Monocyte chemoattractant protein-1 and osteopontin differentially regulate monocytes recruitment in experimental glomerulonephritis

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Background. This study evaluated the mechanisms of monocyte/macrophage (M/M) infiltration in a rat model of anti-glomerular basement membrane glomerulonephritis (GN). We focused on chemokines and osteopontin, which are known regulators of M/M recruitment.

Methods. Using immunohistology, in situ hybridization, and Northern blotting, the expression levels of chemokines and osteopontin were evaluated in isolated glomeruli and tubules 4, 10, and 20 days after the induction of GN. In vivo blocking experiments were performed by application of neutralizing antibodies against osteopontin and monocyte chemoattractant protein-1 (MCP-1).

Results. In nephritic animals, high glomerular MCP-1 and RANTES (regulated upon activation normal T cell expressed and secreted) expression levels were observed on days 4 and 10. The tubular expression of MCP-1, however, was only slightly enhanced. In contrast, tubular osteopontin production was maximally stimulated (day 10) and paralleled with peaks of albuminuria and tubulointerstitial M/M infiltration. Application of an anti-osteopontin antibody ameliorated tubulointerstitial and glomerular M/M recruitment, whereas treatment with an anti-MCP-1 antibody selectively reduced glomerular M/M recruitment. However, tubulointerstitial M/M infiltration remained unchanged.

Conclusion. These studies show that chemokines and osteopontin are differentially expressed in glomeruli and tubules in this model of GN. Chemokines play a primary role in the glomeruli, whereas osteopontin has a predominant role in tubulointerstitial M/M recruitment. The roles of chemokines and osteopontin may thus be dependent on the renal compartment and on the disease model.

Key words: glomerular monocytes, tubulointerstitial monocytes, macrophages, mononuclear cells, progressive renal disease, inflammatory cell recruitment.

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The infiltration of mononuclear cells into the glomerulus and the renal tubulointerstitium are hallmarks of almost any human glomerulonephritis (GN). Tubulointerstitial cell infiltrates in particular correlate with disease progression [1]. The chemokine molecule family plays an important role in the regulation of leukocyte recruitment in inflammatory renal diseases [2–4]. Chemokines are small glycoproteins that stimulate leukocyte trafficking and mediate inflammation [5]. Based on their structural and genetic characteristics, chemokines are divided into different families. The classification, which is based on structural characteristics, is made according to the position of the cysteine residues in the amino acid sequences of the molecules. Four families of chemokines have been characterized to date. The first two cysteines of the CC chemokine family are directly adjacent. The most commonly investigated CC chemokines in renal diseases are monocyte chemoattractant protein-1 (MCP-1) and regulated upon activation normal T-cell expressed and secreted (RANTES). They exert their effects through binding to chemokine receptors that are predominantly expressed on leukocytes. MCP-1 and RANTES-neutralizing approaches have shown to reduce renal cell infiltration and tissue damage in experimental models of GN [6–11].

Although a prominent pathophysiological role of monocyte/macrophage (M/M) migration into the tubulointerstitium is generally accepted, the responsible mechanisms, which lead to inflammatory cell recruitment, have remained largely unknown to date [12]. One hypothesis is that in GN, albumin, filtered by the glomerulus, leads to an increased protein uptake in tubular cells, which may result in the formation of chemoattractants and proinflammatory cytokines. As a result, tubulointerstitial inflammation may occur [13–16]. However, very little in vivo evidence exists describing the responsible mechanisms [13, 14]. The available data are based on mostly in vitro studies [15, 16].
Recently, osteopontin has been suggested to play an important role in the pathophysiology of GN. Osteopontin is a secreted phosphoprotein with potent in vitro and in vivo chemoattractant effects on M/M [17, 18]. Osteopontin blockade inhibits glomerular and tubulointerstitial inflammatory cell recruitment [19, 20]. Thus, chemokines and osteopontin play a central role in the regulation of renal leukocyte infiltration. To characterize these potential mechanisms further, we studied the expression levels of MCP-1, RANTES, and osteopontin in isolated glomeruli and proximal tubules in a rat model of anti-glomerular basement membrane (GBM) GN.

