EFFECT OF THE MCP-1/CCR2 SYSTEM ON NEPHRIN EXPRESSION IN STREPTOZOTOCIN-TREATED MICE AND HUMAN CULTURED PODOCYTES

Short running title: MCP-1/CCR2 system in diabetic nephropathy

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**Objective:** Monocyte Chemoattractant Protein-1 (MCP-1), a chemokine binding to the CCR2 receptor and promoting monocyte infiltration, has been implicated in the pathogenesis of diabetic nephropathy (DN). 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 DN 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 8 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.
Diabetic nephropathy (DN) is characterised by increased glomerular permeability to proteins (1). Recently, much attention has been paid to the role of podocyte injury in glomerular diseases, including DN (2,3), but the precise molecular mechanisms underlying the development of diabetic proteinuria remain unclear.

The slit diaphragm (SD), a junction connecting foot processes of neighbouring podocytes, represents the major restriction site to protein filtration (4). Mutations of the gene encoding for nephrin, a key component of the SD, 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 DN (6-8), and studies in patients with incipient DN 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 DN (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 DN and whether lack of MCP-1 affects proteinuria and/or expression of nephrin in experimental diabetes.

**RESEARCH DESIGN AND METHODS**

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

**In Vitro Study**

**Cell Culture**: Immortalised 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 prior to the study, as we have previously reported (20).

**mRNA Expression**: Total RNA was extracted using the RNaseasy Mini Kit (Qiagen, Chatsworth, CA). Two µg of total RNA were reverse transcribed into cDNA using AMV reverse transcriptase and poly-d(T) primers. Human nephrin, mouse nephrin, and mouse MCP-1 mRNA expression were analysed by real-time PCR using pre-developed TaqMan reagents (Applied-Biosystems). Fluorescence for each cycle was analysed 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, Maaßstraße, Germany) antibody. After rinsing, FITC-conjugated secondary antibodies (SantaCruz Biotecnology, USA) 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 (ROCK) activity:** 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 RIPA 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-Thr853 antibody (Cyclex). Following 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 hours) and 8 control specimens 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 the 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 DN 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 HbA1c by ion-exchange liquid chromatography. Creatinine clearance was estimated using the Cockcroft-Gault formula (24).

**CCR2 Protein Expression and Localisation:** Immunohistochemical staining was performed on 4-µm paraffin sections of formalin-fixed tissue. Following antigen retrieval in citrate buffer, endogenous peroxidase activity quenching with 3% H2O2, and blocking with avidin-biotin and 3% BSA, sections were incubated with a rabbit monoclonal anti-CCR2 antibody (Epitomics, Burlingame, USA) and the specific staining detected using the LSAB+ system-HRP. Sections were visualised 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 analysed 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.
specific podocyte marker (25). After blocking with 3% BSA, sections were incubated with a monoclonal anti-synaptopodin antibody (Progen Biotechnik) for 18 hours 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 hours at 4°C, washed, incubated with a biotinylated swine anti-rabbit IgG for 1h 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, USA) were maintained on a normal 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 ~22g, by intraperitoneal injections of streptozotocin (STZ)-citrate buffer (55 mg/kg body wt/day) for five 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 non-diabetic MCP-1+/+ (n=9) and MCP-1−/− mice (n=4) were studied in parallel. Blood glucose obtained via saphenous vein sampling between 12 p.m. and 1 p.m. on alert 4 hours fasted animals was measured using a glucometer (Glucocard G meter, Menarini Diagnostics). Before sacrifice mice were placed in individual metabolic cages for a period of 18 hours and urinary albumin concentration measured by a mouse albumin ELISA kit (Bethyl Laboratories, USA). After sacrifice mice were euthanized, 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 haemoglobin was measured in whole blood samples obtained via cardiac puncture at the time of sacrifice by quantitative immunoturbidimetric latex determination (Sentinel Diagnostic, Milan, Italy).

Glomerular isolation: Glomeruli were isolated immediately after sacrifice, using the Dynabeads method from Takemoto et al. (27). Briefly, anesthetized mice were perfused with 8x10^7 surface-inactivated Dynabeads® (Invitrogen). The kidneys, removed and minced, were digested in a collagenase A solution containing 100 U/ml deoxyribonuclease 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) till no tubular contamination was found as assessed under light microscopy.

Nephrin, Synaptopodin, and Zonula Occludens-1 (ZO-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-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 digitised 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 mM, 500 mM NaCl pH 7.5) lysis buffer containing 0.5% CHAPS, 5 mM EDTA and protease inhibitors (synaptopodin). Proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes. Following blocking in 5% non-fat milk in TBS, 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 HRP-conjugated antibodies were added for one hour. 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 hours, postfixed in 1% osmium tetroxide for 1 hour, 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 Inc., Tokyo, Japan). Two-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 figure. Data, presented as mean ± SEM, geometric mean (25-75% percentile), or fold change over control, were analysed by Student’s t-test or ANOVA, as appropriate. Newman-Keuls and Pearson tests were used for post hoc comparisons and correlation analysis, respectively. Values for p<0.05 were considered significant.

