MCP-1/CCR2 system is involved in high glucose-induced fibronectin and type IV collagen expression in cultured mesangial cells

Jehyun Park,1ª Dong-Ryeol Ryu,2* Jin Ji Li,1,3 Dong-Sub Jung,1 Seung-Jae Kwak,1 Sun Ha Lee,1 Tae-Hyun Yoo,1 Seung Hyeok Han,1 Jung Eun Lee,1 Dong Ki Kim,1 Sung Jin Moon,1 Kunhong Kim,4 Dae Suk Han,1 and Shin-Wook Kang1

1Department of Internal Medicine, College of Medicine, Yonsei University and 2Department of Internal Medicine, College of Medicine, Ewha Womans University, Seoul; 3Nephrology and Dialysis Unit, Department of Internal Medicine, The Affiliated Hospital, YanBian University Medical College, JiLin, China, and 4Department of Biochemistry and Molecular Biology, College of Medicine, Yonsei University, Seoul, Korea

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MCP-1/CCR2 system is involved in high glucose-induced fibronectin and type IV collagen expression in cultured mesangial cells. Am J Physiol Renal Physiol 295: F749–F757, 2008. First published June 25, 2008; doi:10.1152/ajprenal.00547.2007.—Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that plays an important role in the recruitment of macrophages. Although previous studies have demonstrated the importance of MCP-1 in the pathogenesis of diabetic nephropathy (DN) in terms of inflammation, the role of MCP-1 and its receptor (C-C chemokine receptor 2; CCR2) in extracellular matrix (ECM) accumulation under diabetic conditions has been largely unexplored. This study was undertaken to investigate the functional role of the MCP-1/CCR2 system in high glucose-induced ECM (fibronectin and type IV collagen) protein expression in cultured mesangial cells (MCs). Mouse MCs were exposed to medium containing 5.6 mM glucose (NG), NG + 24.4 mM mannitol (NG+M), or 30 mM glucose (HG) with or without mutant MCP-1 (mMCP-1), CCR2 small interfering (si)RNA, or CCR2 inhibitor (RS102895). To examine the relationship between MCP-1 and transforming growth factor (TGF)-β1, MCs were also treated with TGF-β1 (2 ng/ml) with or without mMCP-1 or CCR2 siRNA. Transient transfection was performed with Lipofectamine 2000 for 24 h. Cell viability was determined by an MTT assay, mouse and human MCP-1 and TGF-β1 levels by ELISA, and CCR2 and ECM protein expression by Western blotting. Transfections of mMCP-1 and CCR2 siRNA increased human MCP-1 levels and inhibited CCR2 expression, respectively. HG-induced ECM protein expression and TGF-β1 levels were significantly attenuated by mMCP-1, CCR2 siRNA, and RS102895 (P < 0.05). MCP-1 directly increased ECM protein expression, and this increase was inhibited by an anti-TGF-β1 antibody. In addition, TGF-β1-induced ECM protein expression was significantly abrogated by the inhibition of the MCP-1/CCR2 system (P < 0.05). These results suggest that an interaction between the MCP-1/CCR2 system and TGF-β1 may contribute to ECM accumulation in DN.

diabetic nephropathy; monocyte chemoattractant protein-1; transforming growth factor-β1; extracellular matrix

MONOCYTES/MACROPHAGES are the principle inflammatory cells found in the diabetic kidney (6, 9, 29). These cells are extravasculated from the bloodstream through a process mediated by chemokines secreted from resident glomerular cells. Chemokines are a family of chemotactic cytokines that induce the migration of various cell types, and to date >40 chemokines have been identified (31). Among them, monocyte chemoattractant protein (MCP)-1 is the most extensively studied chemokine. In the kidney, MCP-1 is expressed in mesangial cells (MCs) and tubular epithelial cells (22, 26) and is known to be involved in the pathogenesis of various renal diseases, including diabetic nephropathy. Previous studies have demonstrated that plasma MCP-1 levels are increased in type 1 diabetes with microalbuminuria (4) and that urinary levels of MCP-1 are also increased in accordance with the extent of albuminuria (1, 20). In addition, it has been reported that glomerular MCP-1 expression is increased in experimental diabetic rats and that this increase is associated with the number of infiltrated monocytes in the glomeruli (5, 6). Moreover, an angiotensin-converting enzyme inhibitor, angiotensin II type 1 receptor blocker, and aldosterone antagonist all ameliorate glomerular injury in experimental diabetic animals via the inhibition of MCP-1 expression and macrophage infiltration (13, 17). Taken together, these findings suggest that MCP-1 plays a critical role in the pathogenesis of diabetic nephropathy via inducing inflammatory cell infiltration.