In our studies, the induction of anti-GBM GN led to a rapid increase in the glomerular expression of MCP-1 and RANTES. In proximal tubules, however, osteopontin expression was predominantly elevated. An osteopontin-neutralizing approach based on the application of an anti-osteopontin antibody reduced tubulointerstitial and, to a certain degree, glomerular M/M recruitment. In contrast, MCP-1 blockade ameliorated glomerular M/M infiltration but did not influence tubulointerstitial M/M recruitment.

In summary, our data suggest a differential glomerular and tubular expression pattern of chemokines and osteopontin. Neutralization of osteopontin primarily reduces tubulointerstitial M/M infiltration, whereas in this model of experimental GN, MCP-1 plays a dominant role in the glomerulus.

METHODS

Induction of glomerulonephritis and experimental design

Anti-glomerular basement membrane GN was induced in male Wistar rats (140 to 160 g/body weight) by an intravenous injection of 0.5 mL/100 g body weight of an anti-GBM antiserum [21]. Pre-immunization was done with 0.5 mg Hunter Titer Max (Sigma, St. Louis, MO, USA) four days before anti-GBM antiserum application. Anti-GBM antiserum was induced in rabbits by repeated immunization with disrupted rat glomeruli. Indirect immunofluorescence revealed a faint linear glomerular staining of IgG. All animal experiments were conducted in accordance with the national guidelines. The following groups of animals were studied (all groups were preimmunized):

1. Control. Animals received 0.5 mL/100 g body weight rabbit IgG.
2. Controls + anti-osteopontin. Animals received 50 μg anti-osteopontin antibody (MPIIIB10 Developmental Hybridoma Studies Institute, University of Iowa, Iowa City IA, USA).
3. Controls + anti-MCP-1. Animals received 1 mL polyclonal anti-MCP-1 antibodies [7].
4. Nephritis. Animals received 0.5 mL/100 g body weight anti-GBM antiserum.
5. Nephritis + anti-osteopontin. Animals received 0.5 mL/100 g body weight anti-GBM antiserum + 50 μg anti-osteopontin antibody.
6. Nephritis + anti-MCP-1. Animals received 0.5 mL/100 g body weight anti-GBM antiserum + 1 mL polyclonal anti-MCP-1 antibodies.
7. Nephritis + anti-osteopontin + anti-MCP-1. Animals received 0.5 mL/100 g body weight anti-GBM antiserum + 50 μg anti-osteopontin antibody + 1 mL polyclonal anti-MCP-1 antibodies.

All animal groups were studied on days 4, 10, and 20 following the induction of GN. The experiments were carried out in three complete separate sets (N = 6 animals for each group and time point, except for anti-osteopontin and anti-MCP-1 experiments, which were evaluated on day 10, only). Albuminuria was quantitated by a urinary rat albumin-specific competitive enzyme-linked immunosorbent assay (WAK Chemie Medical GmbH, Bad Soden, Germany).

Isolation of glomeruli and proximal tubules

Glomeruli were isolated by a fractional sieving technique as described earlier [22]. Microscopic analysis revealed that in approximately 40 to 70%, the Bowman’s capsules remained intact after isolation of the glomeruli (data not shown). Proximal tubules were isolated from rats according to Vinlay, Gougoux, and Lemieux [23]. In brief, kidneys were perfused with 20 mL phosphate-buffered saline (PBS) and 20 mL collagenase (1 mg/mL; Biochrom KG, Berlin, Germany). Minced kidneys were incubated in collagenase 1 mg/mL for 30 minutes at 37°C and gassed with oxygen. Homogenized kidneys were sieved through a 425 μm sieve, and the pellet was washed three times in ice-cold PBS and centrifuged three times at 800 r.p.m. for three minutes. The pellet was resuspended in 50% Percoll solution (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 12,500 r.p.m. for 30 minutes. A glass pipette was used to isolate the proximal tubules. Tubules were centrifuged at 1000 r.p.m. for three minutes and washed twice with PBS. Microscopic examination of the proximal tubules showed that more than 98% of the isolated material contained blue lysosomal inclusions demonstrating isolation of proximal tubule fragments [23].

