Role of Tumor Necrosis Factor-α on Mesangial Cell MCP-1 Expression and Monocyte Migration: Mechanisms Mediated by Signal Transduction

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ABSTRACT

Monocyte chemotactic protein-1 (MCP-1), a specific chemoattractant for monocytes, has been thought to play an important role in the recruitment and accumulation of monocytes within the glomerulus seen in glomerular diseases. This study examined the role of tumor necrosis factor (TNF)-α-mediated cellular signal transduction pathways on mesangial cell MCP-1 gene expression and monocyte migration. Incubation of mesangial cells with TNF-α stimulated MCP-1 mRNA expression in a dose- and time-dependent manner. Phorbol myristate acetate (PMA), a protein kinase C (PKC) activator, increased MCP-1 message by mesangial cells while depletion of PKC decreased MCP-1 gene expression to control levels. Activation of PKC-depleted mesangial cells with PMA but not with TNF-α inhibited MCP-1 mRNA expression. Similarly, calphostin C, a PKC inhibitor, failed to inhibit TNF-α-induced MCP-1 expression. The incubation of mesangial cells with various protein tyrosine kinase inhibitors (PTK, e.g., herbimycin, tyrphostin, genistein) blocked TNF-α-induced MCP-1 mRNA message. Additional experiments examining the role of cAMP on MCP-1 expression indicated that the preincubation of mesangial cells with various cAMP generating substances (pertussis toxin, isoproterenol, dbcAMP) did not induce mesangial cell MCP-1 mRNA transcripts. However, the coincubation of mesangial cells with TNF-α and dbcAMP completely inhibited TNF-α-induced MCP-1 gene expression. Finally, TNF-α-activated mesangial cell media increased monocyte transmigration that could be blocked by neutralizing anti-MCP-1. These studies indicate that TNF-α facilitates monocyte transmigration into the glomerulus mediated by the increased expression of MCP-1 by mesangial cells. TNF-α-induced mesangial cell MCP-1 expression is regulated by signal transduction pathways involving PTK but not those dependent on PKC or cAMP.

Key Words: Mesangial cells, monocyte chemotaxis, MCP-1, TNF-α, signal transduction

Mesangial cells, specialized vascular pericytes within the glomerulus, perform a fundamental role in the homeostasis of glomerular function. Their unique location within the glomerulus, their contractility characteristics, and their ability to produce pseudopodia that project into the glomerular capillary lumen allow mesangial cells to interact with other resident and bone-marrow derived cells and numerous circulating chemical modulators (1). In addition, mesangial cells can express a variety of cytoregulatory peptides, including chemokines, that attract circulating mononuclear leukocytes into the glomerulus (2,3). The adherence and subsequent transmigration of circulating monocytes into the glomerular mesangium occurs early in the development of various forms of renal diseases, including focal and segmental glomerulosclerosis (4,5), membranoproliferative glomerulonephritis (6,7), antiglomerular basement membrane disease (8), immune complex nephritis (9), puromycin aminonucleoside nephritis (10,11), and the glomerular injury associated with hyperlipidemia or cholesterol feeding (12). Although the cellular and molecular mechanisms underlying monocyte infiltration into the glomerulus are poorly understood, monocyte recruitment into the mesangium seems to involve a series of complex receptor-mediated interactions between resident glomerular cells and circulating mononuclear leukocytes. These interactions are intensified by the enhanced expression of glomerular endothelial and mesangial cell-derived adhesion molecules and by the elaborated expression of monocyte chemokines by intrinsic glomerular cells (2,3,13–15).

Monocyte chemotactic protein-1 (MCP-1) belongs to a family of chemokines that may be involved in inflammatory and tissue repair processes, including the chemotraction of mononuclear phagocytes into the mesangium (3,16). MCP-1 is a monomeric polypeptide that corresponds to the early response gene J E of the mouse and is produced by a variety of cells, including endothelial, smooth muscle, and mesangial cells and monocyte/macrophages (17–21). MCP-1 is expressed in response to proinflammatory factors such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IFN-γ (18,22). Although original studies have indicated that
MCP-1 acts as a specific factor for monocyte/macrophage migration (23,24), recent observations suggest that this chemokine can also act as a chemoattractant for T-lymphocytes (25,26). Furthermore, MCP-1 accounts for nearly all of the monocyte chemotactic activity expressed by vascular cells (27).

Although the increased expression of monocyte chemoattractants and the subsequent accumulation of monocyte/macrophages in the mesangium are seen in many experimental models of renal injury, the cellular and molecular mechanisms associated with this inflammatory process are poorly understood. In this regard, many in vitro and in vivo studies have suggested that TNF-α is a fundamental proinflammatory cytokine involved in the pathogenesis of glomerular injury (28,29). Furthermore, in response to activation, both infiltrating macrophages and intrinsic glomerular cells may secrete TNF-α, allowing this peptide to serve as an important proinflammatory cytokine associated with the pathogenesis of glomerular inflammation and injury.

