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Blocking of Monocyte Chemoattractant Protein-1 during Tubulointerstitial Nephritis Resulted in Delayed Neutrophil Clearance

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The chemokine monocyte chemoattractant protein (MCP)-1 has been implicated in the monocyte/macrophage infiltration that occurs during tubulointerstitial nephritis (TIN). We investigated the role of MCP-1 in rats with TIN by administering a neutralizing anti-MCP-1 antibody (Ab). We observed significantly reduced macrophage infiltration and delayed neutrophil clearance in the kidneys of TIN model rats treated with the anti-MCP-1 Ab. To exclude the possibility that an observed immune complex could affect the resolution of apoptotic neutrophils via the Fc receptor, TIN model rats were treated with a peptide-based MCP-1 receptor antagonist (RA). The MCP-1 RA had effects similar to those of the anti-MCP-1 Ab. In addition, MCP-1 did not affect macrophage-mediated phagocytosis of neutrophils *in vitro*. Deposition of the anti-MCP-1 Ab in rat kidneys resulted from its binding to heparan sulfate-immobilized MCP-1, as demonstrated by the detection of MCP-1 in both pull-down and immunoprecipitation assays. We conclude that induction of chemokines, specifically MCP-1, in TIN corresponds with leukocyte infiltration and that the anti-MCP-1 Ab formed an immune complex with heparan sulfate-immobilized MCP-1 in the kidney. Antagonism of MCP-1 in TIN by Ab or RA may alter the pathological process, most likely through delayed removal of apoptotic neutrophils in the inflammatory loci. (Am J Pathol 2005, 167:637–649)

Brown Norway rats that are immunized with bovine tubular basement membrane antigens develop tubulointerstitial nephritis (TIN).^{1,2} In this disease model, the composition of the inflammatory cell infiltrate is characterized by the sequential accumulation of polymorphonuclear leukocytes (PMNs), T lymphocytes, and monocytes/macrophages in the affected kidney. Anti-tubular basement membrane antibody (Ab) deposition in the kidney begins on days 7 to 8 after immunization, and is followed on days 8 to 10 by C3 deposition and infiltration of PMNs. Starting from day 10, the infiltration of monocytes/macrophages is increased. After day 13, the cellular infiltration becomes almost exclusively mononuclear. The recruitment pattern of inflammatory cells in this model involves a switch from neutrophils to monocytes, and is similar to the cellular recruitment patterns seen in other acute inflammatory processes. The recruited neutrophils are thought to be dependent on complement system activation, and the recruited macrophages have been determined to play a central role in the initiation, resolution, and repair processes of inflammation.

In the acute inflammatory response, neutrophils and monocytes migrate from the circulation across the vessel wall into sites of inflammation.³ The leukocyte migration in inflammatory conditions occurs by a multistep process, involving both adhesion molecules and chemokines as well as their receptors on leukocytes and endothelial cells. Transendothelial migration of endothelium-bound neutrophils and mononuclear leukocytes, a critical event in this process, is mediated by members of the large chemokine family. The principle biological function of chemokines is the attraction and activation of leukocytes with the distinction between the C-X-C and C-C chemokine subgroups being their target cells.⁴ The C-X-C family of chemokines includes platelet factor-4 (PF-4), interleu-

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kin-8 (IL-8), GRO/MGSA, KC, and MIP-2. The C-C family of chemokines includes RANTES, MIP-1, and MCP-1. These two subfamilies represent two gene clusters on two different chromosomes. Their structural differences are related to their abilities to mediate distinct leukocyte migration. In general, the migration responses of PMNs involve C-X-C chemokines, whereas mononuclear cells respond most strongly to CC chemokines. During the inflammatory process, the switch in infiltrating cell type, from neutrophils to monocytes, has been correlated with the complex patterns of chemokine expression at different phases of inflammation.

Several groups have identified the role of the C-X-C family of chemokines in acute inflammation by blocking these chemokines with neutralizing Abs.^{5,6} In the model of anti-glomerular basement membrane Ab-induced glomerulonephritis, we and others have shown that MIP-2 and KC guide neutrophil influx.^{7,8} Our previous studies suggest that MCP-1 also plays a major role in the pathogenesis of this model of TIN.⁹ MCP-1 is produced by a wide variety of cell types including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells. Expression of MCP-1 is induced by various stimuli, such as lipopolysaccharide, IL-1 β , tumor necrosis factor- α , platelet-derived growth factor, interferon- γ , and phorbol esters.¹⁰ It is thought that MCP-1 plays a significant role in the monocyte/macrophage infiltration that occurs during TIN, but the mechanism of this mononuclear cell influx has not yet been fully elucidated. The role of locally produced MCP-1 in this process seems to be one of involvement in the initiation and progression of tubulointerstitial damage as observed in studies using MCP-1 knockout mice.¹¹

The rationale for anti-inflammatory therapy based on interference with the chemokine system has been established in *in vitro* systems and in animal models.¹² Although chemokines have been regarded as rational targets for the development of anti-inflammatory reagents, the approach of anti-chemokine therapies has been hampered by the pleiotropy and the redundancy of the chemokine system. Not only are multiple chemokines with overlapping activities frequently induced in inflammatory diseases; but also, many different chemokine receptors are often expressed by activated leukocytes. Molecules that have the capacity to bind and antagonize inflammatory chemokine receptors may provide a rational approach to overcome the difficulties associated with this potential redundancy. Chemokine receptor antagonists (RAs) that attenuate the effects of C-C and C-X-C chemokines have been, and continue to be, studied as viable treatment strategies in inflammatory disease. Inhibition of IL-8-mediated neutrophil activation is being explored as a potential anti-inflammatory treatment. Moser and colleagues¹³ have found that IL-8 analogues, generated by using a truncated version of IL-8 as a template, bind to IL-8 receptors and inhibit IL-8-mediated neutrophil responses. Treatment strategies using the inhibition of RANTES- and MIP-1 α -mediated leukocyte recruitment to sites of inflammation to relieve chronic inflammatory disease are also being studied. RANTES analogs, generated by extension of the NH₂-terminus of this chemokine, have been shown to block both RANTES- and MIP-1 α -

mediated chemotaxis of THP-1 cells and calcium mobilization in THP-1 cells.¹⁴ Attenuation of the proinflammatory effects of SDF-1 via antagonism of the CXCR-4 receptor has been studied as well. SDF-1 analogs, obtained by modification of the first two N-terminal amino acids of SDF-1, were found to be potent RAs that inhibited proinflammatory SDF-1 function as well as SDF-1-mediated HIV-1 replication¹⁵ *in vitro* and *in vivo* in animal models. The anti-inflammatory activity of the broad-spectrum chemokine antagonist, vMIP-II, has been investigated as a potential therapeutic agent to inhibit the proinflammatory effects of a number of chemokines. vMIP-II is a viral protein encoded by human herpesvirus 8, and it was found to have *in vitro* antagonistic activity against the chemokine receptors CXCR-4, CCR-1, CCR-3, CCR-5, and CX₃CR-1. *In vivo*, vMIP-II was found to effectively reduce leukocyte infiltration in the kidneys of anti-glomerular basement membrane glomerulonephritic rats.¹⁶

MCP-1 RAs have been found to have anti-inflammatory activity *in vivo*. In a mouse model of rheumatoid arthritis, MCP-1(9-76) was found to inhibit the onset of arthritis, as well as to reduce the symptoms of the disease and leukocyte infiltration after the disease had already developed.¹⁷⁻¹⁹ In the current study, we hypothesized that neutralizing MCP-1 via a RA could reduce cellular infiltration into the tubulointerstitium and improve TIN. Surprisingly, we found that anti-MCP-1(1-73) Ab-treated animals exhibited an accumulation of apoptotic neutrophils in the tubular regions during TIN. We additionally found that anti-MCP-1(1-73) Ab-treated macrophages exhibited a reduction in the rate of phagocytosis of apoptotic neutrophils *in vivo*. The data also indicate that the delayed clearance of neutrophils may be a direct result of a reduction in the numbers of infiltrating macrophages that are mediated by anti-MCP-1(1-73) Ab. Our findings add new perspective to chemokine biology, indicating that MCP-1-induced macrophage infiltration is involved in the resolution of acute inflammation by clearing apoptotic neutrophils.