RNA isolation and Northern blot hybridization

Cellular RNA from glomeruli and proximal tubules was isolated using the guanidinium isothiocyanate method [24]. For Northern blot analysis, total RNA (20 μg) was size fractionated by electrophoresis on a 1.2% agarose-formaldehyde gel and transferred to nylon membranes (Zetabind; Cuno, Meriden, CT, USA). Equal loading of the lanes was evaluated by ethidium bromide staining of...
the 18S and 28S RNAs. The membranes were hybridized with a rat MCP-1 cDNA fragment, a rat RANTES cDNA, and a rat osteopontin cDNA fragment, previously labeled with α-32P dCTP in hybridization buffer [5 × SSPE, 2 × Denhardt’s, 150 μg/mL sonicated and denatured salmon sperm DNA; Sigma; 0.1% sodium dodecyl sulfate (SDS), 5% dextran sulfate, and 50% formamide] at 42°C for 18 hours as described earlier [22]. The membranes were washed at high stringency in standard saline citrate (SSC)/SDS at 65°C. Autoradiography was performed at −80°C for 2 to 72 hours. Membranes were rehybridized with a cDNA probe of 18S RNA to account for small loading and transfer variabilities. Exposed films were quantitated by using the phosphorimager Bio-Rad-GS-363 (multianalyst software). Northern blot experiments were performed independently in three different complete sets with quantitatively similar results.

**Immunohistochemistry**

Kidney tissues were either fixed in 1% buffered formaldehyde or in Methyl Carnoy’s solution. Tissue sections (2 μm) were stained with the alkaline phosphatase-antialkaline-phosphatase (APAAP) complex following application of primary antibodies for immunohistology. Tissue was stained with a monoclonal anti–ED-1 antibody (Chemicon International, Temecula, CA, USA). For immunolocalization of osteopontin, the monoclonal anti-rat osteopontin antibody MPIIIIB10 was used. All quantitative morphologic analyses were performed in blinded fashion. Evaluation of ED-1–positive cells was performed by counting positive cells in at least 80 glomeruli (range 80 to 110) of at least six kidneys at each time point as described earlier [22]. Tubulointerstitial M/M infiltration was assessed by counting ED-1–positive cells in 20 cortical high-power fields (×400) for each section.

**In situ hybridization**

In situ hybridization was done using a previously published method [25]. Cryostat sections (10 μm thick) of fresh frozen kidneys were thaw mounted onto slides and fixed in 4% paraformaldehyde in PBS for 30 minutes. After washing in PBS, sections were stored in 70% ethanol at 4°C for further treatment. Before use, sections were brought to room temperature, washed twice with PBS, and incubated in 0.1 mol/L triethanolamine, pH 8.0, containing 0.25% acetic anhydride for 20 minutes. Sections were then washed twice in PBS, dehydrated in an ascending alcohol series, and air dried.

Prehybridization was performed overnight at 37°C in a humidified chamber with prehybridization buffer, containing 50% formamide, 25 mmol/L ethylenediaminetetraacetic acid (EDTA), 50 mmol/L Tris-HCl, pH 7.6, 2.5 × Denhardt’s solution, 0.25 mg/mL tRNA (Boehringer Mannheim, Mannheim, Germany), and 20 mmol/L NaCl. Hybridization was performed overnight at 55°C in hybridization buffer [50% formamide, 20 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA, 1 × Denhardt’s solution, 0.5 mg/mL tRNA, 0.1 mg/mL poly (A) RNA (Sigma), 0.1 mol/L dithiothreitol (DTT), and 10% dextran sulfate (Sigma)]. Digoxigenin-labeled cRNA probes of rat osteopontin were used at a concentration of approximately 200 pg/μL hybridization buffer. After hybridization, cryostat sections were washed twice in SSC containing 50% formamide.