Despite several studies showing that TNF-α induces various cytoskeletal reagents by both glomerular and nonglomerular cells, the cellular signaling pathways between TNF-α binding to its cell-surface receptor and subsequent nuclear signals that regulate TNF-α-induced gene expression in glomerular mesangial cells are not clearly understood. Thus, we examined the ability of exogenous TNF-α to influence the gene expression of MCP-1 by murine mesangial cells in an attempt to further elucidate the role of TNF-α in the pathobiology of glomerular injury. Additional studies were performed to understand the multiple signal transduction pathways mediated through protein kinase C (PKC), protein tyrosine kinase (PTK), and cyclic nucleotides that may be involved in TNF-α-induced MCP-1 gene expression. The biological significance of increased mesangial cell synthesis and secretion of MCP-1 was assessed by its ability to modulate monocyte chemotaxis.

MATERIALS AND METHODS

Materials

Dulbecco’s minimal essential media (DMEM), RPMI 1640, dibutyryl (db)AMP, phorbol myristate acetate (PMA), and other tissue culture reagents were obtained from Sigma Chemical Company, St. Louis, MO. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc., Logan, UT. Human recombinant TNF-α was purchased from R & D Systems, Minneapolis, MN. cDNA probes for murine MCP-1 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were procured from American Type Culture Collection (ATCC, Rockville, MD). MSI™ nylon transfer membranes were obtained from Fisher Scientific, Tustin, CA. Calpastatin C, isoproterenol, pertussis toxin, genistein, tyrphostin B46, herbimycin A, and isobutyl-1-methylyanthine (IBMX) were purchased from Calbiochem, La Jolla, CA. Murine anti-MCP-1 was obtained from Pharmingen, San Diego, CA. All other chemicals used were of analytical grade.

Mesangial Cell MCP-1 Gene Expression: Response of TNF-α Dose and Incubation Time

Mesangial cells (5 x 10⁶) were grown in DMEM media containing 5% FBS at 37°C for 24 h to attain 75 to 80% confluence. The media was replaced with DMEM + 5% FBS containing various concentrations of TNF-α (0 to 200 ng/mL) and incubated for 3 h at 37°C. After incubation, the cell monolayer was washed and used for RNA isolation. The incubation time kinetics studies were performed by growing mesangial cells (5 x 10⁶) in DMEM media containing 5% FBS for 24 h. The media was replaced with fresh DMEM + 5% FBS containing 25 ng/mL of TNF-α and incubated at 37°C.

Monocyte Chemotaxis Assay

An ability of mesangial cell conditioned media in response to TNF-α activation to modulate monocyte migration was examined by in vitro monocyte chemotaxis assay as described earlier by Falk et al. (30). TNF-α-activated mesangial cell conditioned media was obtained from mesangial cells incubated with varying concentrations of TNF-α (0 to 200 ng/mL) for 3 h at 37°C. An aliquot of this medium (five-fold concentration) was placed in the lower compartment of a microchemotaxis assembly (Neuro Probe, Inc., Cabin John, MD) with two compartment chambers separated by a 5-μm polycarbonate membrane filter. Murine monocyte/macrophages (J774.A1, 6 x 10⁴ cells) were added to the upper chamber and incubated at 37°C for 3 h in a humidified 5% CO₂ incubator. After incubation, the membrane filter was removed, stained with Diff-Quick solution (Fisher Scientific, Tustin, CA) and mounted on a glass slide. The migrated monocytes were counted on an oil immersion microscope with a 20X magnification containing 10-mm² counting grid. Four grid areas were counted per sample and averaged. Similar chemotaxis assay was performed with control mesangial cell conditioned media and compared with the results obtained by utilizing TNF-α activated mesangial cell conditioned media. Appropriate exogenous TNF-α controls were used to normalize the data obtained from cytokine-activated cell supernates. Neutralizing anti-MCP-1 was used in the chemotaxis assay medium to determine the biological specificity of MCP-1 secreted by mesangial cells to stimulate monocyte migration.

Mesangial Cell Culture

A murine mesangial cell line (MES-13, cloned from mice transgenic for the early region of SV-40 virus, Passage 25) was obtained from ATCC, Rockville, MD. The mesangial cells were grown in DMEM containing 5% FBS, 1% glutamine-streptomycin-penicillin mixture, 44 mM NaHCO₃, and 14 mM N-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES) in an atmosphere of 5% CO₂ and 95% air at 37°C in a humidified incubator. Subcultures were made from confluent stock cultures by trypsinization in phosphate-buffered saline (PBS) containing 0.5 mM EDTA and 0.025% trypsin. A murine monocyte/macrophage cell line (J774.A1) was obtained from ATCC and cultured in RPMI 1640 media supplemented with l-glutamine, penicillin/streptomycin, and 10% FBS.
for varying periods (0.5 to 24 h). After incubation, cell monolayers were washed and used for RNA isolation.

