Effect of the Monocyte Chemoattractant Protein-1/CC Chemokine Receptor 2 System on Nephrin Expression in Streptozotocin-Treated Mice and Human Cultured Podocytes

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OBJECTIVE—Monocyte chemoattractant protein-1 (MCP-1), a chemokine binding to the CC chemokine receptor 2 (CCR2) and promoting monocyte infiltration, has been implicated in the pathogenesis of diabetic nephropathy. To assess the potential relevance of the MCP-1/CCR2 system in the pathogenesis of diabetic proteinuria, we studied in vitro if MCP-1 binding to the CCR2 receptor modulates nephrin expression in cultured podocytes. Moreover, we investigated in vivo if glomerular CCR2 expression is altered in kidney biopsies from patients with diabetic nephropathy and whether lack of MCP-1 affects proteinuria and expression of nephrin in experimental diabetes.

RESEARCH DESIGN AND METHODS—Expression of nephrin was assessed in human podocytes exposed to rh-MCP-1 by immunofluorescence and real-time PCR. Glomerular CCR2 expression was studied in 10 kidney sections from patients with overt nephropathy and eight control subjects by immunohistochemistry. Both wild-type and MCP-1 knockout mice were made diabetic with streptozotocin. Ten weeks after the onset of diabetes, albuminuria and expression of nephrin, synaptopodin, and zonula occludens-1 were examined by immunofluorescence and immunoblotting.

RESULTS—In human podocytes, MCP-1 binding to the CCR2 receptor induced a significant reduction in nephrin both mRNA and protein expression via a Rho-dependent mechanism. The MCP-1 receptor, CCR2, was overexpressed in the glomerular podocytes of patients with overt nephropathy. In experimental diabetes, MCP-1 was overexpressed within the glomeruli and the absence of MCP-1 reduced both albuminuria and downregulation of nephrin and synaptopodin.

CONCLUSIONS—These findings suggest that the MCP-1/CCR2 system may be relevant in the pathogenesis of proteinuria in diabetes. ***Diabetes* 58:2109–2118, 2009**

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Diabetic nephropathy is characterized by increased glomerular permeability to proteins (1). Recently, much attention has been paid to the role of podocyte injury in glomerular diseases, including diabetic nephropathy (2,3), but the precise molecular mechanisms underlying the development of diabetic proteinuria remain unclear.

The slit diaphragm, a junction connecting foot processes of neighboring podocytes, represents the major restriction site to protein filtration (4). Mutations of the gene encoding for nephrin, a key component of the slit diaphragm, are responsible for the congenital nephrotic syndrome of the Finnish type (5). Furthermore, a link between a reduction in nephrin expression and proteinuria has been also reported in acquired proteinuric conditions, including diabetic nephropathy (6–8), and studies in patients with incipient diabetic nephropathy have demonstrated that nephrin downregulation occurs in an early stage of the disease (9).

A number of factors, including high glucose, advanced glycation end products, and hypertension play a role in the pathogenesis of diabetic nephropathy (10). In addition, monocyte chemoattractant protein-1 (MCP-1), a potent mononuclear cell chemoattractant, is overexpressed within the glomeruli in experimental diabetes (11,12) and has been recently implicated in both functional and structural abnormalities of the diabetic kidney (13).

MCP-1 binds to the cognate CC chemokine receptor 2 (CCR2), which is predominantly expressed on monocytes (14), and MCP-1–driven monocyte accrual is considered the predominant mechanism whereby MCP-1 contributes to the glomerular damage. However, the CCR2 receptor has also been shown both in vitro (15,16) and in vivo (17–19) in other cell types besides monocytes, and we have recently demonstrated that both mesangial cells and glomerular podocytes express a functionally active CCR2 receptor (20–22).

To assess the potential relevance of the MCP-1/CCR2 system in the pathogenesis of diabetic proteinuria we studied in vitro if MCP-1 binding to the CCR2 receptor modulates nephrin, expression in podocytes. Moreover, we investigated in vivo if glomerular CCR2 expression is altered in kidney biopsies from patients with diabetic nephropathy and whether lack of MCP-1 affects proteinuria and/or expression of nephrin in experimental diabetes.

RESEARCH DESIGN AND METHODS

All materials were purchased from Sigma-Aldrich (St. Louis, MO) and DAKO (Glostrup, Denmark) unless otherwise stated.

In vitro study

Cell culture. Immortalized human podocytes were established, characterized, and cultured as previously described (7,22). Cells retained their phenotypic characteristics, including expression of nephrin, a specific marker of differentiated podocytes, which was detectable in all cells. Podocyte expression of the CCR2 receptor was assessed by immunoblotting before the study, as we have previously reported (20).

mRNA expression. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). Two micrograms of total RNA were reverse transcribed into cDNA using avian myeloblastosis virus (AMV) reverse transcriptase and poly-d(T) primers. Human nephrin, mouse nephrin, and mouse MCP-1 mRNA expression were analyzed by real-time PCR using predeveloped TaqMan reagents (Applied-Biosystems). Fluorescence for each cycle was analyzed quantitatively and gene expression normalized relative to the expression of the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase and hypoxanthine-phosphoribosyl transferase.

Immunofluorescence. Cells, fixed in 3.5% paraformaldehyde, were incubated with either a guinea pig anti-nephrin or a rabbit anti-synaptopodin (Progen Biotechnik, Heidelberg, Germany) antibody. After rinsing, fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (SantaCruz Biotecnology, Santa Cruz, CA) were added. Fluorescent intensity was assessed on six microscopic fields (~100 cells) by digital analysis (Windows MicroImage, version 3.4; CASTI Imaging) on images obtained using a low-light video camera (Leica-DC100). The background fluorescence was subtracted by digital image analysis. The results, corrected for cell density, were expressed as relative fluorescence intensity (RFI) on a scale from 0 (fluorescence of background) to 255 (fluorescence of standard filter).

Rho-kinase activity. Rho-kinase (ROCK) activity was assessed by determination of the phosphorylation state of myosin phosphatase target subunit 1 (MYPT1), a downstream target of ROCK (23). Cells were lysed in radioimmunoprecipitation assay buffer containing protease/phosphatase inhibitors. Total protein concentration was determined using the DC-Protein Assay (Biorad). Proteins were separated and electrotransferred and subsequently probed with an anti–phospho-MBS/MYPT-Thr^{853} antibody (Cyclex). After detection by enhanced chemiluminescence, membranes were stripped and reprobed for total MYPT using a rabbit anti-MYPT antibody (SantaCruz Biotechnology).

Human study

Human biopsies. The study was performed on 10 renal biopsies from diabetic patients with overt nephropathy (persistent proteinuria >0.5g/24 h) and eight control subjects obtained from normal kidney portions from patients who underwent surgery for hypernephromas and did not have proteinuria or glomerular abnormalities, as detected by light and immunofluorescence microscopy. The study was approved by the ethical committee of Genoa University, the procedures were in accordance with the Helsinki Declaration, and informed consent was obtained from all subjects. Patient biopsies presented classic histological features of diabetic nephropathy, and those with other patterns of injury, such as vascular or interstitial lesions without glomerular diabetic damage, were excluded. Control subjects were selected to be comparable for age and sex, and individuals with diabetes and/or hypertension were excluded. Hypertension was defined as a blood pressure ≥140/90 mmHg on at least three different occasions. Diabetic retinopathy was assessed by direct funduscopic examination. Twenty-four-hour urinary protein content was measured using the pyrogallol-red method in three separate urine collections, plasma creatinine by the kinetic Jaffé method, and A1C by ion-exchange liquid chromatography. Creatinine clearance was estimated using the Cockcroft-Gault formula (24).