On the other hand, several studies have demonstrated that cultured MCs express C-C chemokine receptor 2 (CCR2) (10, 16), a receptor of MCP-1, suggesting that MCP-1 may act directly on these renal cells. Recently, Giunti et al. (10) have shown that MCP-1 induces concentration-dependent ICAM-1 expression in cultured MCs, implying that MCP-1 may further increase the inflammatory response by increasing adhesion molecule expression. In addition, some investigators have provided evidence that MCP-1 may be directly involved in extracellular matrix (ECM) synthesis, using animal models of experimental glomerulonephritis and diabetic nephropathy (11, 30, 36). To our knowledge, however, there has not been a study that has looked at the functional role of the MCP-1/CCR2 system in high glucose-induced ECM protein expression. In this study, we investigated the role of the MCP-1/CCR2 system in high glucose-induced fibronectin and type IV collagen expression and the relationship between the MCP-1/CCR2 system and TGF-β1 in cultured MCs.

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MATERIALS AND METHODS

Cell cultures. Mouse MCs from an SV40 transgenic mouse (MESC-13) were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM (Invitrogen, Gaithersburg, MD) containing 5% FBS (Invitrogen). MCs were maintained and incubated at 37°C in humidified 5% CO₂ in air.

Inhibition of MCP-1/CCR2 system. In this study, three different methods were utilized to block the MCP-1/CCR2 system in cultured MCs. First, mutant MCP-1 (mMCP-1) was used to competitively inhibit native MCP-1 action. The mMCP-1 gene was constructed by a recombinant PCR using a wild-type human MCP-1 cDNA from human peritoneal mesothelial cells as a template, which was then cloned into the BamHI (5’) and XhoI (3’) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen) as described previously (40). Second, predesigned mouse CCR2 small interfering (si)RNA was purchased from Ambion (Austin, TX). The mMCP-1 gene and CCR2 siRNA were transfected with Lipofectamine 2000 according to the manufacturer’s protocol. Briefly, 6 μl of Lipofectamine 2000 was diluted in 1 ml of Opti-MEM I Reduced Serum Medium (Invitrogen), incubated for 15 min at room temperature, and mixed with a variable amount of mMCP-1 (final concentrations: 0.5 and 1.0 μg/ml) and CCR2 siRNA (final concentrations: 10, 25, and 50 nM). After 15-min incubation at room temperature, the mixture was added to each well of MCs, which were plated at a density of 5 × 10⁵ cells/well into six-well plates the day before, and the medium was changed after 24 h. Last, RS102895 (final concentrations: 1 and 10 μM, Sigma, St., Louis, MO), a specific chemical inhibitor of CCR2, was also used.

Transfected MCs were serum restricted for 24 h, after which the medium was replaced by serum-free DMEM containing normal glucose (NG; 5.6 mM), NG + mannitol (NG + M; 24.4 mM), or high glucose (HG; 30 mM). In addition, nontransfected MCs were cultured under NG, NG + M, or HG with or without RS102895 or anti-TGF-β1 antibody (25 μg/ml). Nontransfected MCs were also exposed to medium containing recombinant human MCP-1 (10 ng/ml, R&D Systems, Minneapolis, MN) or recombinant human TGF-β1 (2 ng/ml, R&D Systems). At 24 h after the media change, cells were harvested and the conditioned culture media were collected.

