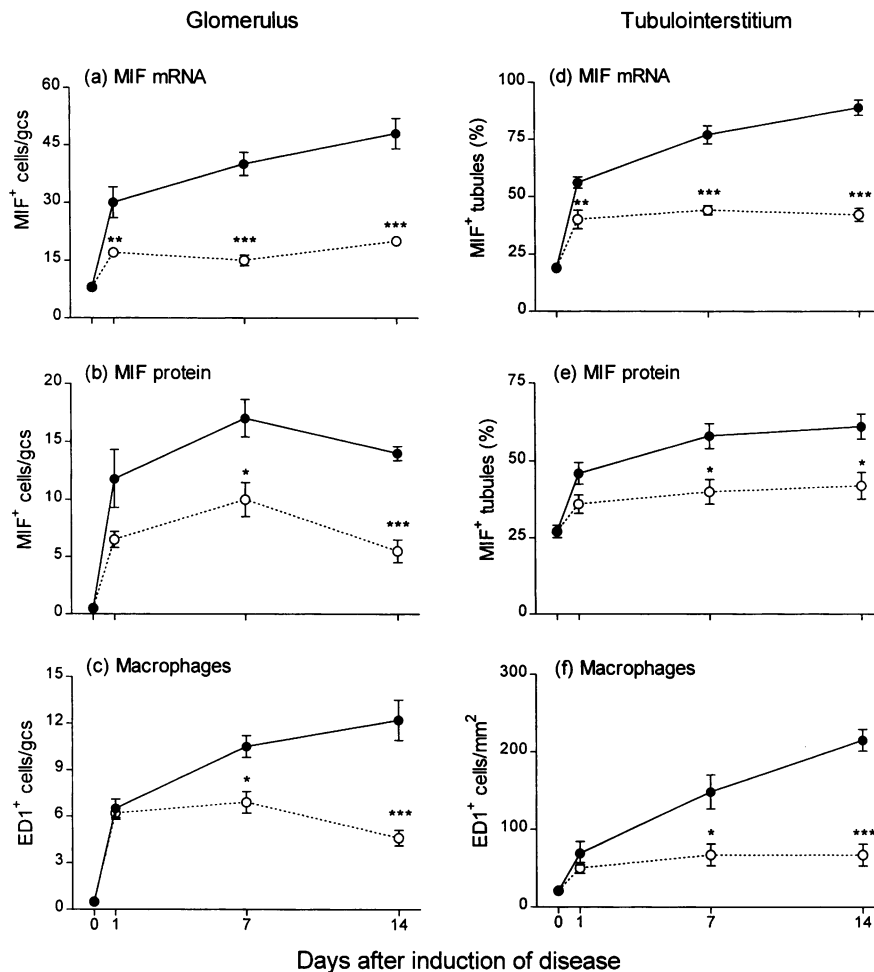


FIG. 1. TNFbp treatment inhibits glomerular and tubular MIF expression and macrophage accumulation in rat anti-GBM glomerulonephritis

(a) Quantitation of glomerular MIF mRNA expression by in situ hybridization; (b) quantitation of glomerular MIF protein expression by immunohistochemistry; (c) quantitation of glomerular ED1-positive macrophages; (d) quantitation of tubular MIF mRNA expression by in situ hybridization; (e) quantitation of tubular MIF protein expression by immunohistochemistry; and (f) quantitation of ED1-positive macrophages within the tubulointerstitium. Saline-treated animals are represented by solid lines and closed circles, TNFbp-treated animals, by dotted lines and open circles. Data are expressed as the mean \pm SEM from groups of six animals. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with saline-treated animals.