Materials and Methods

Induction and Evaluation of Anti-Tubular Basement Membrane Ab-Induced TIN Model on Brown Norway Rat

Homogenized bovine tubular basement membrane was prepared and stored as previously described.²⁰ Twenty-eight Brown Norway rats, purchased from Harlan (Indianapolis, IN), were immunized in the tail base with 100 μ g of the prepared bovine tubular basement membrane in complete Freund's adjuvant with 4 mg of dried *Mycobacterium tuberculosis*, H37Ra strain (Difco, Detroit, MI). The rats also received a *Bordetella pertussis* vaccine (Massachusetts Public Health Biologics Laboratory, Boston, MA) containing $\sim 22.2 \times 10^8$ cells in a separate intradermal injection into the flank. Control animals ($n = 21$) were immunized with ovalbumin (100 μ g) in the same adjuvant. The sensitized and control animals were sacrificed at 3, 7, 8, 9, 10, 12, and 14 days after injection, with four animals at each time point for the sensitized group and

three animals at each time point for the control group. At the time of sacrifice, renal tissue was fixed in liquid nitrogen with Tissue-Tek (Miles Inc., Elkhart, IN) or in formalin solution. Histological examination was performed on paraffin sections stained with periodic-acid Schiff reagent and hematoxylin counterstain (DAKO, Carpinteria, CA).

Probe Generation and RNase Protection Assay

The cDNA fragments coding for rat KC (274 bp), MIP-2 (174 bp), and MCP-1 (239 bp) were cloned by polymerase chain reaction (PCR) and were used for riboprobe generation. A 114-bp probe prepared from rat L32 cDNA was used as an internal control. Rat cytokine multiprobe template sets (BD Biosciences, San Diego, CA) were used in this study to determine the levels of various cytokine mRNAs during tubulointerstitial disease. RNase protection assay was performed as described,²¹ except that Stop Buffer (Torrey Pines Biolabs, Houston, TX), rather than proteinase K, was used to denature RNase A and T₁. Densitometric analysis was performed on each of the resulting specific bands using Scion Image Beta 4.02 Win. The data for each gene mRNA level was presented as a ratio to GAPDH or L32.

Expression of MCP-1 RAs

The coding sequences of both MCP-1 analogues, MCP-1(9-73) and MCP-1(11-73), were generated by PCR. Because the N-terminus of these chemokines could not be deleted or extended, MCP-1 analogues were expressed in 6× histidine (His)- and Factor Xa-fused forms [HIS6-Ile-Glu-Gly-Arg- (MCP-1 analogues)]. To avoid extra amino acids generated from the restriction enzymes at the N-terminus of the MCP-1 analogues, the sense primers of the MCP-1 analogues contained an *Xba*I cleavage site before the Factor Xa sequence and MCP-1-specific sequences, 5'-gcg tct aga atc gaa ggt cgt (MCP-1-specific sequence)-3'. PCR products were digested with *Xba*I and *Xho*I and subcloned into pETM1 (Novagen, Madison, WI). Expression and induction of recombinant proteins were performed as previously described (Qiagen Inc., Valencia, CA). The purified MCP-1 analogues were digested with Factor Xa (New England Biolabs, Beverly, MA) and the 6× His-tag was removed using a Ni-NTA resin. PCR was used to obtain the mammalian expressed form of the MCP-1(9-73) RA peptide. The primers used for generation of the recombinant MCP-1 RA peptides are listed as follows: the sense primer contained an *Eco*RI site, a MCP-1 leader sequence, and a gene-specific sequence (starting from amino acid 9 or amino acid 11), and the anti-sense primer contained an *Xho*I site and a coding sequence ending at amino acid 73. The PCR products were digested with *Eco*RI and *Xho*I, and subcloned into the mammalian expression vector, pCAGGS. The recombinant DNAs were then transfected into subconfluent COS-7 cells using the LipofectAMINE reagent (Invitrogen, San Diego, CA). The anti-sense clones were used to perform a mock transfection as a control. After incubating the transfected cells for 72 hours, the cell culture supernatant was collected for Western blot analysis.

Production of Abs against KC, MIP-2, and MCP-1

The coding sequences for rat KC, MIP-2, MCP-1(1-73), and MCP-1 (full length) were generated by PCR and subcloned into the expression vector, pETM1. pETM1 was modified [using the pET11a vector (Novagen)] by adding the sequence coding for 6× histidine for ease in affinity purification. The host cell strain, BL21 (DE3) (New England Biolabs), was transformed using recombinant plasmids and was cultured in LB broth inoculated with ampicillin (100 µg/ml). Protein expression was induced with 0.5 mmol/L of isopropyl-β-thiogalactopyranoside at 37°C for 4 hours. The cell cultures were centrifuged, and the resulting pellets were stored at -20°C for subsequent purification. A ProBond metal-binding resin was purchased from Invitrogen and was used for protein purification. The purification procedure of the manufacturer was used, with some modifications, as described previously (Qiagen Inc.).

Polyclonal antisera were raised by immunizing rabbits with recombinant chemokines, including KC, MIP-2, MCP-1(1-73), and MCP-1 (full length). An initial dose of 1 mg of each recombinant peptide in complete Freund's adjuvant was injected into the animals subcutaneously. Subsequent doses of 0.5 mg of recombinant peptide in complete Freund's adjuvant were given weekly. The protein levels of KC, MIP-1α, and MCP-1 were analyzed by Western blot analysis, as described below. The sera against these recombinant peptides bound to the expected position of the natural peptides when incubated with the cell culture supernatants of transfected COS-7 cells, but did not bind to the cell culture supernatants of the anti-sense transfectants, as demonstrated by Western blot analysis. Because the anti-MCP-1(1-73) Ab was produced for *in vivo* use, a standard enzyme-linked immunosorbent assay was used to determine the titer of the Ab present in the antiserum. High titers of anti-MCP-1(1-73) Ab were present in antisera dilutions as high as 1:200,000. The neutralizing activity of the anti-MCP-1(1-73) Ab was assessed *in vitro* by evaluating the ability of the antiserum to inhibit MCP-1-induced chemotaxis of rat macrophages as described below.