Methylthiazolyltetrazolium assay. To examine the cytotoxicity of the agents used in this study, MCs were cultured in 96-well culture plates, phenol red-free DMEM with 1 mg/ml of methylthiazolesetrazilum (MTT) added to each well after the experimental periods, and then incubated for 2 h at 37°C in humidified 5% CO₂ in air. Extraction buffer (20% SDS, 50% N,N-dimethylformamide, pH 4.7) was added to the wells, which were further incubated overnight at 37°C. Optical density (OD) was measured with a microplate reader (SpectraMax 340, Molecular Devices) at a wavelength of 562 nm. The OD of the NG cells was assigned a relative value of 100. The experiments were performed in triplicate.

ELISA. The levels of secreted MCP-1 and TGF-β1 under different experimental conditions were determined with conditioned culture media using commercial ELISA kits (R&D Systems) according to the manufacturer’s protocol, and each protein level was normalized with cell numbers. The experiments were performed in triplicate.

The kits for mouse and human MCP-1 were species specific and sensitive up to 2.0 and 5.0 pg/ml, respectively. On the other hand, the minimum sensitivity for mouse TGF-β1 of the kit used in this study was 4.6 pg/ml.

Western blot analysis. Harvested MCs lysed in SDS sample buffer [2% SDS, 10 mM Tris-HCl, pH 6.8, 10% (vol/vol) glycerol] were

Fig. 1. Human (A) and mouse (B) monocyte chemoattractant protein-1 (MCP-1) levels, assessed by ELISA, in mMCP-1-transfected mouse mesangial cells (n = 5). In mutant (m)MCP-1-transfected cells, human MCP-1 levels were significantly increased in a dose-dependent manner, while there was no difference in the levels of mouse MCP-1, suggesting that mMCP-1 was efficiently transfected. Transfection of an empty vector had no effects on the levels of mouse and human MCP-1. *P < 0.0001 vs. control.

Fig. 2. C-C chemokine receptor 2 (CCR2) protein expression, assessed by Western blotting, in CCR2 small interfering (si)RNA-transfected mouse mesangial cells (n = 5). CCR2 protein expression was significantly decreased in CCR2 siRNA-transfected cells in a dose-dependent manner, suggesting that transfected CCR2 siRNA efficiently inhibited the native expression of CCR2. In contrast, a negative control of CCR2 siRNA had no effect on CCR2 protein expression. *P < 0.05 vs. control.
used for CCR2, fibronectin, and type IV collagen protein expression. The samples were treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in a 10% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA). The membrane was then incubated in blocking buffer A (1× PBS, 0.1% Tween 20, and 8% nonfat milk) for 1 h at room temperature, followed by an overnight incubation at 4°C in a 1:1,000 dilution of polyclonal antibodies to CCR2, fibronectin, type IV collagen, or β-actin (Santa Cruz Biotechnology, Santa

Fig. 3. A: fibronectin and type IV collagen protein expression, assessed by Western blotting, in mouse mesangial cells exposed to 10 ng/ml MCP-1 (n = 5). Administration of 10 ng/ml MCP-1 significantly increased the protein expression of fibronectin in cultured mesangial cells, and this increase in fibronectin expression was significantly ameliorated by the inhibition of CCR2 with 10 nM CCR2 siRNA (siRNA) or 10 μM RS102895 (RS). *P < 0.05 vs. other groups. B–D: fibronectin and type IV collagen protein expression, assessed by Western blotting, in mouse mesangial cells exposed to high glucose (HG) with or without mMCP-1, CCR2 siRNA, or RS102895 (n = 5). The protein expression of fibronectin was 2.1-fold higher in mesangial cells cultured under HG conditions than in normal glucose (NG) cells, and this increase in fibronectin expression was abrogated in mMCP-1-transfected cells, CCR2 siRNA-transfected cells, and RS102895-treated cells. *P < 0.05 vs. other groups.

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Cruz, CA). The membrane was then washed once for 15 min and twice for 5 min in 1× PBS with 0.1% Tween 20. Next, the membrane was incubated in buffer A containing a 1:1,000 dilution of horseradish peroxidase-linked goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science, Arlington Heights, IL). The band densities were measured using TINA image software (Raytest, Straubenhardt, Germany), and the changes in the OD of treated cells relative to NG cells were used for analysis.

Statistical analysis. All values are expressed as means ± SE. Statistical analysis was performed using the statistical package SPSS for Windows Ver. 11.0 (SPSS, Chicago, IL). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the Mann-Whitney U-test. P values <0.05 were considered statistically significant.

