Induction of Monocyte Chemoattractant Protein-1 in Proximal Tubule Cells by Urinary Protein

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Abstract. Cytokines play a pivotal role in synthesis and deposition of extracellular matrix in chronic renal failure (CRF). The proinflammatory properties of monocyte chemoattractant protein (MCP)-1 make it an ideal candidate cytokine for the production of interstitial inflammation in CRF. To investigate the possible role of proteinuria in inducing proximal tubular (PT) MCP-1, MCP-1 mRNA levels were measured by Northern blot and reverse transcription PCR in confluent monolayers of PT cells in primary culture in media containing a variety of proteins. PT cells produced MCP-1 mRNA in response to bovine serum albumin (BSA), delipidated BSA (dBSA; 0.5 to 30 mg/ml), holotransferrin, and apotransferrin (1 to 8 mg/ml). Unstimulated PT cells expressed very low levels of MCP-1 mRNA, detectable by reverse transcription PCR but not by Northern blot. The expression of MCP-1 mRNA reached a peak (sixfold greater than control) within 4 h of exposure to dBSA and was maintained for at least 24 h with continued exposure. Removal of dBSA from the media led to a rapid decline in MCP-1 mRNA expression. dBSA-induced MCP-1 expression was inhibited by lysine, an inhibitor of protein uptake, and reproduced by dBSA purified by gel and size-selective filtration. dBSA influenced MCP-1 expression at the level of transcription and probably translation, as evidenced by abrogation of MCP-1 by actinomycin D and superinduction with the protein synthesis inhibitor cycloheximide. The concentration of MCP-1 protein in response to dBSA added to the apical surface of PT cells was 2.4-fold greater in basolateral than in apical media, indicating basolateral secretion of MCP-1 protein. In summary, PT cell MCP-1 mRNA and protein expression are upregulated by albumin and transferrin, in concentrations similar to those of proteinuric urine. This effect could explain the link between proteinuria and interstitial inflammation in CRF. (J Am Soc Nephrol 8: 1537-1545, 1997)

Interstitial inflammation and proteinuria are hallmarks of progressive chronic renal disease (1), and proteinuria has been implicated as an effect of progressive renal scarring, particularly in the tubulointerstitial compartment (2). The degree of proteinuria appears to correlate with the rate of progression of renal failure in both human and experimental renal disease (3).

Previous studies in this laboratory have shown that holotransferrin (Tf-Fe), but not apotransferrin or albumin, is toxic to rat proximal tubule cells via a pH-dependent mechanism that involves cell uptake of Tf-Fe, intracellular iron accumulation, and lipid peroxidation. These studies suggested that the iron moiety of Tf-Fe, by causing tubular cell injury, could lead to interstitial injury in proteinuric chronic renal disease (4). However, the mechanisms by which iron-free proteins such as albumin and apotransferrin, also important components of proteinuric urine, may cause interstitial scarring and progression of renal impairment remain undefined.

Monocytes form an early and prominent component of interstitial infiltrate and appear to play a key role in further evolution of tubulointerstitial inflammation and fibrosis (5). The stimuli responsible for initial monocyte recruitment into and activation in the interstitial compartment are unknown, but almost certainly include chemokines. Monocyte chemoattractant protein (MCP)-1 is a prototype of the β chemokine family, and data from humans (6) and experimental animals (7) suggest strongly that it is one of the chemokines primarily responsible for interstitial monocyte recruitment. MCP-1 is thought to act primarily on monocytes, but recent studies suggest that MCP-1 may also activate lymphocytes, basophils, and eosinophils (8). Its expression can be induced in renal tubular cells after ischemia and after exposure to a variety of cytokines and noxious molecules (9-11). Under appropriate stimuli, tubular cells are able to produce a whole host of cytokines and other potential mediators of inflammation.

It seems reasonable to propose that tubular cells, as the predominant cell type in the kidney, and based on their unique position between the tubular lumen and surrounding interstitium, play an important role in initiating interstitial pathological change. We hypothesized that urinary proteins per se might induce proximal tubular cells to overproduce MCP-1 and thereby cause interstitial inflammation. The aim of this study was to investigate this potential mechanism linking proteinuria to the development of chronic interstitial inflammation.