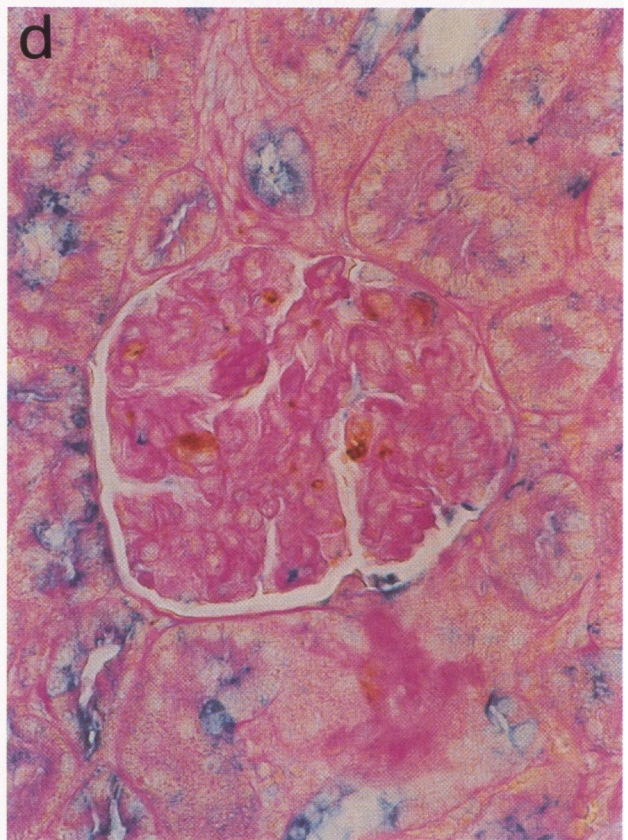
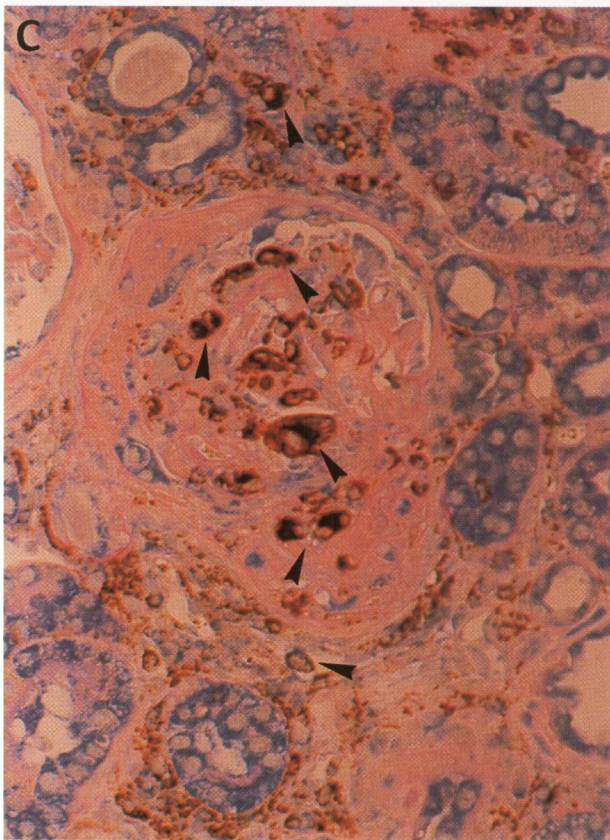
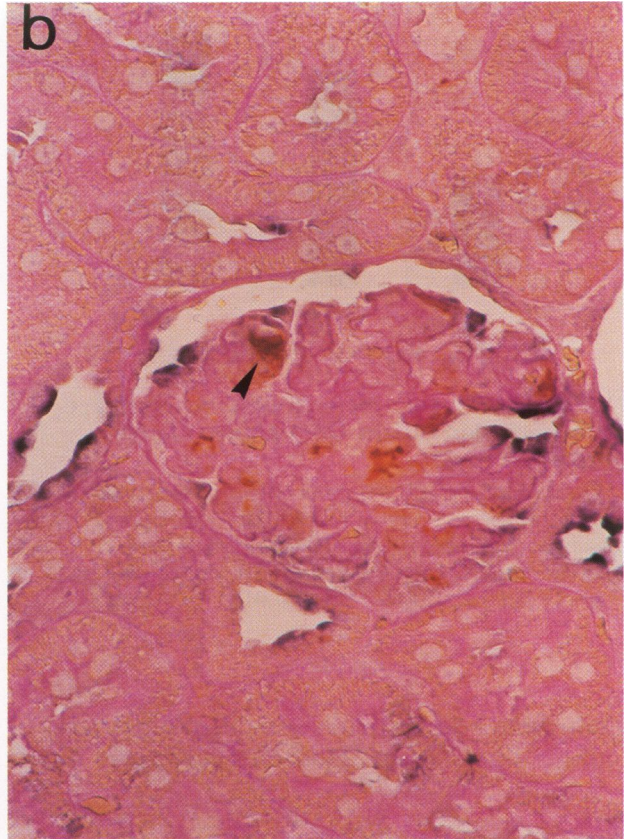
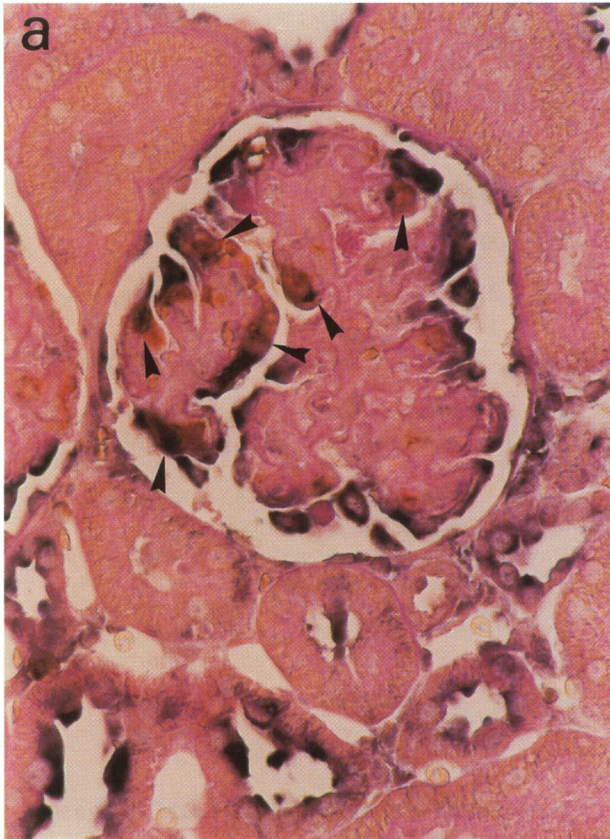
Since TNF- α is known to induce macrophage MIF production in vitro, we used double immunohistochemistry staining to examine the number of macrophages expressing MIF mRNA and protein in anti-GBM disease (Fig. 2). Quantitation of double staining demonstrated that the number of glomerular ED1⁺MIF⁺ macrophages was significantly inhibited by TNFbp treatment (Fig. 3). While this was due in part to an overall reduction in the number of glomerular macrophages seen with TNFbp treatment, there was also a reduction in the percentage of glomerular macrophages positive for MIF (25.8 ± 4.5 versus