CCR2 protein expression and localization. Immunohistochemical staining was performed on 4-μm paraffin sections of formalin-fixed tissue. After antigen retrieval in citrate buffer, endogenous peroxidase activity quenching with 3% H_2O_2, and blocking with avidin-biotin and 3% BSA, sections were incubated with a rabbit monoclonal anti-CCR2 antibody (Epitomics, Burlingame, CA) and the specific staining detected using the LSAB+ system-HRP. Sections were visualized with an Olympus-Bx4I microscope. Normal spleen sections served as positive control. Glomerular immunostaining was quantified by a computer-aided image analysis system (Qwin; Leica). All glomeruli in the sections were analyzed and results were expressed as percentage area of positive staining per glomerulus. Evaluations were performed by two investigators in a blinded fashion.

Double immunofluorescent staining was performed for CCR2 and synaptopodin, a specific podocyte marker (25). After blocking with 3% BSA, sections were incubated with a monoclonal anti-synaptopodin antibody (Progen Biotechnik) for 18 h at 4°C, washed, then incubated with a RPE-conjugated goat anti-mouse IgG-F(ab′)2 fragment. After washing and further blocking in 3% BSA, sections were incubated with the rabbit anti-CCR2 antibody for 18 h at 4°C, washed, incubated with a biotinylated swine anti-rabbit IgG for 1 h and then with FITC-conjugated streptavidin.

Study in experimental diabetes

Animals. MCP-1–intact (MCP-$1^{+/+}$) C57BL6/J and MCP-1–deficient (MCP-$1^{-/-}$) B6.129S4-Ccl2tm1Rol/J mice from Jackson Laboratories (Bar Harbor, ME) were maintained on a normal rodent diet under standard animal house conditions. Diabetes (blood glucose >250 mg/dl) was induced in both MCP-$1^{+/+}$ and MCP-$1^{-/-}$ mice, aged 8 weeks and weighing ~22 g, by intraperitoneal injections of streptozotocin (STZ)-citrate buffer (55 mg/kg body weight per day) for 5 consecutive days (26). Mice sham injected with sodium citrate buffer were used as controls. Groups of MCP-$1^{+/+}$ (n = 6) and MCP-$1^{-/-}$ (n = 5) diabetic mice with equivalent blood glucose levels and control nondiabetic MCP-$1^{+/+}$ (n = 9) and MCP-$1^{-/-}$ mice (n = 4) were studied in parallel. Blood glucose obtained via saphenous vein sampling between 12:00 P.M. and 1:00 P.M. on alert 4-h–fasted animals was measured using a glucometer (Glucocard G meter; Menarini Diagnostics). Before being killed, mice were placed in individual metabolic cages for a period of 18 h and urinary albumin concentration measured by a mouse albumin enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Montgomery, TX). After 10 weeks of diabetes, mice were killed under anesthesia by exsanguination via cardiac puncture. The kidneys were rapidly dissected out and weighed. The right kidney was frozen in liquid nitrogen and then stored at −80°C for mRNA analysis. The left kidney was fixed in 10% PBS-formalin at room temperature then paraffin embedded for light microscopy. Glycated hemoglobin was measured in whole-blood samples obtained via cardiac puncture at the time of death by quantitative immunoturbidimetric latex determination (Sentinel Diagnostic, Milan, Italy).

Glomerular isolation. Glomeruli were isolated immediately after mice were killed, using the Dynabeads method from Takemoto et al. (27). Briefly, anesthetized mice were perfused with 8×10^7 surface-inactivated Dynabeads (Invitrogen). The kidneys, removed and minced, were digested in a collagenase A solution containing 100 units/ml DNase I (Roche Diagnostics, Milan, Italy), then passed twice through a cell strainer. The cell suspension was collected by centrifugation, then glomeruli containing Dynabeads were gathered by the magnetic particle concentrator and washed. The procedure of isolation and washing was repeated (~6–8 times) until no tubular contamination was found as assessed under light microscopy.

Nephrin, synaptopodin, and zonula occludens-1 protein expression. After antigen retrieval and blocking 4-μm kidney paraffin sections were incubated with primary guinea pig anti-nephrin, or monoclonal anti-synaptopodin (Progen Biotechnik), or rabbit anti-zonula occludens (ZO)-1 antibodies (Zymed Laboratories), followed by incubation with secondary FITC-conjugated antibodies against guinea pig IgG, rabbit IgG, or mouse IgG-F(ab′)2 fragment. After counterstaining with DAPI, sections were digitized and quantitated as described above. On average, 20 randomly selected hilar glomerular tuft cross-sections were assessed per mouse. Results were calculated as percentage positively stained tissue within the glomerular tuft. Fluorescence color images were also obtained as TIF files by a confocal laser-scanning microscope LSM-510 (Carl Zeiss, Oberkochen, Germany).

Western blotting. Renal cortex specimens were homogenized in either Laemmli buffer (nephrin, ZO-1) or Tris (20 mmol/l, 500 mmol/l NaCl, pH 7.5) lysis buffer containing 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 5 mmol/l EDTA, and protease inhibitors (synaptopodin). Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. Following blocking in 5% nonfat milk in Tris-buffered saline, membranes were incubated with primary antibodies against nephrin (Progen Biotechnik), synaptopodin (Synaptic Systems), or ZO-1 (Zymed) overnight at 4°C. After washing, secondary anti-rabbit/mouse horseradish peroxidase–conjugated antibodies were added for 1 h. Detection was performed by enhanced chemiluminescence (Amersham) and band intensity quantified by densitometry.

Electron microscopy. Renal cortex specimens were fixed in 3% glutaraldehyde in cacodylate buffer for 2 h, postfixed in 1% osmium tetroxide for 1 h, dehydrated in graded ethanol, washed in acetone, and embedded in Epon 812. Ultrathin sections for ultrastructural examination were stained with uranylacetate and lead citrate and examined with a transmission electron microscope (JEM 100 CX-II; JEOL, Tokyo, Japan). Two to three animals per group were used for the analysis.

Data presentation and statistical analysis. The number of independent experiments, carried out in at least triplicate, is reported in the legend to figures. Data, presented as means ± SE, geometric mean (25–75% percentile), or fold change over control, were analyzed by Student's t test or ANOVA, as appropriate. Newman-Keuls and Pearson tests were used for post hoc comparisons and correlation analysis, respectively. $P < 0.05$ was considered significant.

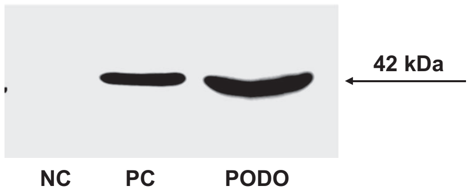

FIG. 1. The CCR2 receptor is expressed by human podocytes. CCR2 protein expression was studied in human cultured podocytes by immunoblotting as described in RESEARCH DESIGN AND METHODS. Total proteins were separated by SDS gel electrophoresis, transferred to nitrocellulose membranes, and probed for the CCR2 receptor by immunoblotting using a rabbit anti-human CCR2 antibody. A representative immunoblot is shown of the specific band for CCR2 at ~42 kDa. NC: negative control obtained by omitting the primary antibody. PC: positive control of total protein extracts from the monocyte cell line THP-1. PODO: total protein extracts from human podocytes.