RESULTS

MCP-1 and CCR2 levels in mMCP-1 and CCR2 siRNA-transfected MCs. The levels of mouse and human MCP-1 were measured in transfected MCs with different concentrations of mMCP-1 (0.5 or 1 µg/ml) to evaluate transfection efficiency. Human MCP-1 levels were significantly increased in mMCP-1-transfected MCs in a dose-dependent manner (P < 0.0001) (Fig. 1A), while there was no difference in the levels of mouse MCP-1 (Fig. 1B). Transfection of the empty vector did not affect the levels of mouse or human MCP-1.

Next, the efficacy of CCR2 siRNA transfection was evaluated by determining the protein expression of CCR2. As shown in Fig. 2, CCR2 protein expression was significantly decreased in CCR2 siRNA-transfected MCs in a dose-dependent manner.
(P < 0.05). In contrast, a negative control of the CCR2 siRNA had no effects on the expression of CCR2 protein.

The concentrations of mMCP-1 (0.5 and 1 μg/ml), CCR2 siRNA (10 and 50 nM), and RS102895 (1 and 10 μM) used in this study did not affect cell viability, which was assessed by an MTT assay (data not shown).

**MCP-1/CCR2 inhibition ameliorates HG-induced fibronectin and type IV collagen expression.** To clarify whether the MCP-1/CCR2 system is involved in HG-induced ECM protein expression, we first examined the effects of MCP-1 on fibronectin and type IV collagen protein expression in MCs. The administration of 10 ng/ml MCP-1 significantly increased the protein expression of fibronectin and type IV collagen in cultured MCs (P < 0.05), and this increase in ECM protein expression was significantly ameliorated by the inhibition of CCR2 with 10 nM CCR2 siRNA and 10 μM RS102895 (Fig. 3A), suggesting that MCP-1 directly induced fibronectin and type IV collagen expression in MCs. Administration of 10 ng/ml MCP-1 significantly increased the protein expression of fibronectin and type IV collagen in HG-stimulated MCs (P < 0.05), and this increase in ECM protein expression was significantly ameliorated by the inhibition of CCR2 with 10 nM CCR2 siRNA or 10 μM RS102895 (Fig. 3A), suggesting that MCP-1 directly induced fibronectin and type IV collagen expression. The protein expression of fibronectin and type IV collagen were 2.1- and 2.0-fold, respectively, higher in MCs cultured under HG conditions relative to NG cells (P < 0.05), and this HG-induced ECM protein expression was abrogated in mMCP-1-transfected MCs (Fig. 3B) and in CCR2 siRNA-transfected MCs (Fig. 3C). Treatment with 1 and 10 μM RS102895 also significantly inhibited fibronectin and type IV collagen protein expression in HG-stimulated MCs (P < 0.05) (Fig. 3D).

**MCP-1/CCR2 inhibition ameliorates HG-induced TGF-β1 levels.** Since TGF-β1 is the most well-known profibrogenic cytokine and its expression is increased under diabetic conditions, we attempted to elucidate whether the MCP-1/CCR2 system was involved in HG-induced TGF-β1 levels. First, the direct effects of MCP-1 on TGF-β1 levels were examined. The administration of 10 ng/ml MCP-1 in MCs significantly increased the levels of TGF-β1 in conditioned media (P < 0.05), and this increase in TGF-β1 concentrations was significantly abrogated by the inhibition of CCR2 with 10 nM CCR2 siRNA or 10 μM RS102895 (Fig. 3A), suggesting that MCP-1 directly induced TGF-β1 levels in MCs via CCR2. Next, we evaluated the role of the MCP-1/CCR2 system in HG-induced TGF-β1 levels. The levels of TGF-β1 in conditioned media were significantly increased by 142.6% compared with NG media, and this increase in TGF-β1 levels was significantly abrogated by the inhibition of CCR2 with 10 nM CCR2 siRNA or 10 μM RS102895 (Fig. 3B). The concentrations of TGF-β1 in conditioned media were significantly increased in HG-stimulated mesangial cells compared with those of NG cells, and this increase in TGF-β1 levels was significantly attenuated by 0.5 μg/ml mMCP-1 (Mu), 10 nM CCR2 siRNA (siRNA), and 10 μM RS102895 (RS). *P < 0.05 vs. other groups.