$58.0 \pm 3.4\%$ in TNFbp- and saline-treated animals, respectively; $p < 0.001$).

Glomerular MIF production was also quantitated by measuring MIF secretion by purified glomeruli during a 24-hr culture period. Substantial levels of MIF were secreted by glomeruli cultured from normal rat kidney (Fig. 4). This was significantly increased on Day 14 of anti-GBM disease in saline-treated animals. TNFbp treatment caused a significant reduction in glomerular MIF secretion compared with that in saline-treated animals. In addition, TNFbp treatment produced a trend towards a reduction in the basal level of

FIG. 2. TNFbp treatment inhibits glomerular and tubular MIF expression and macrophage accumulation in rat anti-GBM glomerulonephritis

Combined in situ hybridization and immunohistochemistry show MIF mRNA expression (blue) by intrinsic kidney cells and by ED1-positive macrophages (brown) on Day 7 of disease with (a) saline or (b) TNFbp treatment. Double immunohistochemistry shows colocalization of strong MIF expression (blue) and ED1-positive macrophages (brown) on Day 14 of disease with (c) saline or (d) TNFbp treatment. Macrophages (brown) expressing MIF mRNA or protein are indicated by arrowheads. Strong MIF expression and the development of crescent formation and tubulitis are seen. Sections were counterstained with PAS minus haematoxylin. Original magnification, $\times 400$.