RESULTS

In vitro study

The CCR2 receptor is constitutively expressed by cultured human podocytes. We have recently demonstrated that human cultured podocytes express the CCR2 receptor at both mRNA and protein level by RT-PCR, cytofluorimetry, and immunocytochemistry (22). This was further confirmed in the podocytes used in this study by Western blotting. Immunoblotting showed a band migrating at ~42 kDa, corresponding to the reported molecular weight of CCR2, and a band of identical molecular weight was seen in protein extracts from THP-1, a monocyte cell line used as positive control (Fig. 1).

Effect of rh-MCP-1 on nephrin mRNA expression. We next tested whether exposure to rh-MCP-1 alters nephrin mRNA expression in cultured podocytes. Analysis by quantitative real-time PCR demonstrated that exposure to rh-MCP-1 at a concentration of 10 ng/ml induced a significant reduction in nephrin mRNA levels after 2 h, with a return to baseline by 4 h (Fig. 2*A*). Endotoxin contamination of the rh-MCP-1 preparation was excluded by the Limulus test assay. Cell viability was comparable in podocytes exposed to either rh-MCP-1 or vehicle as assessed by Trypan Blue exclusion test (98 vs. 99%).

Effect of rh-MCP-1 on nephrin protein expression. Podocytes were exposed to rh-MCP-1 10 ng/ml for 2, 4, 6, 12, and 24 h and to increasing rh-MCP-1 concentrations (0.1, 1, 10, and 100 ng/ml) for 4 h, then nephrin expression assessed by immunofluorescence. Addition of rh-MCP-1 induced a significant decrease over control in nephrin protein expression after 2 h that was sustained up to 24 h and peaked at 4–6 h (Fig. 2*B*, *D*, and *E*). In dose-response experiments, we found that MCP-1 induced nephrin downregulation in a concentration-dependent manner with a minimum effective concentration of 0.1 ng/ml and a maximal response at 10 ng/ml (Fig. 2*C*). On the contrary, as shown in Fig. 3, addition of rh-MCP-1 did not alter synaptopodin protein expression.

MCP-1 induced nephrin downregulation via a CCR2-ROCK-dependent pathway. To test whether nephrin downregulation was a specific effect of MCP-1 occurring via the CCR2 receptor, experiments were repeated either in the presence or in the absence of a highly specific inhibitor of CCR2 signaling, RS102895 (RS 6 μmol/l), added 60 min before rh-MCP-1 (10 ng/ml). RS, a member of the spiropiperidine family, interacts specifically with the CCR2 binding domain and has no significant inhibitory activity on other chemokine receptors (28). RS completely prevented MCP-1–induced downregulation of nephrin mRNA at 2 h and of nephrin protein at 4 h (Fig. 2*G* and *H*). Similarly, the addition of Y27632 (10 μmol/l), a pyridine derivative with a specific inhibitory activity on the ROCK family of protein kinases (29), also abolished MCP-1–induced nephrin mRNA and protein downregulation (Fig. 2*G* and *H*). Furthermore, podocyte exposure to MCP-1 (10 ng/ml) induced a rapid and transient increase in phospho-MYPT1, a specific ROCK substrate (23), and the significant 2.5-fold rise in phospho-MYPT1 levels observed at 10 min was completely abolished by the ROCK inhibitor Y27632 (Fig. 2*F*). Taken together these results indicate that nephrin diminution in response to MCP-1 occurred via a CCR2-ROCK–dependent pathway.

Human study

The CCR2 receptor is overexpressed by glomerular podocytes in patients with diabetic nephropathy. To assess the in vivo relevance of our findings and to exclude that CCR2 receptor expression was solely related to in vitro culture conditions, we studied glomerular CCR2 expression in renal sections from 10 type 2 diabetic patients with overt diabetic nephropathy and 8 control subjects. Clinical and laboratory characteristics of both study patients and controls are showed in Table 1.

In normal renal cortex only few glomerular cells per kidney biopsy, predominantly podocytes and mesangial cells, stained positively for CCR2, as assessed by immunohistochemistry (Fig. 4*A* and *D*). Specificity of the antibody binding was confirmed by disappearance of the signal when the antibody was preabsorbed with a 10-fold excess of control peptide (Fig. 4*C*).

In patients with diabetic nephropathy, CCR2 protein expression was greatly enhanced (Fig. 4*B* and *E*) and semi-quantitative analysis showed that the percentage positive area was ninefold greater than in the controls (19.7 ± 2.94 vs. 2.0 ± 0.43, $P < 0.001$). Furthermore, there was a positive correlation between staining for CCR2 and extent of proteinuria ($P < 0.001$, $r = 0.89$), whereas no correlation was found with other clinical parameters, such as age, diabetes duration, A1C, and creatinine clearance. To clarify which glomerular cell type overexpressed CCR2, double-labeling immunofluorescence was performed in patients with diabetic nephropathy using both CCR2 and synaptopodin, a specific podocyte marker (25). The CCR2 receptor was primarily expressed by glomerular podocytes as CCR2 staining showed a comma-like pattern along the glomerular capillary wall (Fig. 4*B*) and the positive staining for synaptopodin (Fig. 4*G*) colocalized with the CCR2 staining (Fig. 4*H*).

In vivo study

Clinical parameters. As shown in Table 2, after 10 weeks of diabetes intact and deficient MCP-1 mice showed a similar degree of glycemic control. A significant decrease in body weight and a significant increase in kidney weight-to-body weight ratio were observed in the diabetic mice, while these parameters were similar in diabetic MCP-1 intact and deficient mice. The induction of diabetes resulted in a significant increase in albuminuria in MCP-$1^{+/+}$ mice, which was significantly reduced in mice lacking MCP-1. On the contrary, albuminuria was comparable in nondiabetic MCP-$1^{+/+}$ and MCP-$1^{-/-}$ mice.

Glomerular MCP-1 mRNA levels are enhanced in experimental diabetes. There was a significant sixfold increase in glomerular MCP-1 mRNA levels in diabetic mice as compared with controls as assessed by quantitative real-time PCR (diabetic mice: 9.46 ± 2.20; control subjects: $1.49 \pm 0,49$, $P < 0.05$ diabetics vs. control