Chemotaxis Assay

Peritoneal macrophages were isolated as described below. Migration was evaluated using a chemotaxis microchamber technique as previously described.²² Briefly, 25 µl of increasing concentrations of recombinant rat MCP-1 (BD Pharmingen, San Diego, CA) or of vehicle was placed in the lower wells of a chemotaxis chamber (Neuro Probe, Gaithersburg, MD) and separated from 50 µl (1 × 10⁶/ml) of cell suspension in the top of the wells by a 5-mm polycarbonate filter (Neuro Probe). After incubation at 37°C for 2 hours, filters were removed and the migrated cells on the undersurface were fixed with methanol and stained with Diff-Quik (American Scientific Products, McGraw Park, IL). Results are expressed as the

mean number of migrated cells and are representative of $n = 3$ experiments performed in duplicate.

In Vivo Treatment of TIN Model Rats with Neutralizing Antiserum and MCP-1(9-73) RA Peptide

Neutralizing antiserum against a truncated MCP-1 peptide (from amino acids 1 to 73) was administered to animals (0.5 ml of antiserum per rat daily) from day 5 to day 9. MCP-1(9-73) RA peptide was given intravenously beginning on day 3 (200 μ g/rat) and was continued daily for 5 days from day 5 to day 9. Control rats were given the same dose of preimmune rabbit serum and a nonfunctional, truncated MCP-1 analogue [MCP-1(11-73) RA peptide] following the same schedule. Animals were divided into the following four groups: 1) normal rabbit serum treated (NRS, $n = 6$); 2) anti-MCP-1(1-73) Ab treated (α MCP-1, $n = 6$); 3) MCP-1(9-73) RA peptide treated ($n = 6$); and, 4) MCP-1(11-73) RA peptide treated as a control group ($n = 6$).

Histology and Immunohistochemistry

Tissue samples were fixed in Methacarn and embedded in paraffin. Sections were cut at 5 μ m and were stained immunohistochemically as follows: antigen retrieval was achieved using sodium citrate buffer (10 mmol/L, pH 6.0) and microwave treatment of tissue sections. MPO staining of tissues was preceded by treatment of tissue sections with microwave (full power, 10 minutes) to denature any rabbit anti-MCP-1 Ab that had been deposited in the tubulointerstitium. Co-localization of monocytes/macrophages and neutrophils was achieved by staining serial sections of tissue with Abs to ED-1 (Harlan Bioproducts Inc., Indianapolis, IN) and MPO (DAKO), respectively. A similar technique was used to determine macrophage-mediated phagocytosis of apoptotic neutrophils, using heating instead of microwave to retrieve MPO. Areas that stained positively for macrophages or neutrophils were measured using computer-assisted image quantification (Optimas 6.5; Optimas Corp.).

Rabbit IgG deposited in the rat kidney was directly detected using a horseradish peroxidase-labeled goat anti-rabbit Ab. This Ab was incubated with agarose-conjugated rat IgG (Rockland, Gilbertsville, PA) before immunohistochemistry to reduce any cross-reaction between this horseradish peroxidase-labeled Ab and the rat anti-tubular basement membrane Ab present in the tubular region. Tissue sections subjected to immunohistochemistry were developed using the DAKO Envision system (DAKO). Tissue sections were also stained using periodic acid-Schiff reagent as well as Masson's trichrome to evaluate fibrosis of the tubulointerstitial region. Immunohistochemistry was also performed using frozen sections to detect rat anti-tubular basement membrane IgG and C3 in the kidney using fluorescein isothiocyanate-labeled mouse anti-rat IgG (Zymed, South San Francisco, CA) or rabbit anti-C3 (Bethyl, Montgomery, TX) Ab, respectively.

Detection of the Immune Complex of Rabbit Anti-MCP-1 Ab and MCP-1 in Inflamed Kidney of Rats Treated with Anti-MCP-1 Ab

Enrichment of rabbit IgG in kidney tissues was performed as follows: kidney homogenates in 1.0 ml of lysis buffer [25 mmol/L Tris-HCl, 0.25% (w/v) sodium deoxycholate, 1.0% Nonidet P-40, 0.05% (v/v) Tween 20, 0.15 mol/L NaCl (pH 8.1)] were centrifuged at $10,000 \times g$ for 5 minutes at 4°C to remove insoluble debris. Next, 0.34 ml of protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ) in 3.0 ml of lysis buffer [10% (v/v)] suspension was added to the supernatant and the resulting slurry was gently mixed at 4°C for 1 hour. After the beads were washed (twice, in 10 vol of lysis buffer), the Sepharose-bound immune complexes were harvested as follows: Sepharose beads were subjected to centrifugation at $3000 \times g$ for 20 seconds at 4°C and were then washed three times in lysis buffer (4 ml). Elution of the immune complexes from the Sepharose beads was achieved by suspending the beads in 0.5 ml Laemmli sample buffer and heating at 95°C for 1 minute. Elution was repeated once, and the pooled eluate containing the rabbit IgG was further fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electrophoretic transfer to nitrocellulose membrane. The membrane-bound protein was then incubated with a monoclonal Ab against rat MCP-1 (BD Pharmingen).

Immunoprecipitation and Immunoblot

To demonstrate heparan sulfate (HS) binding to MCP-1 during tubulointerstitial disease, kidney lysates were incubated overnight with 3 μ g of anti-HS Ab (10E4; Seikagaku) at 4°C. Negative controls were isotype-matched (mouse IgM) as well as normal kidney lysates. Immunoprecipitation was performed by adding 25 μ l of a 50% (w/v) slurry of rat anti-murine IgM-Sepharose (Zymed) in phosphate-buffered saline (PBS) to the lysates. This was followed by inverted mixing for a further 3 hours at 4°C. After brief centrifugation of the lysate-Sepharose slurry (5 seconds, $13,000 \times g$), the supernatant was decanted from the mixture and saved for immunoblot analysis. The immune complexes that remained bound to the IgM-Sepharose were then washed twice with 500 μ l of wash buffer (50 mmol/L Tris-HCl, pH 8, 500 mmol/L NaCl, 0.1% Triton X-100), briefly centrifuged (5 seconds, 6000 rpm), and the resulting supernatants were decanted and stored for subsequent immunoblot analysis. The remaining pellets were mixed with equal volumes of Laemmli buffer (denaturing, reducing), heated at 95°C for 5 minutes, and fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide). Collected supernatants were subjected to the same denaturing and electrophoresis procedures. After electrophoresis, proteins from all samples were electrotransferred in a semidry set-up to nitrocellulose membranes (Scheleicher and Schuell, Inc., Keene, NH). The blots were then blocked with 5% (w/v) skimmed milk followed by incubation with an anti-MCP-1 polyclonal Ab at a dilution of 1:20,000 (Torrey Pines Biolabs). Detection was performed using a horseradish per-

oxidase-linked anti-rabbit IgG (Sigma, St. Louis, MO) followed by visualization using photoluminescence (ECL reagents; Amersham Pharmacia Biotech, Piscataway, NJ).