**DISCUSSION**

A recent study has provided evidence that MCP-1 is directly involved in ECM synthesis using a mouse model of experimental diabetic nephropathy and cultured human MCs (11). However, the role of the MCP-1/CCR2 system in high glucose-induced fibronectin and type IV collagen expression has never been explored. In this study, we demonstrate for the first time that the MCP-1/CCR2 system plays an important role in high glucose-induced fibronectin and type IV collagen synthesis in cultured MCs. In addition, the results of the present study did not show any additive or synergistic effect on ECM protein expression (Fig. 6).

**TGF-β1 inhibition ameliorates HG-induced MCP-1 concentrations.** The levels of MCP-1 in HG-conditioned media were significantly increased by 142.6% compared with NG media, and this increase in MCP-1 levels was significantly abrogated by treatment with a TGF-β1-neutralizing antibody (P < 0.05) (Fig. 7).

**TGF-β1 inhibition ameliorates MCP-1-induced fibronectin and type IV collagen expression.** The administration of 10 ng/ml MCP-1 significantly increased the protein expression of fibronectin and type IV collagen in cultured MCs (P < 0.05), and this increase in ECM protein expression was significantly abrogated by treatment with a TGF-β1-neutralizing antibody (P < 0.05) (Fig. 8).
show that the MCP-1/CCR2 system and TGF-β1 pathway are involved in ECM protein expression in high glucose-stimulated MCs and are regulated by each other.

Previous clinical and experimental studies have suggested that MCP-1 plays an important role in the pathogenesis of diabetic nephropathy through the recruitment and activation of monocytes/macrophages (1, 5, 20, 34), which are known to release lysosomal enzymes, nitric oxide, and TGF-β1 and, in turn, modify MC biology (24, 39). Since MCs are known to secrete MCP-1, many studies have focused on the expression of MCP-1 in MCs under diabetic conditions. High glucose and advanced glycation end products increased MCP-1 expression in cultured MCs (15, 37). ANG II, an important mediator in the pathogenesis of diabetic nephropathy, also stimulated MCP-1 expression in MCs (27). In addition, mechanical stretch, an in vitro model of glomerular hypertension, induced MCP-1 expression in MCs, and this increase of MCP-1 expression was ameliorated by an NF-κB inhibitor and rosiglitazone (12). In this study, the levels of MCP-1 were increased in high glucose-stimulated MCs, which was in accordance with the results of the aforementioned studies. Moreover, we demonstrated for the first time that the increase in MCP-1 levels might directly induce fibronectin and type IV collagen expression in MCs under diabetic conditions in an autocrine manner, which was in addition to the previously known direct proinflammatory effects of MCP-1 in MCs and tubular epithelial cells.

To inhibit the MCP-1/CCR2 system, three different methods were used in the present study. First, we used mMCP-1, an NH₂-terminal deletion mutant of the human MCP-1 gene (7ND). An in vivo study by Furuichi et al. (8) demonstrated that gene therapy using this mMCP-1 prevented macrophage infiltration and acute tubular necrosis in mice with renal ischemia-reperfusion injury. Administration of mMCP-1 also abrogated the infiltration of inflammatory cells and ECM accumulation in animals with renal fibrosis induced by protein overload (32) and unilateral ureteral obstruction (UUO) (33). The results of this study showed that fibronectin and type IV collagen expression was significantly decreased in mMCP-1-transfected cells exposed to high glucose, providing strong evidence that MCP-1 was directly involved in high glucose-induced ECM production. Second, CCR2 siRNA and RS102895, which is a specific chemical inhibitor of CCR2 and does not affect chemotaxis or postreceptor signaling of any kind, were used. MCP-1 is known to induce functional responses through dimerization with CCR2, a G protein-coupled receptor (25). Previous studies have revealed that inflammatory cell infiltration is significantly reduced in CCR2-deficient mice with renal injury induced by ischemia-reperfusion (7), UUO (18), and immune complex (21), suggesting that MCP-1 exerts its proinflammatory action via CCR2. The administration of RS102895 prevented renal injury induced by ischemia-reperfusion (7) and UUO (18), similar to the results of the experiments with CCR2 knockout mice. In the present study, there were also significant decreases in fibronectin and type IV collagen expression in high glucose-stimulated CCR2 siRNA-transfected MCs and in high glucose-stimulated MCs treated with RS102895, suggesting that MCP-1 mediated ECM protein expression under diabetic conditions via CCR2. On the other hand, two alternatively spliced forms of CCR2, CCR2A and
CCR2B, have been identified in humans (3). In addition, previous studies have demonstrated that functional differences exist between the two isoforms and that the two isoforms are expressed by different cell subsets in idiopathic inflammatory myopathies (2, 28). Based on these findings, there is a possibility that CCR2A and CCR2B may have a different role in the pathogenesis of human diabetic nephropathy, but we could not verify it because mouse MCs were used in this study.