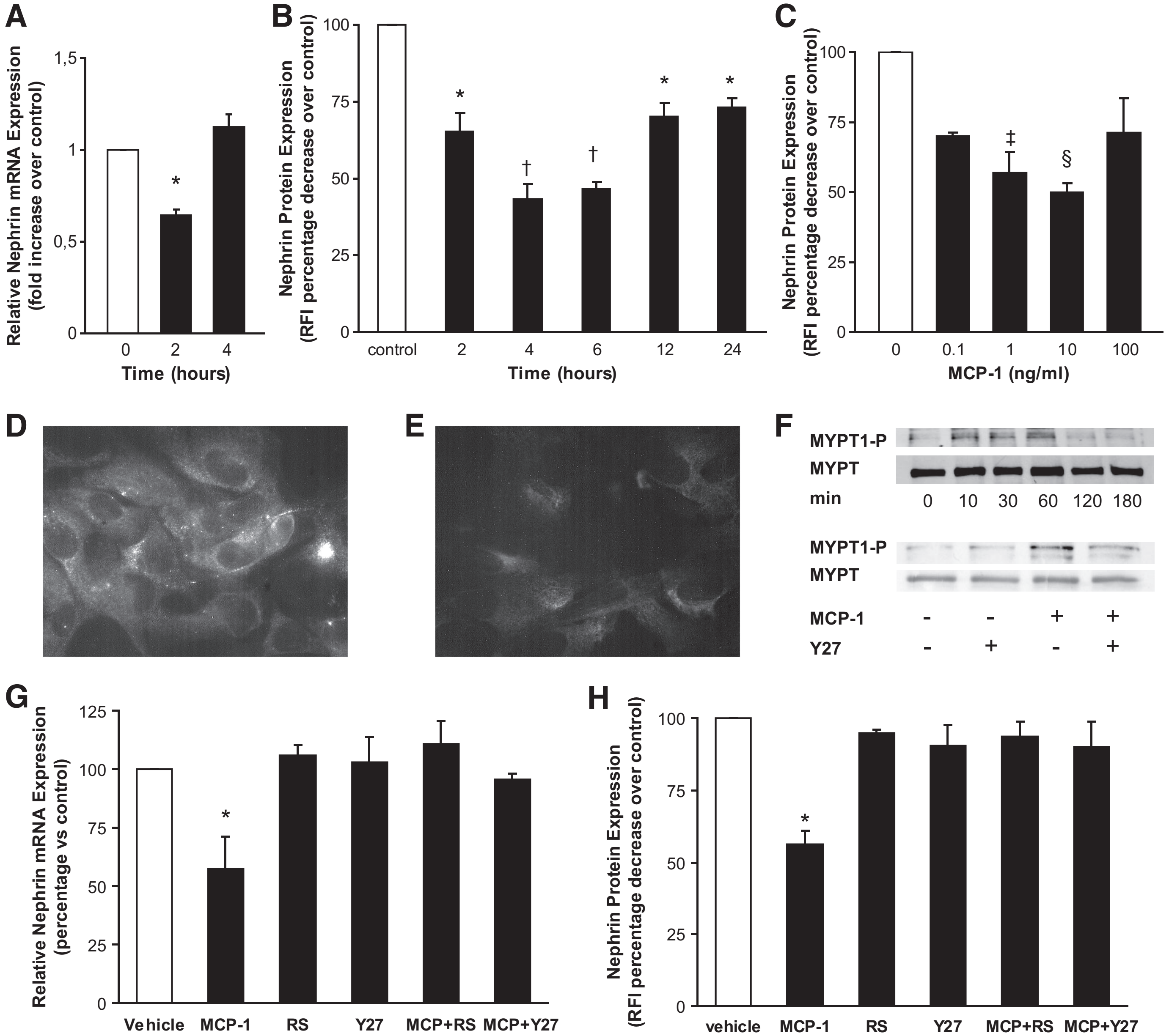

FIG. 2. MCP-1 reduces nephrin mRNA and protein expression via a CCR2-Rho-dependent mechanism in cultured human podocytes. *A*: Nephrin mRNA levels measured by real-time PCR in podocytes exposed to either vehicle or rh-MCP-1 (10 ng/ml) for 2 and 4 h. Results were corrected for the expression of the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase and expressed as percentage decrease as compared with control subjects ($n = 3$, *$P < 0.01$ rh-MCP-1 at 2 h vs. control subjects). *B*: Podocytes were exposed to rh-MCP-1 (10 ng/ml) for 2, 4, 6, 12, and 24 h and (*C*) to rh-MCP-1 (0.1–1 to 10–100 ng/ml) for 4 h. Nephrin expression, assessed by immunofluorescence, was expressed as percentage change in RFI as compared with control subjects ($n = 3$ *$P < 0.01$ rh-MCP-1 at 2, 12, and 24 h over control subjects [□]; †$P < 0.001$ rh-MCP-1 at 4 and 6 h over control subjects; ‡$P < 0.05$ rh-MCP-1 at 1 ng/ml over control subjects; §$P < 0.001$ rh-MCP-1 at 10 ng/ml over control subjects). Representative immunofluorescence images are shown in *D* (vehicle) and *E* (rh-MCP-1 at 10 ng/ml for 4 h). Magnification ×400. *F*: Podocytes were exposed to MCP-1 (10 ng/ml) for 0, 10, 30, 60, 120, and 180 min (*upper panel*) and 10 min in the absence and/or in the presence of Y27632 (Y27 10 μmol/l), a specific ROCK inhibitor (*lower panel*). Both total and phosphorylayed MYPT1 were assessed by immunoblotting on total protein extracts. Representative blottings are shown. *G*: Podocytes were exposed to rh-MCP-1 (10 ng/ml) in the presence and in the absence of RS102895 (RS 6 μmol/l), a CCR2 receptor antagonist, and Y27632 (Y27 10 μmol/l), a specific ROCK inhibitor, added 60 min before rh-MCP-1. After 2 h incubation, nephrin mRNA levels were measured by real-time PCR, corrected for the expression of the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase, and expressed as percentage change over control ($n = 3$, *$P < 0.05$ rh-MCP-1 vs. others). *H*: At 4 h, nephrin protein expression was assessed by indirect immunofluorescence using a low-light video camera and expressed as percentage change in RFI as compared to control subjects ($n = 3$; *$P < 0.05$ rh-MCP-1 vs. others).

subjects). As expected MCP-1 mRNA levels were undetectable in the MCP-1$^{-/-}$ animals.

MCP-1 deficiency prevents both nephrin and synaptopodin downregulation in diabetic mice. To evaluate whether MCP-1 modulates the expression of slit-diaphragm–associated proteins in vivo, in the context of diabetes, we assessed nephrin, synaptopodin, and ZO-1 glomerular expression by immunofluorescence. After 10 weeks of diabetes, there was a significant diminution in both nephrin and synaptopodin expression, which was significantly blunted in MCP-1$^{-/-}$ diabetic mice (Fig. 5*A*–*D*). By contrast, diabetes did not alter glomerular ZO-1 protein expression in either MCP-1$^{+/+}$ or MCP-1$^{-/-}$ mice (Fig. 5*E* and *F*). These results were confirmed by immu-