Materials and Methods
Proximal Tubule Isolation and Culture

Proximal tubular cells were isolated from normal Wistar rats (weighing 250 to 300 g) by a modification of the method of Vinay et
Table 1. Primers for GAPDH and MCP-1

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Size (bp)</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Annealing Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>822</td>
<td>5' AATGGGTGATGCTGGTGCTGA</td>
<td>3' TGGGGGGCTGAGTTGGGATGG</td>
<td>68°C</td>
</tr>
<tr>
<td>MCP-1</td>
<td>595</td>
<td>5' TATGCAGGTCTCTGTCAAGG</td>
<td>3' TATGCAGGTCTCTGTCAAGG</td>
<td>60°C</td>
</tr>
</tbody>
</table>

*Temp., temperature; MCP, monocyte chemoattractant protein.

Figure 1. Representative Northern blot demonstrating expression of monocyte chemoattractant protein (MCP)-1 mRNA in proximal tubule (PT) cells. Cells were treated with 0 to 30 mg/ml bovine serum albumin (BSA; A) or 1 to 8 mg/ml transferrin (Tf; B) for 8 h. MCP-1 was undetectable in the control. Mean ± SD densitometric ratios of MCP-1/β-actin are derived from two separate experiments.

al. (12). Briefly, kidneys were perfused in vivo with Krebs-Henseleit bicarbonate buffer (KHB; Sigma-Aldrich, Sydney, Australia) via the abdominal aorta. Kidney cortices were dissected from medulla, sliced, minced on a coarse metal mesh using a syringe plunger in KHB, and washed twice after brief centrifugation at 1000 x g. Cortices were incubated at 37°C for 20 mm with agitation in KHB containing 0.5 mg of collagenase (Type II, Sigma-Aldrich) per milliliter. The buffer was gassed with 95% oxygen and 5% carbon dioxide in a sealed roller bottle. At the end of digestion, the tissue suspension was passed through a 50-mesh screen (Sigma-Aldrich) and washed in KHB. Proximal tubule cells were gradient-selected using a 32% Percoll buffer solution and spun at 2000 x g for 3 min. The pellet containing proximal tubules was washed thoroughly in KHB and suspended in Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham (DMEM/F-12, 1:1 mixture, Sigma-Aldrich), supplemented with 5% heat-inactivated fetal calf serum (FCS). Transmission electronmicroscopy of freshly isolated and also cultured cells confirmed their proximal tubular origin, with microvilli, lysosomes, and mitochondria.

Fresh proximal tubules were added to 20-mm plastic culture plates coated with rat tail collagen and cultured at 37°C in air containing 5% carbon dioxide. After overnight culture, unattached tubules were washed away and new Dulbecco's F-12 medium was added without FCS, but with epidermal growth factor (10 ng/ml) and insulin (5 μg/ml). Tubules were cultured for an additional 4 d and allowed to grow to confluence.

Purification of Albumin

For some experiments, delipidated bovine serum albumin (dBSA) (lipopolysaccharide [LPS] < 0.1 ng/mg, Sigma) was fractionated by gel filtration (Sephadex G-75, Pharmacia/Biotec, Uppsala, Sweden). Ten-milliliter samples (20 mg/ml) were loaded onto a 2.5 x 75-cm gel column. dBSA fraction was collected by recording the volume at which dBSA displayed a maximum elution peak. Further purification and concentration were carried out using Ultrafilters (Amicon, W. R. Grace Australia, Ltd.) with a YM30 membrane, which filters out
proteins of Mr <30,000 (thus excluding LPS and most cytokines), under 25 psi pressure. The samples were run on sodium dodecyl sulfate-polyacrylamide gels and demonstrated a single band. The amount of purified dBSA was measured using the method of Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Treatments

Cultured confluent proximal tubule cells were treated on the fifth day of culture. The proximal tubule cells were treated with 30 mg/ml bovine serum albumin (BSA), 0.1 to 30 mg/ml dBSA, bovine apotransferrin (Tf; 1 to 8 mg/ml), and holotransferrin (Tf-Fe; 8 mg/ml). These concentrations of proteins were chosen to reflect those of severe proteinuria. In selected experiments, 100 mM lysine (an inhibitor of protein uptake at the brush border membrane), 10 μM actinomycin D (an inhibitor of transcription), and 100 μM cycloheximide (an inhibitor of protein synthesis) were added to the medium with BSA for 8 h in culture. Phorbol myristate acetate (100 nM) and concanavalin A (5 μg/ml) were used as positive controls. To exclude a role for low amounts of LPS in dBSA, proximal cells were treated with 0.1 to 10 μg/ml endotoxin for 8 h under conditions used for dBSA treatment.