After washing, cryostat sections hybridized with digoxigenin-labeled probes were rinsed in SSC and equilibrated with buffer 1 (100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) for 10 minutes, followed by blocking for 30 minutes in buffer 2 [1% Boehringer blocking reagent in buffer 1 + 0.5% bovine serum albumin (BSA)], and incubated overnight at room temperature with α-DIG-AP antibodies at a dilution of 1:500 in modified buffer 2.
Fig. 1. (Continued)
**RESULTS**

**Glomerular and tubular expression of MCP-1, RANTES, and osteopontin**

The induction of anti-GBM nephritis led to increased glomerular expression of MCP-1 and RANTES on days 4 and 10 when assessed by Northern blotting (Fig. 1A, C). The expression levels of both chemokines returned to control levels on day 20. Osteopontin expression in glomeruli was up-regulated on day 10 only (Fig. 1A, C). A moderate increase in the expression of MCP-1 could be found in the tubules on day 10, whereas RANTES expression was unaltered. The tubular expression of osteopontin increased on day 4 and peaked on day 10 (Fig. 1B, C).

**Localization of osteopontin expression**

In situ hybridization of osteopontin revealed low cortical expression in the control group (Fig. 2A). Osteopontin expression increased on day 4 (data not shown) after induction of the disease and reached a maximum on day 10 (Fig. 2B). Osteopontin RNA expression was primarily localized in proximal tubules and, to a lesser degree, in the area of the Bowman’s capsules (Fig. 2B). The sense probe showed no staining (data not shown). Osteopontin expression returned to control levels on day 20 (data not shown). Osteopontin protein expression was studied by immunohistology, which demonstrated almost identical results compared with in situ hybridization (Fig. 3A, B). No osteopontin was detected in glomeruli on day 10. However, proximal tubular cells showed strong positivity for osteopontin in nephritic animals (Fig. 3B).

**Glomerular and tubulointerstitial monocyte/macrophage recruitment**

Induction of GN resulted in a significant increase of glomerular [2.94 ± 0.31 cells/glomerular cross-section (c/gcs)] and tubulointerstitial (2.59 ± 0.59 cells/high-power field) M/M recruitment on day 10 (P < 0.01 vs. control). To
test whether osteopontin and MCP-1 play a role in M/M recruitment, nephritic animals were treated with an anti-osteopontin antibody and an anti–MCP-1 antibody. Chemotactic assay demonstrated that these antibodies blocked MCP-1 or osteopontin-induced M/M migration in vitro (Fig. 4). Blocking of osteopontin reduced glomerular (1.99 ± 0.28 c/gcs; \( P < 0.05 \) vs. nephritis; Figs. 5A and 6E) and tubulointerstitial infiltration of M/M on day 10 (1.4 ± 0.07, \( P < 0.01 \) vs. nephritis; Figs. 5B and 6E). In contrast, application of a neutralizing anti-MCP-1 antibody markedly reduced glomerular M/M infiltration (1.33 ± 0.09, \( P < 0.01 \) vs. nephritis; Figs. 5A and 6F) but did not influence tubulointerstitial M/M recruitment (2.44 ± 0.3; Figs. 5B, 6F). When control animals had received antibodies alone, M/M recruitment (Figs. 5 and 6) remained unchanged.

**Albuminuria**

The induction of anti-GBM nephritis led to a significant increase in albuminuria on day 10 (10.77 mg ± 2.21/24 h, \( P < 0.01 \) vs. control; control group 0.05 mg ± 0.01/24 h). The treatment of nephritic animals with an anti-osteopontin antibody (2.7 mg ± 0.39/24 h, \( P < 0.01 \) vs. nephritis) and an anti-MCP-1 antibody (1.1 mg ± 0.08/24 h, \( P < 0.01 \) vs. nephritis) significantly reduced albuminuria on day 10. Twenty days after induction of nephritis, albuminuria returned to baseline levels (data not shown). When control animals had received antibodies alone, albuminuria remained unchanged (Fig. 7).