Mesangial Cell MCP-1 Gene Expression in Response to TNF-α Activation: Regulation Through Protein Kinases

These experiments were performed to determine the regulatory role of various protein kinases and cyclic-AMP (cAMP) on TNF-α-induced gene expression by mesangial cells. The effect of PKC on mesangial cell gene expression was evaluated by incubating cells with either inducers or inhibitors of PKC. Mesangial cells (5 × 10⁶) were grown in DMEM containing 5% FBS for 24 h to attain 75 to 80% confluence. The media was replaced with fresh DMEM + 5% FBS containing PMA (50 nM), an activator of PKC, and incubated for 3 h at 37°C. In other experiments, intracellular PKC was depleted by incubating mesangial cells with 50 nM PMA for 24 h at 37°C. After predepletion of intracellular PKC, mesangial cells were stimulated by incubating them with either PMA (50 nM) or TNF-α (10 ng/mL) for 3 h at 37°C. After the incubation, mesangial cell monolayers were washed and used for RNA isolation. Further experiments were performed to assess the effect of PKC on cellular gene expression by preincubating mesangial cells (5 × 10⁶) at 37°C for 1 h with a PKC inhibitor, calphostin C (100 nM). After preincubation with calphostin C, mesangial cells were stimulated for 3 h at 37°C with 10 ng/mL TNF-α. After the incubation, cell monolayers were washed and used for RNA isolation.

Additional studies were designed to examine the role of cAMP-mediated cellular signalling pathways in mesangial cell MCP-1 expression. Mesangial cells (5 × 10⁶) were grown in DMEM + 5% FBS for 24 h at 37°C to attain 75 to 80% confluence. The media was replaced with fresh DMEM + 5% FBS containing various cAMP generating agents, including pertussis toxin (50 ng/mL), isoproterenol (5 μM) or dbcAMP (1 mM), and incubated for 3 h at 37°C. Experiments were also performed to determine the effect of cAMP on TNF-α-induced mesangial cell MCP-1 gene expression. Mesangial cells were coincubated with TNF-α and dbcAMP for 3 h. Additional studies were done to examine the role of intracellular cAMP degradation in the observed changes in MCP-1 gene expression. Mesangial cells were incubated for 3 h with 0.5 mM IBMX alone or in the presence of cAMP-generating substances. After the incubation, mesangial cell monolayer was washed, RNA isolated and used for Northern blot analysis.

The role of PTK was examined by preincubating cells with three different PTK inhibitors. Because these structurally different PTK inhibitors used in the study may have different modes of action on tyrosine phosphorylation (31-33), we have used three structurally distinct PTK inhibitors to assess the role of TNF-α in MCP-1 expression. Additionally, because of their structural dissimilarity, previous studies have shown that each inhibitor requires different incubation times to penetrate cells for their optimal activity. Therefore, we have used these different inhibitors at various incubation times as reported earlier by others (34). Mesangial cells (5 × 10⁶) were grown in DMEM containing 5% FBS for 24 h at 37°C to attain 75 to 80% confluence. The media was replaced with fresh DMEM + 5% FBS and preincubated with genistein (25 μg/mL) for 15 min, herbimycin A (10 μM) for 1 h, or tyrophostin B46 (100 μM) for 2 h at 37°C. After preincubation with PTK inhibitors, mesangial cells were stimulated with TNF-α (10 ng/mL) for 3 h at 37°C, cells were washed, and RNA isolated for Northern hybridization.

Mesangial Cell MCP-1 Gene Expression in Response to TNF-α Activation: Effect of Cycloheximide

Additional studies were undertaken to determine the regulatory role of protein synthesis on TNF-α-induced mesangial cell MCP-1 gene expression. Mesangial cells (5 × 10⁶) grown in DMEM containing 5% FBS for 24 h to attain 75 to 80% confluence. The media was replaced with DMEM + 5% FBS, preincubated with cycloheximide (10 μg/mL) for 2 h and stimulated with TNF-α for 3 h. After incubation, cell monolayers were washed, and RNA was isolated and used for Northern blot analysis.