Histochemistry and Immunocytochemistry

Proximal tubule cells in primary culture were plated onto four-chamber slides (Medos Co., Sydney, Australia). Confluent cells were washed twice with phosphate-buffered saline (PBS) and fixed in 2.5% paraformaldehyde for 10 min. For alkaline phosphatase histochemistry, cells were incubated in 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium chloride (BCIP/NBT) in 100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM MgCl$_2$ at room temperature in the dark overnight. For immunocytochemistry, slides were incubated in 0.3% H$_2$O$_2$ methanol for 3 min at −20°C to block endogenous peroxidase activity. After washing three times in PBS, slides were blocked with nonantigenic goat serum. The cells were incubated with individual primary antibodies (anti-vimentin: 1:5, Boehringer Mannheim Australia, Sydney, Australia; ED-1:1:2500, Serotec, Oxford, United Kingdom; anti-MCP-1:1:40, Serotec) for 60 min at room temperature, then incubated with biotinylated secondary antibody, and the avidin-biotin complex was visualized using diaminobenzidine as a chromogen (Sigma).

After BCIP/NBT treatment, more than 95% of cells in primary culture showed extensive purple staining, indicating the presence of alkaline phosphatase enzyme, a specific marker of proximal tubular
Table 2. The effect of lysine to inhibit protein uptake by proximal tubule cells incubated with medium containing dBSA-1\(^{125}\) (0.8 mg/ml) and Tf-1\(^{125}\) (35 pg/ml)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>dBSA-1(^{125}) (µg/dish, n = 6)</th>
<th>Tf-1(^{125}) (pg/dish, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>1.73 ± 0.43</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>Lysine (100 mM)</td>
<td>0.98 ± 0.36(^b)</td>
<td>0.18 ± 0.01(^c)</td>
</tr>
</tbody>
</table>

\(^a\) dBSA, delipidated bovine serum albumin; Tf, transferrin.
\(^b\) P < 0.05.
\(^c\) P < 0.01 versus control.

There were no cells stained with ED-1, and there was minimal staining for vimentin.

**Cytotoxicity**

Cell toxicity was assessed by lactate dehydrogenase release, according to the method of Bergmeyer and Bernt (13), and lipid peroxidation was assessed by production of malondialdehyde, as described previously (14).

**Protein Uptake**

Protein uptake into tubule cells was examined by the method of Trinder et al. (15) for transferrin and the method of Brunskill et al. (16) for albumin. Briefly, the cells were incubated with 35 pg/ml transferrin-1\(^{125}\) for 1 h or with 0.8 mg/ml human albumin-1\(^{125}\) for 15 min in 20-mm dishes. After incubation, cells were washed with PBS, lysed by addition of 0.1N NaOH, and radioactivity was measured by a \(\beta\)-counter (LKB Wallac, Turku, Finland). Protein uptake was examined with and without lysine (100 mmol), an inhibitor of protein uptake (17).

**RNA Analysis**

RNA was extracted from cultured cells using RNA isolation solvent, according to specifications of the manufacturer (Biotecx, Houston, TX). Ten micrograms of total RNA was used for Northern blot, and 1 µg was used for reverse transcription (RT)-PCR.

For Northern blot, RNA was fractionated through 1% agarose gel and blotted onto Hybond-N membrane (Amersham, Buckinghamshire, United Kingdom). A fragment of rat MCP-1 (a gift from Dr. Yoshimura, Kumamoto University School of Medicine, Kumamoto City, Japan) (18) was radiolabeled with a random-priming DNA label kit and used as a probe in these experiments.

For RT-PCR, random-primed first-strand cDNA was synthesized with a kit using 1 µg of total RNA, according to the manufacturer's protocol (Boehringer Mannheim). PCR was performed in a total volume of 50 µl containing 0.25 µl of Taq, 5 µl of 10X PCR buffer, 200 µM dNTP, 40 nM oligonucleotide primer, and 0.5 µl of cDNA mixture. Primers for MCP-1 and GAPDH were designed according to sequences in GenBank. The primers span one or more introns to
Figure 5. Expression of MCP-1 mRNA by RT-PCR in untreated PT cells and cells treated with 100 μM cycloheximide (Cyclo); 15 mg/ml dBSA; 100 μM cycloheximide and 15 mg/ml dBSA; or 10 μM actinomycin D (act D) and 15 mg/ml dBSA. Numbers refer to relative densitometry readings of MCP-1 GAPDH in four separate experiments. *P < 0.05 versus control. Mean ± SD.
Figure 6. MCP-1 mRNA expression by RT-PCR in untreated PT cells and cells treated with purified dBSA (component A), another component of dBSA (component B), or unpurified dBSA (15 mg/ml).