Isolation of Rat Peritoneal Macrophages and Neutrophils for Phagocytosis Assay of Apoptotic Neutrophils

Rats were injected intraperitoneally with 1 ml of sterile 4% Brewer's thioglycolate solution. After 8 hours, neutrophils were recovered by peritoneal lavage using ice-cold PBS and, after 5 days, macrophages were isolated as described previously.²³ After isolation, rat peritoneal macrophages were transferred to a chamber slide (BD Biosciences) and were cultured at 37°C, in a 5% CO₂ atmosphere for 2 hours. Nonadherent cells were then washed away using PBS. Adherent macrophages were washed in RPMI. Apoptotic neutrophils obtained by culture (4 × 10⁶/ml in RPMI, 1% (v/v) autologous serum, 37°C, 5% CO₂, 24 hours) were then added to each well of the chamber slide. Most neutrophils used in the phagocytosis assay were apoptotic as determined by annexin V positivity and trypan blue exclusive assay. Macrophages were incubated with the apoptotic neutrophils for 30 minutes at 37°C. After incubation, the wells were washed four times with ice-cold PBS, fixed in 2.5% glutaraldehyde/PBS for 10 minutes, and then stained for myeloperoxidase (MPO).²⁴ The percentage of macrophages containing MPO-positive apoptotic neutrophils was quantified microscopically by examining randomly selected fields and counting at least 500 cells/well.

Statistical Analysis

Data are presented as mean ± SEM. Analysis of variance was used to compare mean values of cell number and protein levels between different groups or different time points. A value of *P* < 0.05 was considered significant.

Results

Escherichia coli and Mammalian Expression of Rat KC, MIP-2, MCP-1 Analogues, and Ab Production for E. coli Expressed MCP-1 N-Terminal and C-Terminal Peptides

Chemokine constructs were expressed as fusion proteins with a 6× histidine tag at the N-terminus in the BL21 (DE3) host cell strain under the control of T₇ RNA polymerase. Figure 1 shows Western blots of the purified proteins in which both *E. coli* and COS-7 forms were recognized by the specific Ab generated against *E. coli*-expressed chemokines. Preimmune serum did not react with any of these chemokines (data not shown). Mammalian-expressed forms of KC, MIP-2, and MCP-1 were used to verify the specificity of the Abs used in the Western blots to rule out the possibility that the Abs were reacting with the 6× histidine tag or with any other se-

Figure 1. Characterization of Abs against MIP-2, KC, and MCP-1. Expression of MIP-2 (**a**), KC (**b**), and MCP-1 (**c**) was detected in transfectants using rabbit anti-rat polyclonal Abs generated from recombinant proteins expressed in *E. coli*. Plasmids (20 µg), expressing full-length chemokine cDNA, were transfected into the human kidney embryo cell line 293T using electroporation. After 72 hours of culture, supernatants were collected and analyzed by Western blot: **lane 1**, MIP-2 transfectant; **lane 2**, KC transfectant; **lane 3**, MCP-1 transfectant. Expression of MIP-2 (**left**), KC (**middle**), and MCP-1 (**right**) was observed in the transfectants. **d**: Coomassie blue staining of purified MCP-1 (full length), MCP-1 RA(9-73), and MCP-1(11-73). Recombinant MCP-1 analogues were generated in *E. coli*. Purification was performed by affinity chromatography using a nickel column. **Left lane**: protein molecular weight markers; **lane 1**, 1 µg of MCP-1(11-73); **lane 2**, 1 µg of MCP-1RA(9-73); **lane 3**: 1 µg of MCP-1 (full length).

quences that could have been added to these chemokines as a result of their expression in *E. coli*.

MCP-1(9-73) RA Peptide and Anti-MCP-1(1-73) Ab Inhibited MCP-1-Mediated Chemotaxis in Vitro

The capability of MCP-1 RA peptides as well as anti-MCP-1(1-73) Ab to block MCP-1-mediated chemotaxis was tested *in vitro* using chemotaxis assays. As shown in Figure 2a, MCP-1-induced chemotaxis of peritoneal macrophages occurs in a dose-dependent manner. The maximal effect of MCP-1 was seen at 10 nmol/L in our experimental system. We tested the effect of the anti-MCP-1(1-73) Ab and the MCP-1(9-73) RA on MCP-1-mediated chemotaxis. Our results show that the Ab generated against the chemotactic (N-terminal) domain of MCP-1 [anti-MCP-1(1-73) Ab] significantly inhibited peritoneal macrophage migration in response to MCP-1 at dilutions as high as 1:1000 as shown in Figure 2a (***P* < 0.01). The MCP-1(9-73) RA peptide also inhibited MCP-1-mediated chemotaxis of peritoneal macrophages in a dose-dependent manner, but the MCP-1(11-73) peptide did not (Figure 2b). The binding epitope for the Ab generated

Figure 2. Evaluation of peritoneal macrophage migration mediated by MCP-1 and effect of anti-MCP-1 Ab and MCP-1 RA on MCP-1-mediated chemotaxis *in vitro*. Recombinant rat MCP-1 mediated the migration of peritoneal macrophages in a dose-dependent manner. Anti-MCP-1(1-73) Ab (**a**) and MCP-1 RA(9-73) (**b**) inhibit chemotaxis of peritoneal macrophage induced by MCP-1. Control experiments were performed with preimmune rabbit serum and MCP-1(11-73) peptide. Each graph shown represents one set of experiments ($n = 3$). All results were verified on two further occasions. The total number of migrating cells in five high-power fields is indicated on the y axis (mean $\pm$ SD).

against full-length MCP-1 [anti-MCP-1(1-125) Ab] had been found to be in the C-terminus of MCP-1 rather than along the chemotactic domain and, as expected, this Ab had a very low inhibitory effect on MCP-1 chemotactic activity (data not shown).

Induction of MCP-1, KC, and MIP-2 in the Kidneys of Rats with TIN

Total RNA samples from TIN model rat kidneys were probed for expression of MCP-1, MIP-2, and KC mRNA. The results are shown in Figure 3. As can be seen in Figure 3b, KC mRNA expression appeared as early as day 7, and peak expression for both KC and MIP-2 mRNA was on day 8. Both KC and MIP-2 expression diminished from day 9 onwards. The expression of these C-X-C chemokines was associated with an early onset of inflammatory cell influx; with our data showing the greatest degree of PMN infiltration occurring on days 7 and 8. KC protein expression was detected by immunoblot (Figure 3c) as a band of 8 kd in TIN model kidney lysates from days 7 and 8. MCP-1 mRNA expression levels remained elevated for a longer period of time than did that of KC and MIP-2. After day 9, MCP-1 mRNA expression declined slowly, but the expression levels remained higher than those in control kidney as late as day 13 (Figure 3a). The expression of MCP-1 mRNA occurred concomitantly with ED1+ cellular infiltration (Figure 4j).

Reduction of Macrophage Infiltration and Delay of Neutrophil Clearance Mediated by Antagonism to MCP-1 Using a Neutralizing Ab or MCP-1(9-73)RA Peptide

The results of the chemotactic assay performed *in vitro* (see Materials and Methods) indicated that the anti-MCP-1(1-73) Ab would provide greater protection against MCP-1-mediated chemotaxis *in vivo* than would the anti-

Figure 3. Assessment of MCP-1, MIP-2, and KC expression in the TIN model kidney by RPA and Western blotting. Experiments were conducted following the protocol mentioned in the Materials and Methods section. **a** and **b**: Renal total RNA (6 μ g) was used for each assay. **Left lane** of each panel contains probes for MCP-1 (239 bp), MIP-2 (174 bp), and KC (274 bp). Housekeeping gene L32 was used as a loading control. The time points (days after injection of bovine tubular basement membrane) are marked. The protected bands are shorter than the probes due to the unhybridized sequences in the polylinker regions of the probes were digested by RNase A and T1, and undigested KC probe is marked with an asterisk. **c**: Immunoblots assessing chemokine expression (MCP-1, MIP-2, and KC) in homogenates from the TIN model kidney.