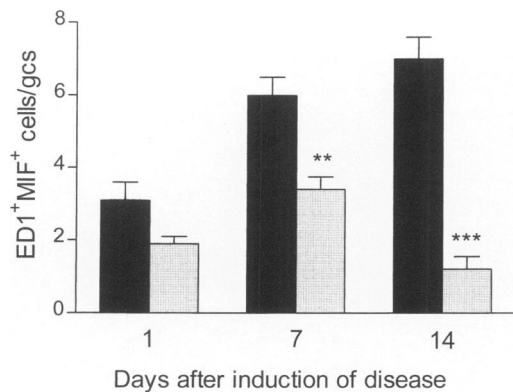


FIG. 3. TNFbp treatment inhibits macrophage MIF expression in anti-GBM glomerulonephritis

Quantitation of double immunohistochemistry staining shows the number of MIF⁺ED1⁺ macrophages in saline- (solid bars) and TNFbp- (dotted bars) treated animals. Each bar represents groups of six animals (mean \pm SEM). ** $p < 0.01$, *** $p < 0.001$.

glomerular MIF secretion seen in normal rats, but this did not reach statistical significance.

Tubular MIF Expression and Interstitial Macrophage Accumulation

Tubular epithelial cells are the main source of MIF in normal kidney, with up to 30% of cortical tubules being positive for MIF mRNA and protein expression. As in glomeruli, there was a rapid up-regulation in tubular MIF mRNA and protein on Day 1 of saline-treated anti-GBM disease, which was further increased over the course of the disease (Figs. 1 and 2a, c). A significant interstitial macrophage infiltration occurred in parallel with the increase in MIF expression (Fig. 1f). Tubular MIF expression was prominent within focal tubulointerstitial lesions and the development of tubulitis. Double-labeling studies showed that most interstitial ED1⁺ macrophages were present in and around tubulointerstitial lesions with strong tubular MIF expression (Fig. 2a, c).

Consistent with the effects seen in glomeruli, TNFbp treatment caused only a partial reduction in tubular MIF expression on Day 1, but it prevented any further increase in tubular MIF mRNA and protein expression over Days 7 and 14 of the disease (Fig. 1d, e). This was associated with a marked suppression of both interstitial macrophage infiltration and a complete inhibition of tubulointerstitial damage (Table 1, Figs. 1 and 2).

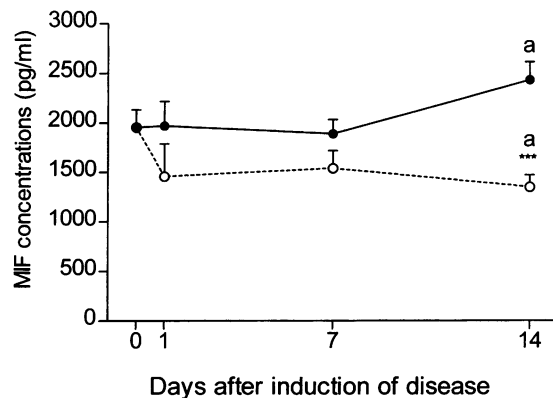


FIG. 4. TNFbp treatment inhibits glomerular MIF secretion in rat anti-GBM glomerulonephritis

Secretion of MIF by cultured glomeruli over a 24-hr period was quantitated by ELISA in normal rats (Day 0) and in saline- (solid circles) and TNFbp- (open circles, dotted line) treated animals. Data are expressed as the mean \pm SEM from groups of six animals. *** $p < 0.001$, compared with saline-treated animals; a, $p < 0.05$, compared with normal animals (Day 0).