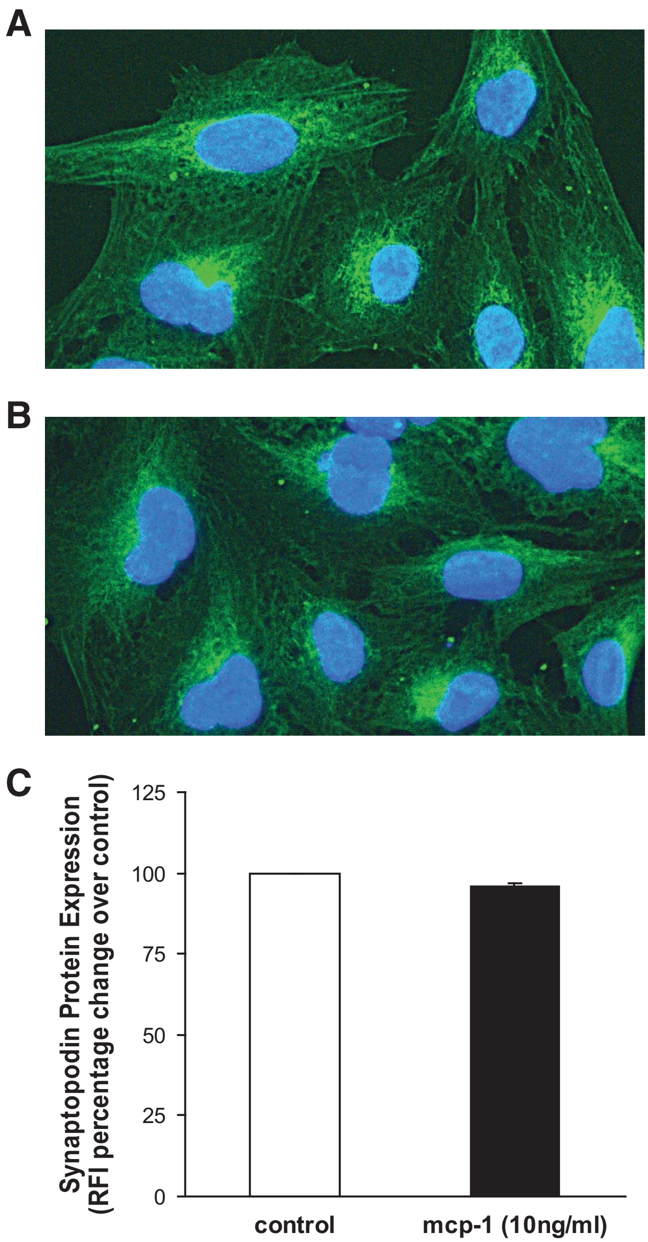

FIG. 3. MCP-1 effect on synaptopodin expression in cultured human podocytes. Podocytes were exposed either to rh-MCP-1 (10 ng/ml) (*A*) or vehicle (*B*) for 4 h, then synaptopodin expression assessed by immunofluorescence. Representative immunofluorescence images are shown (magnification ×800). *C*: Results were expressed as percentage change in RFI as compared with control subjects (*n* = 3 *NS rh-MCP-1 vs. control subjects). (A high-quality digital representation of this figure is available in the online issue.)

noblotting of total protein extracts from renal cortex (Fig. 6). Furthermore, we found that the diabetes-induced reduction in nephrin mRNA levels was significantly diminished in mice lacking MCP-1 (diabetic MCP-$1^{+/+}$: 66.44 ± 7.25; diabetic MCP-$1^{-/-}$: 18.33 ± 12.85, percentage reduction vs. control; $P < 0.01$ diabetic MCP-$1^{+/+}$ vs. control; NS diabetic MCP-$1^{-/-}$ vs. control).

Electron microscopy analysis. Electron microscopy was performed to assess whether there were early signs of podocyte damage in the diabetic animals that were prevented by the absence of MCP-1. As shown in Fig. 7 the normal arrangement of interdigitating foot processes was maintained in all groups and podocyte foot processes appeared tall and narrow in both diabetic MCP-$1^{+/+}$ and MCP-$1^{-/-}$ mice, indicating that changes in podocyte morphology were not yet present in this early phase of experimental diabetes.

TABLE 1
Control subjects and patients with diabetic nephropathy: clinical parameters

	Type 2 diabetes	Control subjects
n	10	8
Age (years)	58.2 ± 2.4	65.4 ± 6.3
Sex (male/female)	6/4	6/2
Diabetes duration (years)	12.5 ± 2.5	—
A1C (%)	7.5 ± 0.6	—
Creatinine (mg/dl)	2.0 ± 0.4	1 ± 0.1
Creatinine clearance (ml/min)	46 ± 6.9	—
Proteinuria (g/24 h)	4.08 ± 0.6	—
Retinopathy (%)	90	—
Hypertension (%)	100	0

Data are means ± SE.

DISCUSSION

The MCP-1/CCR2 system has been implicated in the pathogenesis of diabetic glomerular sclerosis (13,21,30,31). The results, herein reported, showing *1)* overexpression of CCR2 in kidney biopsies from patients with diabetic nephropathy, *2)* overexpression of MCP-1 in the glomeruli from diabetic animals, *3)* prevention of both albuminuria and nephrin downregulation in diabetic MCP-1 deficient mice, and *4)* decreased nephrin expression in cultured podocyte exposed to recombinant MCP-1, indicate that the MCP-1/CCR2 system is also of relevance in the pathogenesis of the diabetic proteinuria.

MCP-1 binding to the CCR2 receptor induced a significant downregulation of both nephrin mRNA and protein expression. The effect was seen at a MCP-1 dose as low as 0.1 ng/ml and reached a peak 57% decrease at 10 ng/ml. This concentration is within the higher physiological range as it is comparable with that measured in vitro in cultured podocytes exposed to high glucose (32) and in vivo at sites of inflammation (33). The magnitude of nephrin downregulation was comparable to that previously reported in podocytes exposed to glycated albumin (9), angiotensin II (9), and oxidized LDL (34). Furthermore, nephrin downregulation has been shown to occur to a comparable extent in proteinuric conditions in humans (35). The prompt decrease in nephrin mRNA levels may be a result of a rapid change in transcriptional activity (36). However, posttranscriptional mechanisms may also be involved as a AU-rich element, which is typical of genes under posttranscriptional regulation, is present in the 3′ untranslated region of the nephrin gene (37). The significant reduction in nephrin protein at later time points, despite the rapid return of the mRNA levels to baseline, suggests that additional mechanisms of nephrin protein reduction, such as ubiquitination and shedding, may also take place. MCP-1–induced cytotoxicity is an unlikely explanation as podocytes exposed to MCP-1 were vital and MCP-1 induces a small increase in cell proliferation in this cell type (22).

MCP-1–induced nephrin downregulation occurred via a CCR2-Rho-kinase–dependent mechanism as podocyte exposure to MCP-1 enhanced ROCK activity and blockade of