MCP-1(1-125) Ab. Administration of these antisera to TIN model rats followed by histological study of harvested kidneys provided evidence that the anti-MCP-1(1-73) Ab indeed proved more effective at blocking MCP-1-activity than did the anti-MCP-1(1-125) Ab (data not shown). As shown in Figure 4, macrophage infiltration during TIN was decreased by 30% on day 8 and 50% on day 10 in samples from rats treated with the anti-MCP-1(1-73) Ab as compared to samples from NRS-treated rats. We also observed a significantly delayed clearance of apoptotic neutrophils in the anti-MCP-1(1-73) Ab-treated rat kidney samples. This accumulation of neutrophils was especially apparent in the tubulointerstitial regions, which showed a twofold to threefold increase in apoptotic neutrophils remaining in the kidney when compared to the NRS-treated rat kidney samples. We hypothesized that the decrease of phagocytic macrophage influx caused by administration of the anti-MCP-1(1-73) Ab might be responsible for the increased numbers of apoptotic neutrophils residing in the kidney. However, we had to rule out the possibility that an immune complex formed between the anti-MCP-1 Ab and MCP-1 might mediate a complement-dependent neutrophil infiltration because we indeed found the immune complex formed in the kidney (data shown later). To this end, we used an MCP-1(11-73) peptide to determine the role of MCP-1 during TIN. The MCP-1 RA(9-73) peptide affected MCP-1-mediated chemotaxis in a manner similar to the anti-MCP-1(1-73) Ab (Figure 2b). This data suggests that the increased numbers of neutrophils

Figure 4. The effects of anti-MCP-1(1-73) Ab (**c, d**) and MCP-1 RA(9-73) peptide (**g, h**) on neutrophil and macrophage infiltration in rat TIN model kidney (10 days). Neutrophils were identified (**left**, red staining) using the anti-MPO Ab. Macrophages were identified (**right**, brown staining) using the ED1 Ab. Sham animals treated with NRS exhibit a high degree of macrophage infiltration (**b**) and substantial neutrophil clearance (**a**) in the area of inflammation; whereas, animals treated with anti MCP-1(1-73) Ab exhibit decreased macrophage infiltration (**d**) and a concomitant accumulation of vast numbers of neutrophils (**c**) in the injured tissue. Animals treated with the MCP-1 RA(9-73) peptide exhibit a similar delay in neutrophil clearance (**g**) corresponding to a low degree of macrophage infiltration (**h**) in the area of injury; whereas, animals treated with an inactive form of the MCP-1(11-73) peptide exhibit neutrophil clearance (**e**) and macrophage infiltration (**f**) similar to sham animals. The number of MPO+ and ED1+ cells were calculated in 10 fields of tubulointerstitial area in each sample (**i, j**). Original magnifications, $\times 200$.

remaining in the TIN model kidney after apoptosis were the result of the decreased macrophage infiltration mediated by MCP-1 chemotactic signaling and were not a result of the formation of an immune complex. In an additional experiment, immunofluorescence staining for the complement component C3 was performed on TIN model kidney samples for both the anti-MCP-1(1-73) Ab- and NRS-treated animals. No significant differences were found in the presence and intensity of C3 deposition in the two groups (Figure 5).

MCP-1 Did Not Affect Phagocytic Activity of Macrophages for Apoptotic Neutrophils

MCP-1 is a potent macrophage chemoattractant factor, and it is hypothesized to be an enhancer of the phagocytic activity of macrophages. However, macrophage-mediated phagocytosis of apoptotic neutrophils was not altered in the presence or absence of MCP-1, MCP-1(11-

Figure 5. Immunofluorescent staining of C3 in kidneys of TIN rats treated with anti-MCP-1(1-73) Ab or NRS. The level of complement component C3 deposition in the anti-MCP-1(1-73) Ab-treated TIN model kidney. Rat kidneys were processed with cryosectioning ($0.5 \mu\text{m}$), followed by probing with fluorescein isothiocyanate-conjugated rabbit anti-rat C3 Ab, which recognizes an epitope expressed by C3. Immunofluorescent staining revealed that C3 protein was localized on the outer surface of the proximal and distal tubules in both NRS-treated group (**a**) and in anti-MCP-1 Ab-treated animals (**b**). Original magnifications, $\times 200$.

Figure 6. The process of phagocytosis of macrophages in TIN model rats treated with anti-MCP-1(1-73) Ab. **A** and **B**: Phagocytosis *in vivo*. Consecutive sections of rat kidneys with TIN were stained either for neutrophils, using the anti-MPO Ab (**left**), or for monocytes/macrophages using the ED1 Ab (**right**). Neutrophil staining is intensely purple while macrophage staining is dark brown. **Top**: **A** and **B** demonstrate the fully formed (area marked with asterisk) or partially formed (areas labeled with **a**, **a'**, **b**, and **b'**) multinuclear giant cells as macrophages engulf and ingest neutrophils in the area of inflammatory loci. **Bottom**: **a**, **a'**, **b**, and **b'** illustrate this process at higher magnification, with neutrophils that are being engulfed indicated by arrows. **c** and **d**: Phagocytosis *in vitro*. Neutrophils within macrophages are readily identifiable by positive staining (brown) for MPO. Two representative images of macrophages treated with vehicle (**a**) or MCP-1 (**b**). The experiment was done three times. Original magnifications: $\times 100$ (**A**, **B**); $\times 200$ (**a-d**).

73) peptide, or the MCP-1 neutralizing Ab as shown in Figure 6, c and d.

Effects of MCP-1 Blockade on the Profile of Cytokine Expression and the Progression of Fibrosis during Intubulointerstitial Nephritis

Macrophages participate in the development of inflammatory injury and in the regulation of acute inflammation through the production of inflammatory mediators and cytokines. To determine whether treatment of animals with the anti-MCP-1(1-73) Ab influences the course of TIN, we compared the dynamic change of cytokine expression and the fibrotic process in treated animals to that of untreated animals. Using rat cytokine multiprobe

Figure 7. RNase protection analysis of cytokine mRNA expression in the kidney of rats treated with anti-MCP-1 Ab or NRS. Each lane represents a single rat sampled. Probes contain polylinker regions and are longer than the protected bands. Rat ribosomal L32 and GAPDH genes were used as a housekeeping gene.

template sets, we observed that IL-1 β , IL-1 α , IL-1RA, IL-6, Lt β , transforming growth factor (TGF)- β 1, and TGF- β 3 expression were significantly up-regulated in a time-dependent manner during intubulointerstitial nephritis. The expression of cytokines reached their highest levels on days 7 and 8. This was followed by a gradual decline (as shown in Figure 7, a and b) in these expression levels in untreated rats. In the anti-MCP-1 Ab-treated rat kidneys, however, mRNA levels of IL-1 β , IL-1 α , IL-6, Lt β , TGF- β 1, and TGF- β 3 were significantly reduced on day 7 as compared with the NRS-treated kidneys. Quantitative analysis revealed a relative up-regulation in the levels of TGF- β 1 and IL-1 β in the anti-MCP-1(1-73) Ab-treated rats on days 9 and 10 (data not shown). Figure 8 illustrates a Masson's trichrome staining of TIN model renal tissues (day 10) that revealed no significant differences in the extent of fibrosis among the three groups when analyzed using computer-assisted image quantification (Optimas 6.5, Optimas Corp) (data not shown). These results demonstrate that although the blockade of macrophage infiltration induced by the anti-MCP-1(1-73) Ab changed the pattern of cytokine expression in the

Figure 8. Representative Masson's trichrome staining of renal sections from a normal rat (**a**), TIN rat (**b**), anti-MCP-1 Ab-treated rat (**c**), and MCP-1 RA-treated rat (**d**). Three dyes are used selectively to stain collagen (blue), nuclei (black), and background (red). Original magnifications, $\times 200$.

early stages of TIN, the delayed clearance of neutrophils might not alter the course of the disease or the fibrotic process in the TIN model kidney.