Northern Blot Analysis

After the media was removed, mesangial cells were collected and washed three times with PBS. Total RNA was isolated from the cells using the protocol described by Chomczynski and Sacchi (35). In brief, cells were homogenized with 4 M guanidium thiocyanate total protein and DNA extracted with acid phenol, and RNA precipitated with isopropanol. After they were washed with ethanol, samples were dried under vacuum centrifugation and the amount of RNA was quantitated by measuring the absorbance at 260 nm using a spectrophotometer. Thirty μg of total RNA was loaded into individual wells of a 1.2% agarose gel containing formaldehyde and electrophoresis performed (36). The RNA from the gel was transferred onto MSi nylon membranes using a capillary transblotting technique. The nylon membrane was ultraviolet (UV)-linked using a UV Crosslinker (Fisher Scientific, Pittsburgh, PA). The CDNA probes for MCP-1 and GAPDH were used for hybridization after [32P]dCTP-labeling by random oligonucleotide priming. The membrane was hybridized using a [32P]-labeled murine MCP-1 CDNA probe. The membranes were washed three times for 30 min: first in 2X standard saline citrate (SSC) with 0.1% SDS at 25°C; second in 0.2× SSC with 0.1% SDS at 25°C; and third in 0.2× SSC with 0.1% SDS at 55°C. Autoradiography was performed by exposing the blots to Kodak X-ray film (Eastman Kodak, Rochester, NY) with intensifying screens at ~70°C. Blots were then rehybridized with a [32P]-labeled human GAPDH cDNA probe as an internal control to assess RNA quantity and integrity. Quantitation of mRNA signals was performed by densitometric scanning of autoradiographs and normalized with the GAPDH mRNA signal.

Statistical Analysis

Results are presented as mean values ± standard error (SE) for 3 to 4 separate experiments. The t-test was used to compare the means and a P value of less than 0.05 was considered significant.

RESULTS

In this study, we have used SV-40 transformed murine mesangial cells as an in vitro model to examine the role of TNF-α on glomerular mesangial cell MCP-1 gene expression. These transformed cells were developed by cloning mesangial cells from transgenic mice for the early region of SV-40 virus and have been shown to exhibit similar characteristics to those of primary cultures of murine mesangial cells (37). In addition to maintaining normal differentiation characteristics, transformed mesangial cells exhibit contractile properties in response to vasoactive sub-
stances and produce various cytokines analogous to primary cultures of mesangial cells (38,39), including MCP-1.

Utilizing these transformed murine mesangial cells, studies were performed to examine the effect of exogenous TNF-α on MCP-1 steady-state mRNA expression. The incubation of mesangial cells with TNF-α for 3 h stimulated MCP-1 mRNA expression in a dose-dependent fashion (Figure 1). TNF-α, as low as 0.5 ng/mL, induced mesangial cell MCP-1 mRNA transcripts and reached a maximal level at 25 ng/mL of TNF-α in the incubation media. Quantitative analysis of MCP-1 gene expression was performed by densitometric scanning of autoradiographs and was normalized with a GAPDH mRNA signal. This showed a dose-dependent increase in MCP-1 mRNA gene expression by TNF-α-treated mesangial cells: concentration of TNF-α, 0, 0.5, 1, 5, 25 ng/mL; respective arbitrary densitometric values were 0.11 ± 0.001, 0.73 ± 0.005 (P < 0.0001), 0.85 ± 0.006 (P = 0.003), 1.06 ± 0.006 (P < 0.0001), 1.09 ± 0.16 (P < 0.0003), 1.45 ± 0.004 (P < 0.0001); all P values were calculated by comparing the experimental values with control. At higher TNF-α concentrations (50 to 200 ng/mL), no further induction of mesangial cell MCP-1 mRNA expression was seen when compared with 25 ng/mL of TNF-α (data not shown). Although a significant induction of MCP-1 was noted at 30 min (arbitrary densitometric values for 30 min control, 0.07 ± 0.005; TNF-α, 0.22 ± 0.001; P = 0.004), the maximal effect of TNF-α on mesangial cell MCP-1 mRNA gene expression was observed at 3 h (arbitrary densitometric values for 3 h control, 0.13 ± 0.004; TNF-α, 1.24 ± 0.03, P = 0.0001) of incubation and the message remained stable at least up to 24 h of incubation (Figure 2, arbitrary densitometric values for 24 h control, 0.04 ± 0.0001; TNF-α, 1.0 ± 0.07; P = 0.0001). On the basis of these observations, mesangial cell-activation with TNF-α was performed for 3 h in all subsequent studies.