TGF-β1 is a well-known mediator of ECM accumulation in diabetic nephropathy (14). Indeed, previous studies have shown that there may be an intrinsic regulatory loop between MCP-1 and TGF-β1 in resident glomerular cells (30, 36). Schneider et al. (30) demonstrated that the increases in type IV collagen and TGF-β1 mRNA and protein expression in glomeruli isolated from rats with experimental glomerulonephritis were abrogated by the neutralization of MCP-1. In another study, the increase in glomerular TGF-β1 expression in a model of glomerular immune injury was also inhibited by the administration of anti-MCP-1 antiserum in the isolated, perfused kidney, which was performed to minimize infiltrating inflammatory cells (36). Moreover, a recent study by Giunti et al. (11) revealed that MCP-1 directly increased fibronectin production and TGF-β1 levels in cultured MCs and that the increase in fibronectin levels was abrogated by an anti-TGF-β1-blocking antibody. These findings suggest that MCP-1 may have a direct profibrogenic effect through TGF-β1 induction. In this study, we also found that MCP-1 induced fibronectin and type IV collagen expression. However, addition of TGF-β1 did not exert any additive or synergistic effect on MCP-1-induced ECM protein expression, suggesting that MCP-1 and TGF-β1 are closely interrelated in ECM synthesis. In addition, the results of this study showed for the first time that the MCP-1/CCR2 system mediated high glucose-induced TGF-β1 expression.

Furthermore, TGF-β1 is known to modulate MCP-1 expression in various cells. TGF-β1 induced MCP-1 mRNA and
protein expression in human synovial cells via the extracellular signal-regulated kinase pathway (38), while *Pneumocystis* stimulated MCP-1 production by alveolar epithelial cells through a c-Jun NH2-amino terminal kinase-dependent mechanism (35). MCP-1 expression was also increased in proximal tubular cells and MCs by TGF-β1 (19, 23). Meanwhile, the administration of an anti-TGF-β1 antibody in the isolated, perfused rat kidney in the absence of infiltrating inflammatory cells aggravated the increase in glomerular MCP-1 in experimental glomerulonephritis (36). Although the reasons for these discrepant effects of TGF-β1 on MCP-1 expression remain unclear, differences in experimental conditions (in vitro vs. in vivo) may contribute. The present study revealed an increase in MCP-1 expression by TGF-β1, which was consistent with the previous MC studies. In addition, TGF-β1-induced fibronectin and type IV collagen expression was mediated by the MCP-1/CCR2 system and MCP-1-induced ECM production was mediated by TGF-β1. Taken together, we propose that a close interaction between the MCP-1/CCR2 system and TGF-β1 may contribute to increased ECM synthesis in MCs under diabetic conditions.

GRANTS

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Fig. 8. Fibronectin and type IV collagen protein expression, assessed by Western blotting, in mouse mesangial cells exposed to MCP-1 with or without a TGF-β1-neutralizing antibody (*n* = 5). Administration of 10 ng/ml MCP-1 significantly increased the protein expression of fibronectin in cultured mesangial cells, and this increment in fibronectin expression was significantly abrogated by treatment with a TGF-β1-neutralizing antibody. *P* < 0.05 vs. other groups.