Immunolocalization of Rabbit IgG and HS in TIN Model Kidney

To assess the accumulation of neutrophils in the anti-MCP-1(1-73) Ab-treated kidneys, we used a rabbit anti-rat MPO Ab. Unexpectedly, strong tubular interstitial staining was observed in all animals treated with the anti-MCP-1(1-73) Ab, but not in the NRS-treated group (Figure 9, a and b). This evidence suggested that the rabbit IgG used for blocking MCP-1 was captured in the local area. The first molecule that we hypothesized was locally up-regulated was MCP-1. But, we still needed to answer how MCP-1 was accumulated in the local area and which molecule was involved in this process. To answer this question, we performed immunohistochemical detection of HS in the TIN model kidneys (Figure 9, c and d). The results of this experiment revealed that HS was constitutively expressed in normal rat kidney (Figure 9a), especially in the basement membrane of Bowman's capsule, in the vessels, and in the distant tubular cells. There was basolateral staining of HS in the tubular cells, but staining in the normal glomeruli was negative. HS expression was significantly increased in the TIN model kidneys, with its expression being tightly associated with inflammatory cell infiltration (Figure 9b). These results suggest that the expression of HS was up-regulated in the TIN model kidneys and that this up-regulation might be involved in the deposition of MCP-1 and the formation of an anti-MCP-1 Ab:MCP-1 immune complex in the local areas.

Figure 9. Comparison of rabbit IgG and HS distribution in TIN model rats. **a** and **b**: Localization of rabbit IgG in the anti-MCP-1(1-73) Ab-treated TIN model kidney. Rabbit IgG was directly detected by a horseradish peroxidase-labeled goat anti-rabbit Ab in NRS-treated group (**a**) and anti MCP-1(1-73) Ab-treated group (**b**). **c** and **d**: Induction of HS in the TIN model. **a**: Rat kidney stained with anti-HS Ab against 10E4 epitope shows that the expression of HS is mainly located to the lumen of the proximal and distal tubules and to Bowman's capsule of the glomeruli. **b**: TIN model rat kidney shows extensive staining of HS in the vascular endothelial cells as well as in the Bowman's capsule of the glomeruli and in the epithelial cells of the interstitial tubules. Numerous infiltrated inflammatory cells in the interstitial lesions were observed when counterstaining (blue) was performed. Original magnifications, $\times 200$.

The Formation of an Anti-MCP-1 Ab:MCP-1 Immune Complex in the Kidney Was Due to HS-Mediated Immobilization of MCP-1

To further confirm our hypothesis, we determined that MCP-1 was expressed and that rabbit IgG was deposited in the inflammatory loci in TIN model kidneys. Immunoprecipitation and immunoblot experiments revealed that the expressed MCP-1 was immobilized in an immune complex with HS. Protein A pull-down precipitates were prepared using kidney homogenates from TIN model rats. The resulting data demonstrated that the kidneys from the anti-MCP-1(1-73) Ab-treated rats, but not from NRS-treated rats, contained MCP-1. Because MCP-1 is a highly soluble, small, secreted peptide, we hypothesized that it was immobilized on cell surfaces in the inflammatory loci to regulate inflammatory cell chemotaxis. We further speculated that the most probable binding partner to immobilize MCP-1 *in vivo* was HS. Immunoprecipitation experiments performed by probing with a monoclonal Ab against HS (10E4) contained MCP-1 (Figure 10), as detected by probing with an anti-MCP-1 Ab. This indicated that MCP-1 in the nephritic kidney was associated with HS and suggested that it could be immobilized in the inflammatory loci. In our histological studies, rabbit IgG was found to be deposited only in the kidneys of the anti-MCP-1(1-73) Ab-treated rats. These results, when taken with the detection of MCP-1 in the protein A pull-down complexes, suggest that our rabbit anti-rat MCP-1(1-73) Ab formed an immune complex with the HS-immobilized MCP-1 in the kidney.

Figure 10. Protein interaction between MCP-1, rabbit anti-MCP-1 Ab, and HS. The immune complex of MCP-1 and rabbit anti-MCP-1 Ab was detected using pull-down and immunoprecipitation assays (**left**). **Left:** Pull-down experiments were performed using protein A beads followed by immunoblotting for MCP-1. Kidney homogenates from TIN models day 8 and day 9 were subjected to pull-down using protein A beads and were tested for the presence of MCP-1 by rabbit anti-rat MCP-1 Ab. **Right:** Immunoprecipitation was performed using mouse anti-rat HS Ab 10E4 followed by immunoblotting for MCP-1. Kidney cell lysate from normal and TIN model kidneys were subjected to immunoprecipitation using the 10E4 Ab (**lanes 1 and 2**). A sample of cell lysate from TIN model kidney was subjected to digestion with heparanase (**lane 3**) after the immunoprecipitation with 10E4. An immunoblot for MCP-1 was then performed on all three samples.

Discussion

Numerous publications have demonstrated the role of C-X-C and C-C chemokine expression in pathologically inflamed tissues.^{5–7,25} Many of these studies suggest that chemokines are the main source of chemotactic signaling which guides inflammatory cells into affected sites, but further studies are required to determine their pathogenetic role in disease. Various cellular infiltrates are involved in the development of TIN, and it is interesting to note that C-X-C and C-C chemokines are expressed concomitantly in this model of disease. The influx of both neutrophils and mononuclear cells in this model may be a result of this concomitant expression of chemokines from two different subfamilies.

Brown Norway rats immunized with bovine tubular basement membrane exhibited no histological changes in the kidney tissue by day 3, but developed focal, irregularly distributed renal cortical cellular infiltrates consisting of ~65% PMNs and 35% mononuclear cells by day 7, as shown in Figure 4, i and j. As previously reported, anti-tubulobasement membrane Ab deposition begins in the kidney 7 or 8 days after immunization of Brown Norway rats with bovine tubulobasement membrane antigens and is followed by C3 deposition and prominent, transient infiltration of PMNs. This sequence is typical of lesions produced by passive administration of an Ab, such as that seen during anti-glomerular basement membrane glomerular nephritis.²⁶ Consistent with previous

reports² we observed PMNs as the predominant infiltrating cell (70 to 80% of total cellular infiltrates) on day 8 and day 9. The activity of the neutrophil marker enzyme MPO correlated well with this peak in PMN infiltration (Figure 4). Our immunohistochemical and RNase protection assay experiments examining the mRNA expression levels of different chemokines up-regulated in the TIN model kidney yielded data that support the conclusion that MCP-1 mRNA expression levels were higher and of longer duration than those of other chemokines involved. MCP-1 is produced by a wide variety of cell types, including monocytes, fibroblasts, vascular endothelial cells, and smooth muscle cells. It is produced in response to stimuli, such as lipopolysaccharide, IL-1, and tumor necrosis factor- α . The mechanism of monocyte infiltration into injured tissues has not been fully elucidated. MCP-1 is thought to play a significant role in this event, and recently it was reported that T lymphocytes were responsive to MCP-1.²⁷ It has been observed that the time course of expression of MCP-1, MIP-1 α , and MIP-1 β correlated well with the observed lymphocytic infiltrates.² We suggest that perhaps expression of MCP-1, as well as MIP-1 α and MIP-1 β could be responsible for the influx of T lymphocytes into the area of inflammation in this model.