Additional studies were performed to identify the cellular signal transduction pathways associated with TNF-α-induced mesangial cell MCP-1 gene expression. Incubation of mesangial cells with PMA, an activator of PKC, for 3 h induced the steady-state mRNA expression of MCP-1 (Figure 3, Lane 3 as compared with Lane 1: arbitrary densitometric values for control, 0.17 ± 0.02; PMA, 1.17 ± 0.15; P = 0.003). The depletion of mesangial cell PKC by prolonged incubation with PMA (24 h) completely blocked the MCP-1 mRNA message and the signal was comparable to control cells without any activation (Figure 3, Lane 4 compared with Lane 3: arbitrary densitometric values for PKC-depletion, 0.30 ± 0.002; PMA, 1.17 ± 0.15; P = 0.005). Additional studies indicated that the activation of PKC-depleted mesangial cells with PMA did not induce mesangial cell MCP-1 expression, suggesting the regulatory role of PKC in mesangial cell

![Figure 1](image1.png)

**Figure 1.** Effect of TNF-α on murine mesangial cell steady-state mRNA expression of MCP-1 by Northern blot analysis. After incubation of mesangial cells with TNF-α (0 to 25 ng/mL) for 3 h, cell monolayers were washed with PBS and RNA isolated, electrophoresed, and transferred onto nylon membranes. Northern blots were hybridized with 32P-labeled murine cDNA probe for MCP-1 and then rehybridized with cDNA probe for GAPDH. Autoradiographs were developed by exposing blots to X-ray films. Northern blot is representative of three separate experiments.

![Figure 2](image2.png)

**Figure 2.** Effect of TNF-α on the murine mesangial cell time kinetics of steady-state MCP-1 mRNA expression by Northern blot analysis. Mesangial cells were incubated in the presence or absence of TNF-α for varying time periods (0.5 to 24 h) and cells were analyzed for MCP-1 mRNA expression as described in the Methods section. Northern blot is representative of four separate experiments.

![Figure 3](image3.png)

**Figure 3.** Role of PKC in basal and TNF-α-induced mesangial cell MCP-1 gene expression. Mesangial cells were incubated with the following substances: control (Lane 1), TNF-α for 3 h (Lane 2), PMA for 3 h (Lane 3), PMA for 24 h (Lane 4), PMA for 24 h and activation with TNF-α for 3 h (Lane 5), PMA for 24 h and activation with PMA for 3 h (Lane 6), TNF-α for 3 h (Lane 7), and TNF-α plus calphostin C for 3 h (Lane 8). All lanes in this figure were derived from a single gel and graphically rearranged for clarity. For more details, see Methods section. After incubation, mesangial cell monolayers were washed, RNA isolated, and used for Northern blot analysis. The Northern blot is representative of four separate experiments.
MCP-1 gene expression (Figure 3, Lane 6 as compared with Lane 4: arbitrary densitometric values for PKC depletions, 0.30 ± 0.002; PKC-depletion + PMA, 0.50 ± 0.08). However, the incubation of PKC-depleted mesangial cells with TNF-α had no effect on the inhibition of mesangial cell MCP-1 mRNA signal and the message was similar to TNF-α activation of non-PKC-depleted cells (Figure 3, Lanes 5 and 2: arbitrary densitometric values for PKC-depletion + TNF-α, 0.75 ± 0.33; TNF-α, 1.14 ± 0.13). Further confirmatory experiments regarding the role of PKC on TNF-α-induced mesangial cell MCP-1 expression were performed using calphostin C, a specific PKC inhibitor. Data obtained from these studies indicated that the preincubation of mesangial cells with calphostin C did not significantly inhibit TNF-α-induced mRNA expression of MCP-1 when compared with the stimulation with TNF-α (Figure 3, Lanes 8 and 7, respectively: arbitrary densitometric values for TNF-α, 1.14 ± 0.13; TNF-α + calphostin C, 1.083 ± 0.03).

The role of PTK in mesangial cell mRNA expression of MCP-1 was examined by utilizing various PTK inhibitors (e.g., genistein, herbimycin, or tyrphostin). As shown in Figure 4, preincubation of mesangial cells with genistein, herbimycin, or tyrphostin caused a marked inhibition of TNF-α-induced MCP-1 expression (Figure 4, Lanes 3, 4, and 5 as compared with Lane 2: arbitrary densitometric values for TNF-α, 2.17 ± 0.12; genistein + TNF-α, 0.14 ± 0.01; herbimycin + TNF-α, 0.15 ± 0.02; tyrphostin + TNF-α, 0.13 ± 0.006; P < 0.0001 for all experimental values compared with TNF-α control).

We further investigated the role of cAMP as a second messenger in mesangial cell MCP-1 mRNA expression by utilizing cAMP generating substances (i.e., pertussis toxin or isoproterenol) and the cell permeable analog of cAMP (dbcAMP). Preincubation of mesangial cells with pertussis toxin, isoproterenol, or dbcAMP for 3 h did not cause any induction in mesangial cell MCP-1 message when compared with controls (Figure 5, Lanes 3, 4, 5 as compared with Lane 1, arbitrary densitometric values for control, 0.70 ± 0.005; pertussis toxin, 0.55 ± 0.03; isoproterenol, 0.30 ± 0.002; dbcAMP, 0.30 ± 0.03). Furthermore, studies were designed to examine whether an increase in intracellular cAMP would have any effect on TNF-α-induced mesangial cell MCP-1 mRNA expression. Coincubation of mesangial cells with dbcAMP and TNF-α for 3 h resulted in an inhibition of TNF-α-induced mesangial cell MCP-1 message (Figure 5, Lane 6 as compared with Lane 2: arbitrary densitometric values for TNF-α + dbcAMP, 0.13 ± 0.01; TNF-α, 1.22 ± 0.16; P = 0.002). Additional experiments were performed to examine the effects of cAMP-generating substances in the presence of IBMX, a phosphodiesterase inhibitor, to determine whether cAMP-degradation may have any influence on mesangial cell MCP-1 expression. The data from these experiments showed that the incubation of mesangial cells with cAMP-generating substances also did not stimulate mesangial cell MCP-1 mRNA transcripts in the presence of IBMX (data not shown).