Discussion

These studies have demonstrated that after apical exposure to proteins, including BSA, dBSA, apotransferrin, and holotransferrin, cultured proximal tubule cells produce MCP-1 mRNA and protein, and secrete MCP-1 protein across the basolateral cell surface. Purification of BSA demonstrated that it was the albumin component of BSA and not a contaminant that was responsible for this effect. The concentrations of protein that induced MCP-1 mRNA were similar to those found in heavy proteinuria, suggesting that these in vitro results could provide an explanation for the close association between proteinuria and interstitial inflammation in progressive chronic renal disease.

Progressive renal disease is characterized histologically by an interstitial infiltrate of mononuclear cells and lymphocytes and by the gradual destruction of normal tubulointerstitial architecture with accumulation of extracellular matrix protein.
Recruitment of mononuclear phagocytes from blood compartment into tissues is a pivotal process in inflammatory and immune reactions, and a large number of inflammatory mediators are potentially responsible for this process (19,20). The mechanisms responsible for the chemotraction of circulating blood monocytes into the renal interstitium in progressive renal disease are not clear. Evidence suggests that MCP-1, a chemottractant cytokine specific for monocytes, may play an important role in mediating infiltration of inflammatory cells into the renal interstitium (21).

The normal kidney expresses small amounts of MCP-1. MCP-1 expression is increased in a number of renal diseases and animal models, including ischemia (10), hydropnepherosis (22), glomerulonephritis (23,24), diabetic nephropathy (25), lupus nephritis (26), renovascular hypertension (27), and partial nephrectomy (28).

Renal MCP-1 was found to arise from glomerular cells in anti-glomerular basement membrane glomerulonephritis (29) and mesangial cells in streptozotocin-induced diabetes and anti-Thy-1 nephritis (30). In contrast, Eddy and Giachelli reported that MCP-1 protein was localized within tubular cells in rats with protein-overload proteinuria (31) and puromycin aminonucleoside nephrosis (32), although failure of MCP-1-neutralizing antibody to alter interstitial macrophages argued against a primary role for MCP-1, at least in these models. Prodjosudjadi et al. showed that MCP-1 expression is increased in renal tubular epithelial cells in various types of glomerular disease and in human proximal tubular epithelial cell lines in response to interleukin (IL)-1β and tumor necrosis factor (TNF)-α (33). However, it is still unknown whether tubular cells per se produce MCP-1 or whether MCP-1 is reabsorbed into tubular cells after its release from different sites. Furthermore, although several chemicals have been shown to upregulate tubular MCP-1 in vitro (11,33), it is unclear which stimuli are responsible for tubular cell MCP-1 production in vivo, and whether a component of proteinuric urine may be responsible.

The present study demonstrated that MCP-1 is produced by tubular cells after exposure to albumin and transferrin, two important components of proteinuric urine. Moreover, MCP-1 expression was seen with exposure of the apical surface of the proximal tubule cell to these proteins, analogous to the in vivo situation in which proteinuric tubular fluid bathes the luminal cell membrane. dBSA and lipidated BSA increased MCP-1 mRNA to a similar extent, a result that contrasts with the contention of Kees-Foits et al. (34), who hold that a fatty acid component of albumin is necessary for its induction of a chemotactic factor by proximal tubule segments. However, the chemotactic factor of those experiments was a nonpolar lipid, and so clearly not MCP-1.

If proximal tubular MCP-1 production is to be accepted as a mediator of macrophage infiltration into the interstitium, then MCP-1 protein needs to gain access to that compartment. The present study demonstrated a 2.4-fold greater concentration of MCP-1 protein in basolateral than in apical media, indicating that MCP-1 protein is secreted across the basolateral membrane of cultured proximal tubule cells, the cell membrane that faces the renal interstitium in vivo.