We were surprised to find that MCP-1 expressed by mammalian cells appeared to have three different molecular weights (Figure 1c). Western blot analysis of the supernatant of MCP-1-transfected COS-7 cells revealed three major bands (18, 21, and 23 kd) that were detected by a monoclonal B4 Ab. An additional 16-kd peptide was observed in the tissue extracts from the diseased kidneys (Figure 3c). We believe that this may be due to posttranslational modifications of the carbohydrate moieties of MCP-1. The 16- to 18-kd products correspond to the predicted molecular weights of nonglycosylated and glycosylated rat MCP-1. Previous studies have demonstrated that both rat²⁸ and murine MCP-1 produced in COS-7 cells contain O-linked carbohydrate moieties;^{29,30} however, there is very little information about the molecular weight and glycosylation state of rat or murine MCP-1 peptide *in vivo*. Rat MCP-1 with a molecular mass of 30 kd has been detected in immunoprecipitates (using clone B4 Ab) generated from the culture supernatant of Con A-stimulated rat spleen cells,²⁸ as well as from the supernatant of rat MCP-1-transfected COS-7 cells (data not shown). A 17-kd form of rat MCP-1 peptide was detected in rat spinal cord tissue extracts from an *in vivo* study using a mouse anti-human MCP-1 Ab in Western blot analysis.³¹ We cannot explain the discrepancy among the observed molecular weights of rat MCP-1 at present; however, we have confirmed that rat MCP-1 was translated and also heavily glycosylated and correlated with MCP-1 transcripts.

Our most intriguing finding is that the anti-MCP-1(1-73) Ab formed an immune complex with MCP-1 that had been immobilized in the diseased kidney. We stumbled on this data in attempting to perform immunohistochemical staining of MPO to localize neutrophils in the kidney. On examination of the MPO-stained tissues, we were surprised to observe significant amounts of rabbit IgG

that bound to the conjugated secondary goat anti-rabbit Ab in the inflamed tubulointerstitial region. Because rabbit IgG was present only in the kidney of the rats treated with the rabbit anti-MCP-1(1-73) Ab, we postulated that the rabbit IgG we detected might be part of an immune complex formed by the rabbit anti-rat anti-MCP-1 Ab binding to MCP-1 in the inflammatory loci. To examine whether rabbit IgG was complexed with MCP-1, we enriched rabbit IgG in kidney lysates using protein A-conjugated Sepharose, and then subjected the samples to immunoblotting. As shown in Figure 8, MCP-1 was detected in the protein A-pull-down precipitates only in the rabbit anti-MCP-1(1-73) Ab-treated rat kidneys but not in the NRS-treated kidneys. Our laboratory has treated a variety of chemokine-dependent renal inflammatory diseases with anti-chemokine Abs, and this is the first time we have observed the formation of a neutralizing Ab: chemokine immune complex *in situ*. Because a large amount of rabbit IgG was deposited in the tubulointerstitial region where neutrophils had accumulated, we had to determine what effect, if any, the observed immune complex had on reduced macrophage infiltration into areas of injury and also whether or not the delay of apoptotic neutrophil clearance from the inflammatory loci was a result of the deposition of the observed immune complex. It is well known that immune complexes play a major role in complement-dependent leukocyte infiltration. It has also been reported that immune complexes have triggered a marked stimulation of neutrophil apoptosis via Fc γ RII, while soluble immune complexes significantly delay spontaneous neutrophil apoptosis.^{32,33} To determine the role that the neutralizing-Ab:chemokine immune complex might have played in macrophage infiltration and in neutrophil clearance in TIN injury, a nonimmunoglobulin MCP-1 RA [MCP-1(9-73) RA peptide] was used to block MCP-1.

We observed that a similar phenotype of injury was observed when the MCP-1 RA(9-73) peptide was administered to TIN model animals with neutrophil clearance being significantly delayed from day 8 through day 10. Positive neutrophil staining was observed in ~50% of the injured tissue (see Materials and Methods) in both NRS and in the MCP-1(11-73) peptide-treated animals on day 8 and rapidly decreased from day 9 onwards. By day 10, neutrophils were hardly detectable in the NRS-treated rat kidneys. This decrease in observed neutrophil staining was associated with a concomitant increase in the number of macrophages exhibiting positive immunohistochemical staining. Positive neutrophil staining in the neutralizing Ab-treated and in the nonIgG-MCP-1 RA(9-73) peptide-treated rats, however, did not decrease until day 11 (10% of the scanned area in contrast to 50% on day 10). The kidneys of anti-MCP-1(1-73) Ab-treated rats did not exhibit a significant increase in the numbers of neutrophils compared to those rats that were treated with the MCP-1(9-73) RA peptide; however, we cannot completely rule out the possibility that the immune complex might contribute to the increase in the neutrophil density in the inflamed tissue. Although immunofluorescent staining of complement 3 (C3) did not reveal an increase in C3 deposition in rats treated with the anti-MCP-1(1-73) Ab

(Figure 5), whether an immune complex of MCP-1:anti-MCP-1 Ab was involved in the observed neutrophil accumulation in rats treated with the anti-MCP-1(1-73) Ab remains to be further clarified.

In addition to other researchers, we have found that the therapeutic antagonism of chemokines may improve renal inflammation. However, in most of these studies only one chemokine was targeted, resulting in the chemotaxis of only one type of cell being blocked. This strategy is acceptable when only mononuclear cells are involved in inflammation, such as in anti-glomerular basement membrane glomerulonephritis in WKY rats. Blocking of CD8+ lymphocytes or of macrophages in anti-glomerular basement membrane glomerulonephritis via antagonism of C-C chemokines results in a decrease of observable renal inflammation as demonstrated in our laboratory previously. In the TIN model, however, both neutrophils and monocytes are involved in inflammation as they simultaneously infiltrate the injured kidney. Targeting only the chemotaxis of monocytes would limit the efficacy in controlling the infiltration of neutrophils involved in this model and would also decrease monocytic engulfment of unwanted neutrophils. The role of monocytes in the clearance of apoptotic neutrophils has been revealed in these experiments, demonstrating the need to examine the role of more than a single chemotactic agent when investigating renal inflammation. A study by Gong and colleagues¹⁷ further demonstrates this need. This study reports that targeting the infiltration of both monocytes and neutrophils has a more pronounced therapeutic effect during inflammation than does targeting of either cell type alone.