**DISCUSSION**

The occurrence of monocytes/macrophages and T cells in the glomerulus and renal tubulointerstitium is a typical morphological feature of most types of GN. In animal models of GN, chemokine-neutralizing or chemokine receptor-blocking approaches lead to a reduction of glomerular mononuclear cell infiltrates [6–11]. This emphasizes the important role of locally produced chemokines in immune-mediated glomerular injuries. However, much less is known about the mechanisms that lead to tubulointerstitial inflammatory cell infiltration in GN, although tubulointerstitial leukocyte recruitment is a good predictor of disease progression [1]. One hypothesis is that in GN, the pathologic traffic of proteins that are filtered
Fig. 7. Albuminuria. The induction of anti-GBM nephritis led to a significant increase of albuminuria on day 10. Treatment with the anti-osteopontin antibody and the anti-MCP-1 antibody reduced albuminuria. Results are expressed as means ± SEM (N = 6). #P < 0.01 vs. control; *P < 0.01 vs. nephritis.

Fig. 6. Immunohistological staining of glomerular and tubulointerstitial monocyte/macrophage (M/M) infiltration. Immunohistological staining of glomerular and tubulointerstitial M/M infiltration with a monoclonal ED-1 antibody revealed a marked increase in ED-1-positive cells 10 days after nephritis induction (D) in comparison to the control group (A) and to control animals that had received the anti-osteopontin antibody (B) or anti-MCP-1 antibody (C). When nephritic animals had received the anti-osteopontin antibody, glomerular and tubulointerstitial ED-1-positive cells were substantially reduced (E), whereas application of the neutralizing anti-MCP-1 antibody reduced glomerular M/M recruitment but did not influence tubulointerstitial M/M infiltration (F). Original magnification ×400.

Our data show that a substantial up-regulation of glomerular MCP-1, RANTES, and osteopontin expression levels occurs in anti-GBM nephritis on day 10. In contrast to the glomerulus, only a weak expression of MCP-1 was detected in tubules isolated from nephritic rats on day 10. No expression of tubular RANTES was detected at any time point examined. Since the animals developed marked proteinuria, it is very unlikely that RANTES plays a role in tubular cell-mediated M/M recruitment in this model of GN. While RANTES and MCP-1 expression levels were only low or absent, the tubular expression of osteopontin was markedly increased on day 4 and peaked on day 10. In comparison to a recent study of nephrotoxic serum nephritis in mice [29], the different expression patterns of MCP-1 are at least in part attributable to the use of a different model of renal injury and suggest a model- and compartment-dependent expression and function of MCP-1. In situ hybridization showed that increased osteopontin expression was in fact localized in tubules and not in tubulointerstitial infiltrating cells or blood vessels. Immunohistological analysis confirmed these mRNA expression data and revealed selective expression of osteopontin in tubules.

In contrast to mRNA data from in situ hybridization studies of glomerular osteopontin expression, only a very low amount of osteopontin protein was detected in glomeruli by immunohistology, which may be indicative of differences in the sensitivity of the respective methods.

In earlier studies, osteopontin, which is a secreted glycosylated phosphoprotein, was shown to be colocalized with glomerular and tubular macrophages [13, 30]. It was postulated that osteopontin plays a role in the migration of M/M into the glomerulus and tubulointerstitium in inflammatory and noninflammatory renal diseases [13, 26–28, 31–34]. A recent study demonstrated increased glomerular osteopontin expression in a rat model of crescentic GN [34]. However, glomerular osteopontin expression was predominantly localized to crescentic lesions, which were absent in this model of GN.
To investigate further whether osteopontin and MCP-1 might in fact play a role in M/M recruitment in this model of anti-GBM-nephritis, the animals were treated with an anti-osteopontin antibody and an anti-MCP-1 antibody. Treatment of nephritic rats with the anti-osteopontin antibody reduced albuminuria and glomerular and tubulointerstitial infiltration of M/M. In contrast, application of an anti-MCP-1 antibody reduced albuminuria and glomerular M/M recruitment but did not influence tubulointerstitial M/M infiltration.

In summary, our data show a sequential increase in chemokine and osteopontin expression levels in glomeruli and proximal tubules. Tubular osteopontin expression is paralleled with the maximum concentration of proteinuria and the occurrence of tubulointerstitial M/M infiltration. The markedly reduced infiltration of M/M into the tubulointerstitium after osteopontin neutralization demonstrates a unique role of osteopontin in tubulo-interstitial inflammatory cell recruitment, whereas in this model of experimental GN, MCP-1 function on monocytes plays a dominant role in the glomerulus.

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