Other investigators have shown that cultured human renal epithelial cells may upregulate MCP-1 mRNA in response to interferon, IL-1, TNF-α, and LPS (11). This raises the possibility that it was a contaminant of the BSA solution, and not protein itself, which activated tubule cells to produce MCP-1.
The dBSA used in these experiments had a very low level of LPS and was purified further by gel filtration and membrane filtration. This reduced the possibility of contamination with the above cytokines, which have a molecular weight ranging from 17 to 25 kb. Lysine, a potent inhibitor of albumin uptake (17) across the luminal membrane of the proximal tubular cell, reduced the upregulation of MCP-1 by dBSA, further supporting the contention that it was the protein component of BSA that upregulated MCP-1 in proximal tubular cells. The expression of MCP-1 mRNA level showed a rapid response to dBSA stimulation and declined quickly after withdrawal of dBSA, indicating that continued exposure to protein is essential for maintenance of MCP-1 upregulation.

Ischemia and other noxious events can increase tubular cell MCP-1 expression (10), and superoxide anions, whether generated by such events, can activate MCP-1 gene in tubular cells. In the present experiments, there was no evidence of cytotoxicity or lipid peroxidation in response to dBSA and apotransferrin. This suggests that MCP-1 mRNA expression was due directly to protein rather than a secondary response to cytotoxicity or lipid peroxidation.

Although a strong correlation exists between the degree of proteinuria and the rate of progression of renal failure, proteinuria is not always associated with progressive renal injury. In minimal change disease, proteinuria—predominantly albuminuria—is heavy, yet tubulointerstitial scarring is absent. It is possible that tubulointerstitial damage does not occur in minimal change disease because proteinuria is episodic, either spontaneously or in response to steroid treatment. For example, tubular cell injury, as evidenced by the excretion of the tubular cell protein N-acetyl glucosaminidase, has been shown during periods of heavy proteinuria in minimal change disease, but not after resolution of the proteinuria (35). Alternatively, an additional stimulus may be required for in vivo tubular cell production of MCP-1 in response to urinary protein. The requirement for such an additional stimulus is unproven and its nature is unknown, but deserves investigation.

The role of protein in MCP-1 gene transcription and translation was examined, using inhibitors of RNA and protein synthesis. It has been reported that actinomycin D and cycloheximide blocked IL-1, TNF-α, and LPS-induced MCP-1 gene expression in monocytes (36). However, in the present experiment, dBSA-induced MCP-1 mRNA expression was abrogated by actinomycin D but was not inhibited by cycloheximide. In fact, cycloheximide increased MCP-1 mRNA expression in response to dBSA, implicating an effect of dBSA on a cellular protein involved in downregulating MCP-1 transcription. Alternatively, these results could be explained by an effect of cycloheximide to stabilize RNA transcripts, as has been shown for other genes such as that of interferon gamma. (37). This lack of inhibition of MCP-1 by cycloheximide has been described in other epithelial cells (38).

In conclusion, MCP-1 production in renal tubular cells is enhanced by exposure to albumin and transferrin in concentrations found in proteinuric urine. This raises the possibility that interstitial inflammation may be triggered by a novel pathway, involving a direct effect of urinary proteins to increase proximal tubular cell production of inflammatory mediators such as MCP-1.

Acknowledgments

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References


Table 3. Toxicity, as assessed by LDH release from and MDA levels in monolayer tubule cells exposed to treatment for 8 h on day 5 of culturea

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>LDH Leakage (%)</th>
<th>MDA (pmol/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>7.4</td>
<td>5.50 ± 0.55</td>
<td>0.78 ± 0.40</td>
</tr>
<tr>
<td>Nil</td>
<td>6.0</td>
<td>5.50 ± 0.55</td>
<td>0.57 ± 0.33</td>
</tr>
<tr>
<td>dBSA (30 mg/ml)</td>
<td>7.4</td>
<td>5.42 ± 0.98</td>
<td>0.57 ± 0.21</td>
</tr>
<tr>
<td>dBSA (30 mg/ml)</td>
<td>6.0</td>
<td>6.02 ± 0.59</td>
<td>0.79 ± 0.25</td>
</tr>
<tr>
<td>Tf (8 mg/ml)</td>
<td>7.4</td>
<td>5.23 ± 0.04</td>
<td>0.69 ± 0.36</td>
</tr>
<tr>
<td>Tf-Fe (8 mg/ml)</td>
<td>7.4</td>
<td>4.01 ± 0.23</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td>Tf-Fe (8 mg/ml)</td>
<td>6.0</td>
<td>10.43 ± 2.93b</td>
<td>3.03 ± 1.56b</td>
</tr>
</tbody>
</table>

a LDH, lactate dehydrogenase; MDA, malondialdehyde; Tf-Fe, holotransferrin. Other abbreviations as in Table 2.
b P < 0.001 from control by ANOVA.