In studies from another laboratory, it was found that blocking of RANTES-mediated macrophage infiltration by RAs aggravates glomerular inflammation.³⁴ The authors concluded that the inhibition of monocyte-mediated removal of apoptotic cells could be responsible for this phenomenon. It has also been reported that CCR1 deficiency in nephrotic mice enhances glomerular injury during nephrotoxic nephritis.³⁵ These findings, when taken together, suggest that rather than simply promoting leukocyte recruitment during nephrotoxic nephritis, chemokines may alter the effector phase of glomerulonephritis. Thus, therapeutic targeting of chemokine receptors may sometimes exacerbate an underlying disease.

The fate of apoptotic neutrophils during glomerulonephritis has been described earlier.^{36–38} Rapid, efficient, and tightly regulated recruitment and clearance of neutrophils at inflammatory loci are essential components of effective host defense. Clearance of neutrophils that are capable of releasing potentially toxic components that activate the inflammatory response is critical in the resolution of inflammation and in the prevention of excessive tissue necrosis for a host.³⁹ Evidence from *in vitro* models and from histopathology suggests that tissue damage mediated by neutrophils is limited by apoptosis and subsequent phagocytosis of the apoptotic neutrophils by macrophages, as well as by nonprofessional phagocytes.^{40,41}

Chemokines are the major mediators of leukocyte extravasation and migration into areas of inflammation. The

glycosaminoglycan HS binds to and modifies the function of multiple molecules involved in inflammatory events under physiological and pathological conditions. These highly anionic polysaccharides are thought to tether chemokines on the cell surface and in the extracellular matrix. Lys-58 and His-66 residues in the C-terminal α -helix of MCP-1 are essential for HS binding.⁴² Interaction between MCP-1 and HS was confirmed by immunoprecipitation and immunoblot as described in Figure 8. We hypothesized that the syndecan family of proteins could be the HSPG core proteins that present HS. However, we found no evidence to support this hypothesis when testing syndecan-1 to -4 (data not shown). Further investigation is required to determine which HSPG(s) are involved in binding MCP-1 in the kidney.

We believe that we are the first group to provide evidence that a chemokine and a chemokine receptor-targeting therapeutic strategy may delay the clearance of apoptotic neutrophils due to inhibition of macrophage chemotaxis by MCP-1 during TIN. However, delayed clearance of neutrophils did not affect disease severity significantly as determined by fibrotic lesion because Mason's Trichrome staining did not reveal any marked differences between the two groups (Figure 8). We also analyzed whether MCP-1 blockade affected cytokine profile by RPA. As shown in Figure 7, all of the cytokines detected were expressed until day 8, with IL-1 α , IL-1 β , and IL-6 expression levels significantly decreasing on day 8 in the rats treated with the anti-MCP-1 Ab. No difference between the two groups was observed on day 10, when neutrophils were the major infiltrates in the rats treated with the anti-MCP-1 Ab. TGF- β 1, TGF- β 3, and Lt β were dramatically decreased on day 7 in rats treated with the anti-MCP-1 Ab, suggesting that blocking of MCP-1 resulted in an inhibition of macrophage influx and, in turn a blockage in cytokine production. However, these cytokines, especially TGF- β 1 and IL-1 β , were relatively increased on days 9 and 10, when neutrophil accumulation was much higher in the group with MCP-1 blockade than in the group treated with vehicle. Nonetheless, whether an increase in TGF- β 1 expression affects fibrotic lesions remains to be further investigated because Trichrome staining did not reveal any change in collagen accumulation.

In conclusion, both C-X-C and C-C chemokines play an important role in leukocyte infiltration; MCP-1 is a key chemokine involved in macrophage chemotaxis *in vivo* in the TIN model; and blocking MCP-1 reduced monocyte infiltration, but also delayed clearance of apoptotic neutrophils. Our surprising finding that the anti-MCP-1 Ab formed an immune complex with MCP-1 in the inflammatory loci suggests that care must be taken when Ab-based antagonism of chemokines is used as a therapeutic approach.

It has been reported that MCP-1 enhances phagocytosis and killing of bacteria, is responsible for eliminating bacteria, and thus enhances the survival rate of the host.⁴³ Therefore, inhibition of MCP-1 activity might directly affect MCP-1-mediated phagocytosis. We further examined whether MCP-1 had a direct effect on macrophage-mediated ingestion of apoptotic neutrophils *in vitro*. As shown in Figure 6, c and d, MCP-1 did not seem to affect macrophage-mediated phagocytosis, suggesting that the reduction of macrophages in the inflamma-

tory loci might be the key factor for delayed clearance of neutrophils in the kidney.

Granulomatous interstitial nephritis is a rare condition whose pathogenesis is poorly understood.⁴⁴ Although foreign body giant cells, Langhans' giant cells, and osteoclasts are derived from monocytes or monocyte progenitor cells, the ways in which they are formed, whether they are induced by cytokines, receptors, or biological activity, are markedly different. It has long been suspected that the formation of multinucleate giant cells in the kidney during TIN might result from cell fusion. Development of multinucleate giant cells always proceeds in accordance with a given mode, namely simultaneous phagocytosis-fusion-unordered multinucleating-ordered multinucleating. Actively phagocytosing macrophages may have a tendency to form the multinucleate giant cells.⁴⁵ Our data reported here suggest that *in vivo*, multinucleate giant cells might be fused due to joint ingestion of apoptotic neutrophils by adjacent multiple macrophages. As shown in Figure 6a, ED1+ cells that were adjacent to one another engulfed neutrophils and came to form semimultinucleated cells. The observed decrease in multinucleated cell density in the kidneys of rats treated with the anti-MCP-1(1-73)-neutralizing Ab and with the MCP-1(9-73) RA peptide might be a result of the inhibition of mononuclear infiltration in the kidney. The number of accumulated neutrophils in the Ab- or in the MCP-1(9-73) RA peptide-treated rats started to decrease on day 11 and thereafter. How the nonphagocytosed neutrophils were finally removed from the injured tissue remains to be determined. It has been reported in the literature that the cells of the proximal tubule are capable of phagocytosing erythrocytes, bacteria, and apoptotic neutrophils.^{46,47} It is possible that phagocytosis by the proximal tubule cells and/or other resident renal cells may play a role in the disposal of inflammatory neutrophils.

One of our most interesting findings is that Ab therapy may lead to the formation of an immune complex. Therefore, care must be taken to analyze the biological effects of Ab therapy. Neutralizing Abs used in *in vivo* studies occasionally enhance inflammation, possibly via the inhibition of the functional activity of the target molecules. The observed HS-mediated immobilization of MCP-1 suggests that other soluble cytokines and chemokines may be also bind to HS, and that immobilization of the soluble growth factors may contribute not only to chemotactic gradient formation, but also to the biological enhancement of chemotaxis.

We conclude that 1) induction of C-X-C and C-C chemokines in TIN corresponds to neutrophil and monocyte/macrophage infiltration, respectively; 2) MCP-1 is a key macrophage chemoattractant factor in TIN; 3) anti-MCP-1(1-73) Ab formed an immune complex with the MCP-1 that was immobilized by HS in TIN model kidney; and 4) antagonism of MCP-1 in TIN using a neutralizing Ab or a peptide-based RA may alter the pathological process, most likely due to a delayed removal of apoptotic neutrophils that may result from reduction of phagocytic macrophages by antagonism against MCP-1 in the inflammatory loci.

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