To examine whether de novo protein synthesis is necessary for TNF-α-induced MCP-1 gene expression, mesangial cells were preincubated with cycloheximide (10 µg/mL) for 2 h before the activation of these cells with TNF-α for 3 h. The inhibition of cellular protein synthesis by cycloheximide inhibited TNF-α-induced mesangial cell MCP-1 gene expression without altering GAPDH message (Figure 6: arbitrary densitometric values for TNF-α, 1.05 ± 0.11; TNF-α + cycloheximide, 0.070 ± 0.002; P = 0.0009).

Because the results of the Northern hybridization indicated that the activation of mesangial cells with TNF-α stimulate the steady-state mRNA expression of MCP-1, further studies were performed to examine the ability of TNF-α-mediated secretion of MCP-1 to stim-
ulate the migration of monocytes by using a chemotaxis assay. Conditioned media obtained by incubating mesangial cells with TNF-α increased monocyte migration in a dose-dependent manner (Figure 7). The addition of neutralizing anti-MCP-1 to the chemotaxis chamber attenuated the migration of monocytes during basol as well as TNF-α-activated conditions (Figure 7). Additional experiments were performed to examine the effect of exogenous TNF-α on monocyte chemotaxis. Results from these experiments indicated that the addition of TNF-α (25 ng/mL) to the chemotaxis incubation media did not alter monocyte migration when compared with the appropriate controls (data not shown). These data indicated that any exogenous TNF-α present in the conditioned media used for determining monocyte chemotaxis was not associated with biological activity to induce monocyte migration.

**DISCUSSION**

In this study, we have delineated mesangial cell signal transduction pathways involved in TNF-α-mediated MCP-1 expression and monocyte migration. Increased glomerular localization of MCP-1 mRNA message and protein was shown to be associated with monocyte/macrophage accumulation within the mesangium of human and experimental glomerular diseases (40,41). The chemotaxis of circulating mononuclear phagocytes within the mesangium, an early pathological cellular event seen in glomerular injury, often precedes the development of functional and structural changes associated with both immune and nonimmune-mediated renal injury (4–12). Furthermore, monocyte infiltration into the glomerulus has been shown to be associated with proteinuria and declining renal function in human subjects and experimental animals (6,42,43). In addition to the association between the increased accumulation of monocytes and glomerular injury, the depletion of renal cortical macrophages in experimental animals by essential fatty acid diet or by carefully timed systemic X-irradiation decreased the progression of glomerular disease suggesting a critical regulatory role for monocytes in the pathogenesis of renal disease (8,44). On the basis of both in vivo and in vitro studies, it was suggested that monocyte-mediated renal injury occurs, at least in part, through the enhanced synthesis and secretion of various cytoregulatory factors (e.g., cytokines, prostanoids, reactive oxygen species, proteolytic enzymes, etc.) that regulate glomerular cell proliferation, extracellular matrix production, and the functional and structural integrity of the glomerulus (4,45).

Although various endogenous mediators can initiate cellular activation within the glomerulus, TNF-α has been considered a primary proinflammatory cytokine involved in the pathogenesis of glomerular injury (46,47). Supporting evidence for the involvement of TNF-α in the pathogenesis of glomerulonephritis has been derived primarily from in vivo studies indicating that the administration of exogenous TNF-α or agents that induce the release of endogenous TNF-α (e.g., endotoxin) increases the severity of experimental glomerular injury (28). Furthermore, the use of specific neutralizing antibodies or receptor-antagonists to inhibit the action of TNF-α has been shown to significantly attenuate the progression of glomerular disease (28,48,49). Although these studies suggest a pathobiological role for TNF-α in the genesis of glomerulosclerosis, the role of this cytokine in the cellular responses within the glomerulus that activate signal transduction pathways and stimulate various cellular genes associated with monocyte infiltration are not clearly defined.

