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 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

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.
MCP-1/CCR2 SYSTEM IN DIABETIC NEPHROPATHY

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 examined in all cells and immunofluorescence 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 Biotechnology, Santa Cruz, CA) were added. Fluorescence 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-MyPT/H11001 antibody (Cycles). After detection by enhanced chemiluminescence (Amersham) and band intensity quantified by computer-aided image analysis system (Qwin; Leica). Two to three animals per group were used for the analysis.

Data presentation and statistical analysis. The left kidney was fixed in 10% PBS-formalin at room temperature and 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 Diagnostik, Milan, Italy).

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Animals. Mice with the genotype C57Bl/6J and C57Bl/6J–deficient (MCP1−/−) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a normal rodent diet under standard animal house conditions. Diabetes (blood glucose >250 mg/dL) was induced in both MCP1−/− and MCP1+/- 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 MCP1−/− (n = 6) and MCP1+/- (n = 5) diabetic mice with equivalent blood glucose levels and nondiabetic MCP1−/− (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-starved 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 and 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 Diagnostik, Milan, Italy).

Nephritis, synaptopodin, and zonula occludens-1 protein expression. After overnight 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. Finally, streptavidin-HRP was added and visualized as described above. On average, 20 randomly selected hilar glomeruli per kidney 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).

Renal cortex specimens were homogenized in either Laemml buffer (nephritis, ZO-1) or Tris (20 mmol/L, 500 mmol/L NaCl, pH 7.4) lysis buffer containing 0.5% 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulphonate, 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 (Syntec 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 each figure as N = n. Experiments were considered significant when P < 0.05. Comparison of two groups was done by unpaired 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.
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 monocye 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. 2A). 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 protein 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. 2B, 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. 2C). 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 spiroperidinede 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. 2G 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. 2G 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. 2F). 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. 4A 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. 4C).

In patients with diabetic nephropathy, CCR2 protein expression was greatly enhanced (Fig. 4B 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. 4B) and the positive staining for synaptopodin (Fig. 4G) colocalized with the CCR2 staining (Fig. 4H).

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 ± 0.49, P < 0.05 diabetes vs. control
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. 5A–D). By contrast, diabetes did not alter glomerular ZO-1 protein expression in either MCP-1−/− or MCP-1+/− mice (Fig. 5E and F). These results were confirmed by immu-
noblotted 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.

**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 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 an AU-rich element, which is typical of genes under posttranscriptional mechanisms may also be involved as 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 recombinant MCP-1 were vital and MCP-1 induced 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...
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 (25–75th 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), synaptopodin (∗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.)
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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−/− mice and control 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−/− mice. On the contrary, no changes in ZO-1 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-
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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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.


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