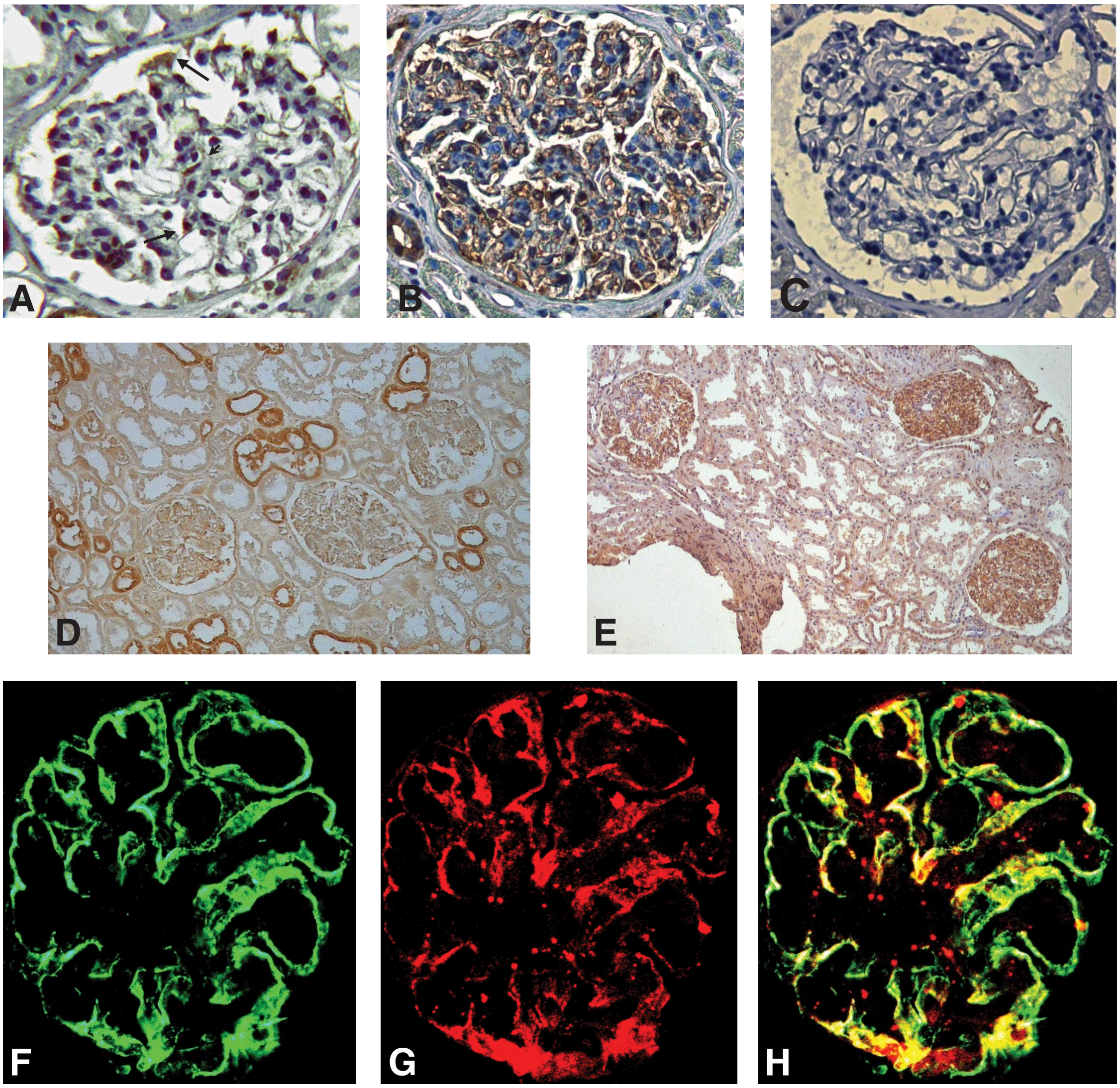

FIG. 4. CCR2 staining of human glomeruli from control subjects and patients with diabetic nephropathy. CCR2 protein expression was evaluated in human glomeruli from control subjects (*A* and *D*) and diabetic patients with overt nephropathy (*B* and *E*) by immunohistochemistry as described in RESEARCH DESIGN AND METHODS. *C*: Nonspecific staining was determined by preabsorbing the anti-CCR2 antibody with a 10-fold excess of control peptide. *F*: Double immunofluorescence for CCR2 (*F*) and (*G*) the podocyte marker synaptopodin performed on the diabetic glomeruli showed colocalisation of the positive staining, as demonstrated by merging (*H*). Magnification ×400 (×80 *D* and *E*). Arrows and arrowhead indicate podocytes and mesangial cells, respectively. (A high-quality digital representation of this figure is available in the online issue.)

both CCR2 and ROCK prevented MCP-1–induced nephrin downregulation. Similarly, in endothelial cells MCP-1–induced loss of tight junction proteins is mediated by a CCR2-Rho–dependent pathway (38). Interestingly, recent in vivo studies have shown that ROCK inhibition ameliorates proteinuria in experimental models of both type 1 and 2 diabetes (39,40).

To assess whether these in vitro findings were relevant to in vivo pathophysiological conditions, we also studied by immunohistochemistry CCR2 expression in both normal

TABLE 2
Characteristics of experimental animals

	Nondiabetic MCP-1$^{+/+}$	Diabetic MCP-1$^{+/+}$	Nondiabetic MCP-1$^{-/-}$	Diabetic MCP-1$^{-/-}$
Animals (*n*)	9	6	4	5
Blood glucose levels (mg/dl)	69 ± 3	329 ± 23*	70 ± 6	372 ± 29*
GHb (%)	3.89 ± 0.30	11.65 ± 0.11*	3.80 ± 0.24	11.82 ± 0.17*
Body weight (g)	28.32 ± 0.57	21.63 ± 1.12*	26.70 ± 0.31	21.94 ± 0.48*
Kidney weight/body weight ratio	5.31 ± 0.08	7.60 ± 0.41*	5.76 ± 0.17	7.94 ± 0.33*
Urinary albumin (μg/18 h)	13.80 (7.88–21.87)	55.69 (35.57–86.67)†	15.57 (9.04–27.70)	26.23 (20.6–35.83)

Data are expressed as means ± SE or median (25–75% percentile). *$P < 0.001$ diabetic vs. nondiabetic mice; †$P < 0.01$ diabetic MCP-1$^{+/+}$ mice vs. nondiabetic mice and vs. diabetic MCP-1$^{-/-}$.