Because MCP-1 acts as a specific chemotaxiant
for the migration of monocytes into the glomerulus, we examined the cellular signal transduction pathways associated with TNF-α-mediated mesangial cell MCP-1 gene expression and monocyte migration. The activation of mesangial cells with TNF-α induced MCP-1 mRNA transcripts in a dose- and time-dependent manner. The results obtained from these studies supported earlier observations made in this and other laboratories that the activation of a variety of different cell types (including mesangial, smooth muscle, endothelial, tubular cells, etc.) in the presence of TNF-α can induce prototypic cytokinergic peptides (e.g., adhesion molecules, colony-stimulating factors, and monocyte chemoattractants) associated with monocyte adhesion and subsequent transmigration into the glomerulus (34,50–53). Additionally, the conditioned media obtained from TNF-α-activated mesangial cells increased monocyte migration as determined by a chemotaxis assay. Because the monocyte migration could be attenuated by neutralizing anti-MCP-1, these data suggested that MCP-1 was a primary factor responsible for the observed monocyte chemotactic activity induced by TNF-α-activation of mesangial cells. The pathobiological effects of the increased expression of MCP-1 associated with TNF-α-activation of mesangial cells may partially explain the increased glomerular adhesion and accumulation of monocytes seen in rabbits that received intravenous infusions of TNF-α or in nephrotoxic nephritis rats treated with exogenous TNF-α (49,55).

Although the cellular mechanisms that regulate glomerular cytokine synthesis and secretion are not clearly understood, it appears that TNF-α can be secreted by a variety of cells in response to either immune- or nonimmune-mediated stimuli, such as bacterial lipopolysaccharide, interferon-γ, and immune complexes (28,46). We have recently observed that atherogenic lipoproteins may also induce mesangial cell TNF-α expression (H. Ha, R. Pal, V.S. Kamanna, M.A. Kirschenbaum, unpublished observations). Thus, the pleiotropic nature of TNF-α derived from activated resident glomerular cells and from infiltrating mononuclear cells may synergistically initiate a cascade of cellular events culminating in the development of glomerular injury.

Although TNF-α receptors have not been clearly shown to possess intrinsic protein kinase activity, the phosphorylation of several distinct proteins in various cell types was shown to occur within minutes after the exposure of cells to TNF-α (47,56). These cellular signals in response to TNF-α activation are likely the result of the activation of several major cellular kinases including PKC, cAMP-dependent protein kinase A, PTK, and mitogen-activated protein kinases. Despite these studies, relatively little is known of the TNF-α-mediated signal transduction pathways involved in mesangial cell MCP-1 expression.

Utilizing gene analysis techniques, endothelial cells were shown to exhibit a phorbol ester-responsive element at the 5′-flanking region of the MCP-1 gene, suggesting an involvement of PKC in the regulation of MCP-1 gene expression (57). In agreement with these studies, we have also shown in the study presented here that the activation of mesangial cells with PMA, an activator of PKC, stimulated mRNA transcripts for MCP-1 indicating the regulatory role of PKC in MCP-1 gene expression. Additionally, the depletion of mesangial cell PKC by prolonged incubation with PMA nearly completely inhibited MCP-1 message further confirming the role of PKC in mesangial cell MCP-1 expression. Prolonged treatment with PMA in endothelial and other cell types resulted in the down regulation of PKC and a concomitant loss of phorbol ester responsiveness that rendered MCP-1 gene expression insensitive to PMA stimulation (58). Similarly, we have noted that mesangial cells treated with PMA for a prolonged period of time were insensitive to PMA activation, suggesting that the phorbol ester-related mechanisms leading to MCP-1 gene expression were also operating in mesangial cells. These PKC-desensitized cells, unresponsive to PMA stimulation, were used to distinguish between PKC-dependent or PKC-independent effects of TNF-α-mediated mesangial cell MCP-1 gene expression. Utilizing this approach, we examined whether TNF-α-induced mesangial cell MCP-1 expression was mediated through PKC or through other PKC-independent mechanisms. The incubation of PKC-desensitized mesangial cells with TNF-α for 3 h did not inhibit MCP-1 mRNA transcripts when compared with TNF-α-activation of control mesangial cells. These data suggested that although PKC activation stimulated mesangial cell MCP-1 mRNA message, the TNF-α-induced MCP-1 mRNA transcripts were independent of PKC-mediated pathways. Similarly, recent studies in mesangial cells also showed PKC-independent mechanisms for interleukin-1 (IL-1)-induced MCP-1 expression (34). In contrast, TNF-α-mediated MCP-1 message appeared to be PKC-dependent in fibroblasts, liver fat-storing cells, and vascular endothelial cells (59,60), suggesting that mesangial cells may utilize PKC-independent pathways to regulate cytokine gene expression.