Serum MIF Levels in Anti-GBM Disease

To compare the effects of TNFbp treatment on systemic MIF production during anti-GBM disease, we examined serum MIF levels by Western blotting. MIF was detected in normal rat serum and MIF levels were substantially increased during the course of saline-treated anti-GBM disease (Fig. 5). In contrast, TNFbp treatment resulted in undetectable levels of serum MIF on Day 1 and markedly reduced serum MIF levels over Days 3 to 14, compared with saline-treated animals.

DISCUSSION

This study is the first to demonstrate that TNF- α up-regulates both local and systemic MIF production in an immunologically induced disease. In previous studies, we described an excellent correlation between up-regulation of renal MIF expression and the progression of renal injury in rat crescentic glomerulonephritis (5). Furthermore, it has recently been reported that treatment of this disease model with a neutralizing anti-MIF antibody substantially blocks macrophage accumulation and progressive renal injury, demonstrating a pathogenic role for MIF in macrophage-mediated glomerulonephritis (20). Blockade of TNF- α action by administration of



FIG. 5. TNFbp treatment reduces serum MIF levels during the anti-GBM glomerulonephritis

Through Western blot analysis, the presence of serum MIF protein was detected in normal rats, which is the same size as the recombinant protein. TNFbp treatment caused a marked reduction in the level of serum MIF protein over the entire disease time course, compared with that in saline-treated animals.

the TNFbp caused a profound suppression of renal MIF production, which was associated with inhibition of macrophage infiltration, reduction in renal injury, prevention of renal function impairment, and histological damage. This confirms the importance of TNF- α in the development of anti-GBM glomerulonephritis (10,11). Furthermore, it suggests that up-regulation of MIF production is a pathological manifestation of progressive renal functional and histologic injury, including glomerular crescent formation. However, TNF- α may also act to promote macrophage accumulation through other mechanisms. For example, TNF- α can induce expression of interleukin-1, monocyte chemoattractant protein-1, and intercellular adhesion molecule-1, all of which have been shown to contribute to leukocytic infiltration and subsequent renal injury in rat anti-GBM disease (13,21,22).

The ability of TNFbp treatment to inhibit macrophage MIF mRNA and protein expression during crescentic glomerulonephritis confirms the relevance of *in vitro* studies in which TNF- α was found to induce macrophage MIF production (4). In addition, the ability of TNFbp to inhibit MIF mRNA and protein expression by intrinsic renal cells, such as glomerular and tubular epithelial cells, demonstrates that TNF- α can also induce MIF expression in cell types other than leukocytes. Furthermore, TNFbp treatment reduced serum MIF levels. Although the cellular source of circulating MIF has yet to be identified, this study has shown that TNF- α up-regulates the systemic MIF response in inflammatory disease. It should be pointed out that while TNFbp treatment suppressed both local and systemic MIF expression, levels of MIF expression were not reduced below those seen in

normal animals, demonstrating that TNF- α does not regulate constitutive MIF expression.

These data support the existence of an amplification loop between TNF- α and MIF that exerts a potent proinflammatory effect *in vivo*. This pathway may operate through the transcription factor NF- κ B. Expression of the TNF- α gene is strongly induced by NF- κ B, and TNF- α stimulation of target cells results in activation of NF- κ B (23,24). Furthermore, an NF- κ B site has been identified within the promoter of the mouse MIF gene (17). Thus, it is feasible that both TNF- α and MIF act to mutually up-regulate each other through the activation of NF- κ B. This may also be the mechanism of the counter-regulatory relationship between MIF and glucocorticoids, since glucocorticoids have recently been shown to exert their anti-inflammatory action by inhibiting activation of NF- κ B (25). Nevertheless, the presence of endocrine-responsive elements within the MIF gene promoter also indicates that the regulation of MIF transcription may be more complex (17).

In summary, this study has demonstrated that TNF- α -induced up-regulation of local and systemic MIF expression may be one of the important mechanisms mediating renal injury in experimental crescentic glomerulonephritis. Furthermore, this provides the first *in vivo* evidence that TNF- α and MIF act together in a proinflammatory amplification loop in the pathogenesis of immune/inflammatory disease.

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