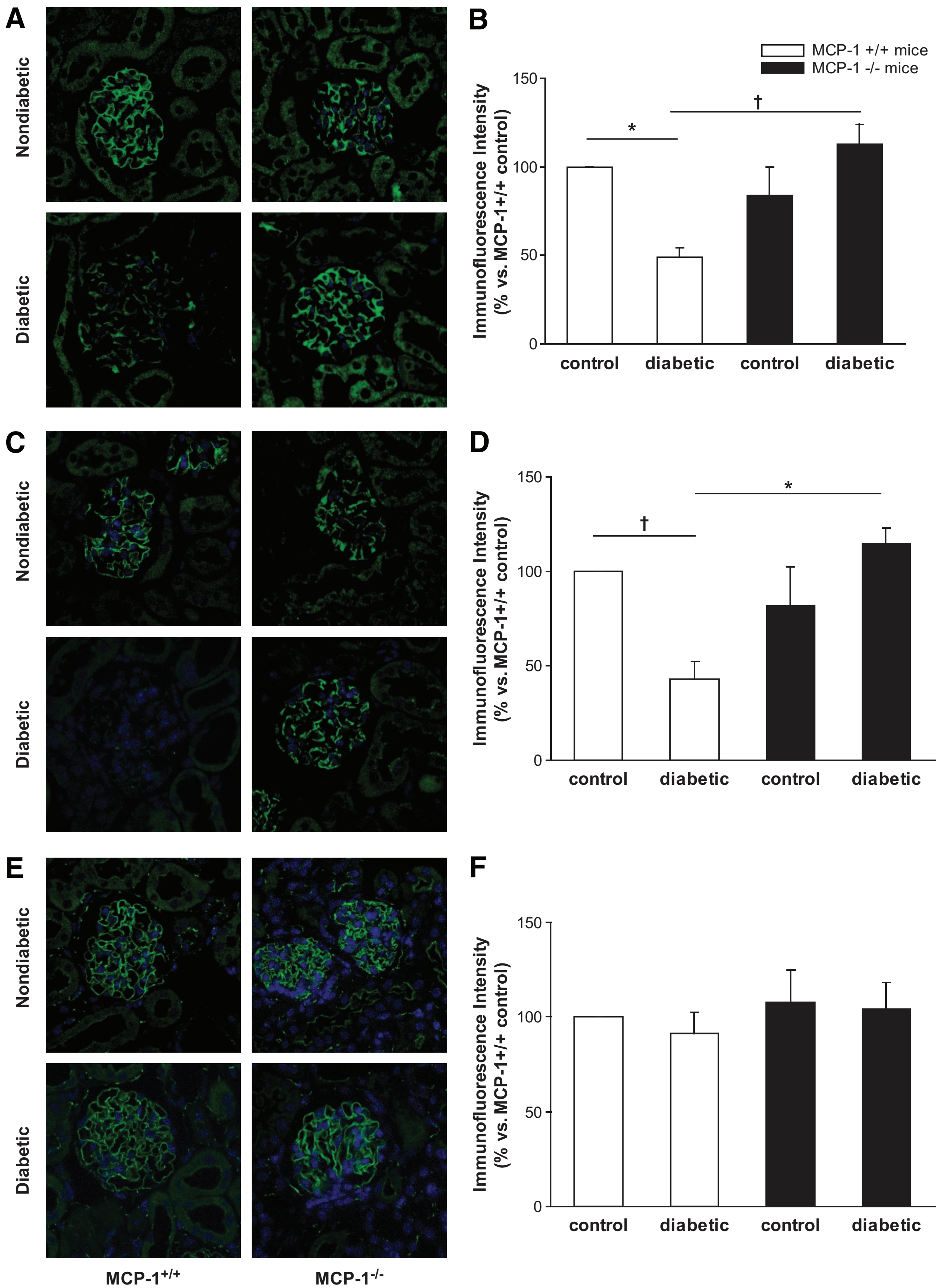

FIG. 5. Glomerular staining for nephrin, synaptopodin, and ZO-1 in diabetic wild-type and MCP-1 knockout mice. Kidney paraffin sections from both diabetic and nondiabetic MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice were stained for nephrin, synaptopodin, and ZO-1 by immunofluorescence as described in RESEARCH DESIGN AND METHODS. *B*, *D*, and *F*: Quantification of glomerular staining for nephrin (*P < 0.01 diabetic MCP-1$^{+/+}$ vs. nondiabetic MCP-1$^{+/+}$ mice; †P < 0.001 diabetic MCP-1$^{-/-}$ vs. diabetic MCP-1$^{+/+}$ mice), synaptopodin (*P < 0.01 diabetic MCP-1$^{-/-}$ vs. diabetic MCP-1$^{+/+}$ mice; †P < 0.05 diabetic MCP-1$^{+/+}$ vs. nondiabetic MCP-1$^{+/+}$ mice), and ZO-1 (P = NS). *A*, *C*, and *E*: Representative figures of nephrin, synaptopodin, and ZO-1 glomerular staining. Magnification ×400. (A high-quality digital representation of this figure is available in the online issue.)

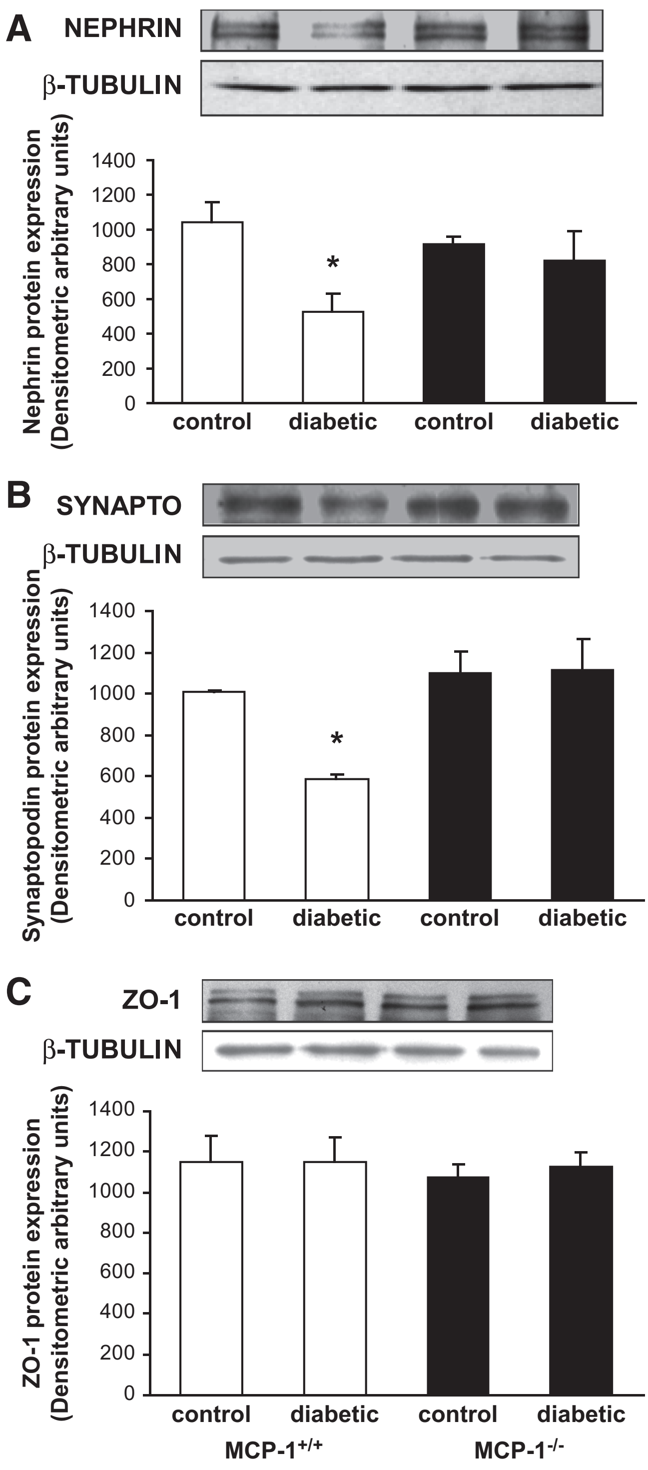

FIG. 6. Nephrin, synaptopodin, and ZO-1 expression in the renal cortex from diabetic wild-type and MCP-1 knockout mice. Nephrin (*A*), synaptopodin (*B*), and ZO-1 (*C*) expression was studied in renal cortex from both diabetic and nondiabetic MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice by immunoblotting as described in RESEARCH DESIGN AND METHODS. Densitometry analysis and representative immunoblots are shown. *P < 0.05 diabetic versus others.

renal cortex and kidney biopsies from patients with type 2 diabetes and overt diabetic nephropathy. In normal kidneys, only a few glomerular cells stained positively for CCR2 in a predominantly podocyte/mesangial cell distribution. However, in patients with diabetic nephropathy there was a ninefold increase in glomerular CCR2 expression as compared to controls and both pattern of staining and colocalization with the podocyte marker synaptopodin strongly indicate that CCR2 was primarily overexpressed by podocytes.

In the kidney, CCR2 expression by glomerular podocytes has been previously reported in a mouse model of Alport syndrome (41) and we have recently demonstrated CCR2 in crescentic glomerulonephritis in humans (22). This is, however, the first report of CCR2 overexpression by podocytes in human diabetic nephropathy. Although we acknowledge that biopsies from type 1 microalbuminuric patients would have been a more appropriate match for our in vivo study in early STZ-induced diabetes, these biopsies are rarely performed for clinically indicated diagnostic purposes and their use in research is restricted by ethical reasons. The underlying mechanism of CCR2 induction in diabetic nephropathy remains elusive; however, both high glucose and hemodynamic stretch are known to downregulate the CCR2 receptor and it is, thus, unlikely a direct role of these insults. The observation that CCR2 expression is enhanced in a variety of glomerulopathies characterized by podocyte damage raises the hypothesis that CCR2 is induced in response to podocyte injury.