Because the activation of cells with inflammatory cytokines (including TNF-α and IL-1) stimulates protein tyrosine phosphorylation (61), we proposed that cellular mechanisms dependent on PTK may serve as second messenger pathways for TNF-α-induced mesangial cell MCP-1 expression. In the study presented here, the preincubation of mesangial cells with various PTK inhibitors markedly inhibited TNF-α-stimulated mesangial cell MCP-1 gene expression. These results indicated that mesangial cell MCP-1 expression in response to TNF-α activation was regulated through PTK-mediated pathways. In agreement with these observations, it has been shown that IL-1-induced mesangial cell MCP-1 expression was mediated through PTK pathways (34). Apart from the role of PTK in the ability of mesangial cells to produce monocyte chemoattractants, the activation of monocytes by various biologically active molecules (e.g., LTB₄, GM-
CSF, TNF-α, etc.) also appears to be mediated through PTK-dependent cellular pathways (62–64). Thus, cellular signal transduction pathways involving PTK may play a critical role in monocyte/macrophage pathobiology and activation processes including oxidative burst, adherence and chemotaxis and the upregulation of various cellular gene products associated with early inflammatory reactions (65–67).

We further examined the role of cAMP as a second messenger to stimulate mesangial cell MCP-1 expression. The preincubation of mesangial cells with pertussis toxin (a cAMP generating substance that catalyzes ADP-ribosylation of a subunit of the G protein and activates adenylate cyclase leading to elevated intracellular cAMP) did not stimulate mesangial cell MCP-1 mRNA expression. Additionally, isoproterenol, an activator of receptors coupled to G protein and adenylate cyclase, and dbcAMP, a cell permeable analog of cAMP, also had no effect on mesangial cell MCP-1 message. As intracellular cAMP may undergo degradation, further experiments were performed to examine the effect of cAMP-generating substances on mesangial cell MCP-1 expression in the presence of IBMX, a potent inhibitor of the phosphodiesterase that degrades cAMP. The data from these studies indicated that the presence of IBMX alone or IBMX plus cAMP generating substances did not influence mesangial cell MCP-1 signal when compared with their respective controls. Thus, the results from these studies indicated that increased mesangial cell cAMP had no stimulatory effect on MCP-1 steady-state mRNA expression. Because the basal mesangial cell MCP-1 steady-state mRNA message was minimally expressed, we examined whether cAMP would influence TNF-α-induced MCP-1 gene expression. The coinucubation of dbcAMP and TNF-α for 3 h with mesangial cells completely abolished the TNF-α-induced mesangial cell MCP-1 mRNA signal. Similarly, earlier studies also showed inhibitory effects of forskolin, a cAMP-generating substance, on TNF-α-induced mesangial cell MCP-1 mRNA expression (68). Taken together, these data indicate that cAMP has an inhibitory role in TNF-α-induced MCP-1 expression.

Apart from these signal transduction mechanisms, recent studies have indicated that reactive oxygen species can act as second messengers in TNF-α-induced MCP-1 and M-CSF expression (38). In this regard, Satriano et al. (38) showed that increased mesangial cell MCP-1 expression in response to TNF-α-activation can be attenuated by free-radical scavengers. These investigators also indicated that TNF-α-induced MCP-1 expression was inhibited by a NAPDH-dependent oxidase inhibitor, suggesting an involvement of reactive oxygen species in TNF-α-stimulated mesangial cell MCP-1 gene expression. Furthermore, Satriano and Schlondorff (69) reported that the increased reactive oxygen species in TNF-α-stimulated mesangial induced MCP-1 gene expression through activation of NF-κB, a transcriptional factor involved in gene transcription.

Additional studies examining the role of protein synthesis on TNF-α-induced mesangial cell MCP-1 expression indicated that the preincubation of mesangial cells with cycloheximide, an inhibitor of protein synthesis, completely blocked TNF-α-mediated increase in mesangial cell MCP-1 mRNA signal, suggesting that intact protein synthetic machinery was required for TNF-α-induced MCP-1 expression.

In summary, we have shown that the activation of mesangial cells with TNF-α stimulated MCP-1 mRNA transcripts and that the MCP-1 secreted into the culture media enhanced monocyte migration as assessed by the use of specific neutralizing antibodies. The TNF-α-induced mesangial cell MCP-1 gene expression was regulated by signal transduction pathways mediated by PTK but not PKC. Increasing intracellular cAMP by the use of activators of adenylate cyclase and a cAMP-regulated analog of cAMP did not induce basal mesangial cell MCP-1 expression. However, elevated intracellular cAMP induced by the incubation with dbcAMP completely inhibited TNF-α-induced mesangial cell MCP-1 mRNA message. On the basis of these and previous studies, we suggest that agents that augment intracellular cAMP may provide potential tools to downregulate the sustained expression of adhesion molecules and monocyte chemoattractants and limit monocyte recruitment and accumulation within the glomerulus in inflammatory cytokine-mediated glomerular injury.

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