To further test the hypothesis of a link between the MCP-1/CCR2 system and enhanced glomerular permeability in diabetic nephropathy, we studied diabetic MCP-1 knockout mice. The induction of diabetes by STZ in this model has been previously established and we and others have shown reduction in macrophage infiltration, overexpression of both fibronectin and transforming growth factor-β1, and albuminuria in this model (13,21), although specific assessment of a potential link between amelioration of albuminuria and preservation of podocyte structural proteins was not examined.

After 10 weeks of diabetes, albuminuria was significantly greater in diabetic than in control mice. This was paralleled by a significant reduction in both nephrin mRNA and protein expression. In the diabetic MCP-1$^{-/-}$ mice, these effects were significantly suppressed, suggesting that in experimental diabetes MCP-1 contributes to both nephrin downregulation and enhanced glomerular permeability. In keeping with this hypothesis, we found that MCP-1 was overexpressed in the glomeruli isolated from the diabetic animals. Blood glucose levels and glycated hemoglobin were similar in diabetic MCP-1$^{-/-}$ and MCP-1$^{+/+}$ mice, consistent with the beneficial effect of MCP-1 deficiency observed in these mice being independent of the glycemic factor. Furthermore, there was no difference in nephrin expression between nondiabetic MCP-1$^{+/+}$ and MCP-1$^{-/-}$ mice, suggesting that the absence of MCP-1 specifically affects diabetes-induced nephrin expression and does not play an important role in the absence of hyperglycemia.

Synaptopodin, an actin-associated protein with preferential localization in podocyte foot processes (25), was also downregulated in diabetic MCP$^{+/+}$ mice and rescued in diabetic MCP-1$^{-/-}$ mice. On the contrary, no changes in ZO-1 glomerular expression were observed in the diabetic animals and our data, thus, do not confirm a previous report showing ZO-1 downregulation in both STZ-induced diabetic rats and type 2 diabetic mice (42). Differences in species/strain may explain this discrepancy.

Previous studies in diabetic mice have shown that nephrin loss and proteinuria are paralleled by podocyte foot process effacement, an early marker of podocyte injury (43–46). However, in our study downregulation of nephrin and synaptopodin were unlikely because of podo-

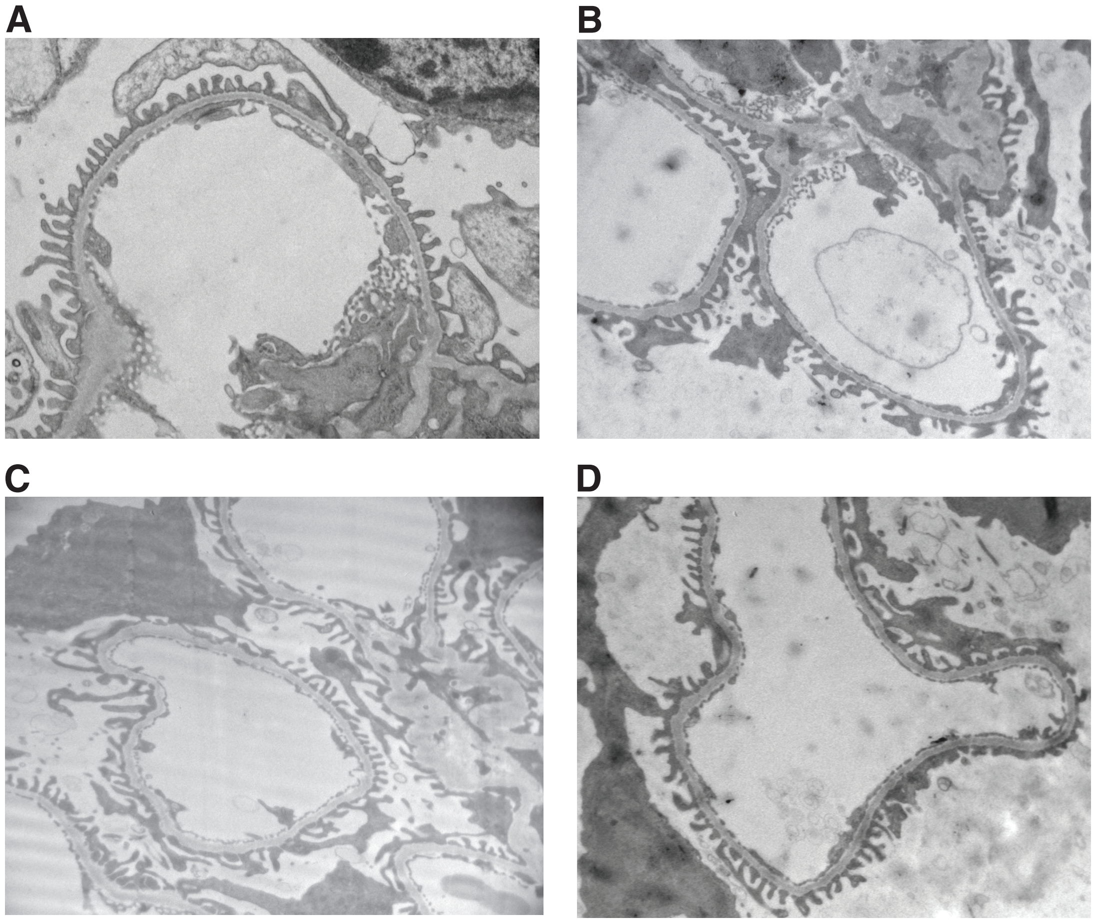

FIG. 7. Morphology of podocyte foot process (transmission electron microscopy, ×7,000) in nondiabetic MCP-1$^{+/+}$ (*A*), diabetic MCP-1$^{+/+}$ (*B*), diabetic MCP-1$^{-/-}$ (*C*), and nondiabetic MCP-1$^{-/-}$ (*D*) mice 10 weeks after the onset of STZ-induced diabetes.

cyte damage as no evidence of podocyte foot process effacement was found at the ultrastructural level in the diabetic animals. This may also suggest that podocyte damage is not strictly required for the loss of nephrin and the development of proteinuria. Consistently with this view, proteinuria occurs, in nephrin knockout animals, even in the absence of any defects in the podocyte foot processes (47).

Strategies preventing glomerular macrophage infiltration have proven beneficial in experimental diabetes (48,49) and reduced glomerular recruitment of macrophages may also be implicated in the protective effects observed in the diabetic MCP-1$^{-/-}$ mice. In particular, the protective effect of MCP-1 deficiency on synaptopodin, which was not affected in vitro in podocytes exposed to MCP-1, may be explained by a macrophage-dependent mechanism.

In conclusion, our findings may have important implications for diabetic nephropathy in humans. Proteinuria is a characteristic feature of diabetic nephropathy and a key determinant of progression (1). Nephrin is downregulated in early diabetic nephropathy and this has been implicated in the pathogenesis of the diabetic proteinuria (9). Our data showing an effect of the MCP-1/CCR2 on both albuminuria and nephrin support the hypothesis of a pathogenic role of this system in the development of the diabetic proteinuria and makes it an attractive target for developing new strategies directed toward reducing proteinuria in diabetic and other nephrotic conditions.

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No potential conflicts of interest relevant to this article were reported.

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