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**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 (Figure 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 hours with a return to baseline by 4 hours (Figure 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 hours and to increasing rh-MCP-1 concentrations (0.1, 1, 10, and 100 ng/ml) for 4 hours, then nephrin expression assessed by immunofluorescence. Addition of rh-MCP-1 induced a significant decrease over control in nephrin protein expression after 2 hours that was sustained up to 24 hours and peaked at 4-6 hours (Figure 2 B,D,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 (Figure 2C). On the contrary, as shown in Figure 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 signalling, RS102895 (RS 6µM), added 60 min prior to 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 hours and of nephrin protein at 4 hours (Figure 2GH). Similarly, the addition of Y27632 (10 µM), 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 (Figure 2GH). 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 minutes was completely abolished by the ROCK inhibitor Y27632 (Figure 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 DN:* 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 DN 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 (Figure 4A,D). Specificity of the antibody binding was confirmed by disappearance of the signal when the antibody was pre-absorbed with a ten-fold excess of control peptide (Figure 4C). In patients with DN, CCR2 protein expression was greatly enhanced (Figure 4B,E) and semi-quantitative analysis showed that the percentage positive area was nine-fold 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), while no correlation was found with other clinical parameters, such as age, diabetes duration, HbA1c, and creatinine clearance. To clarify which glomerular cell type overexpressed CCR2, double-labelling immunofluorescence was performed in patients with DN 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 (Figure 4B) and the positive staining for synaptopodin (Figure 4G) colocalized with the CCR2 staining (Figure 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 was 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 non-diabetic MCP-1+/+ and MCP-1−/− mice.

**Glomerular MCP-1 mRNA levels are enhanced in experimental diabetes:** There was a significant six-fold increase in glomerular MCP-1 mRNA levels in diabetic mice as compared to controls as assessed by quantitative real-time PCR (diabetic mice: 9.46±2.20; controls: 1.49±0.49, p<0.05 diabetics vs. controls). 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 (Figure 5A-D). By contrast, diabetes did not alter glomerular ZO-1 protein expression in either MCP-1+/+ or MCP-1−/− mice (Figure 5E,F). These results were confirmed by immunoblotting of total protein extracts from renal cortex (Figure 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; p=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 Figure 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 DN, 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 due to a rapid change in transcriptional activity (36). However, post-transcriptional mechanisms may also be involved as a AU-rich element, which is typical of genes under post-transcriptional regulation, is present in the 3′ UTR 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 Rho-kinase activity and blockade of both CCR2 and Rho-kinase 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 Rho-kinase 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 renal cortex and kidney biopsies from patients with type 2 diabetes and overt DN. In normal kidneys only a few glomerular cells stained positively for CCR2 in a predominantly podocyte/mesangial cell distribution. However, in patients with DN there was a nine-fold increase in glomerular CCR2 expression as compared to controls and both pattern of staining and co-localisation 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 DN. Although we acknowledge that biopsies from type 1 microalbuminuric patients would have been a more appropriate match for our in vivo study in early streptozotocin-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 DN remains elusive; however, both high glucose and haemodynamic 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 DN, 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 TGF-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 ten 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 haemoglobin 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 glycaemic factor. Furthermore, there was no difference in nephrin expression between non-diabetic 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 hyperglycaemia.

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 streptozotocin-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 due to podocyte 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 \textit{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 DN in humans. Proteinuria is a characteristic feature of DN and a key determinant of progression (1). Nephrin is downregulated in early DN 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 towards reducing proteinuria in diabetic and other nephrotic conditions.

**ACKNOWLEDGMENTS**

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### Table 1. Controls and Patients with Diabetic Nephropathy: Clinical Parameters

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<td><strong>Age (years)</strong></td>
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<td><strong>Sex (M/F)</strong></td>
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<td>6/2</td>
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<td><strong>Diabetes duration (years)</strong></td>
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<td><strong>HbA₁c (%)</strong></td>
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<td><strong>Creatinine (mg/dl)</strong></td>
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<td><strong>Creatinine clearance (ml/min)</strong></td>
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<td><strong>Proteinuria (g/24h)</strong></td>
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### Table 2. Characteristics of Experimental Animals

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<th>Non-Diabetic MCP-1⁺⁺⁺⁺</th>
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<td><strong>Blood glucose levels (mg/dl)</strong></td>
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<td>329 ± 23*</td>
<td>70 ± 6</td>
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<td><strong>Glycated haemoglobin (%)</strong></td>
<td>3.89 ± 0.30</td>
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<td>3.80 ± 0.24</td>
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<td>26.70 ± 0.31</td>
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<td><strong>Kidney weight/body weight ratio</strong></td>
<td>5.31 ± 0.08</td>
<td>7.60 ± 0.41*</td>
<td>5.76 ± 0.17</td>
<td>7.94 ± 0.33*</td>
</tr>
<tr>
<td><strong>Urinary albumin (µg/18 h)</strong></td>
<td>13.80 (7.88-21.87)</td>
<td>55.69 (35.57-86.67)*</td>
<td>15.57 (9.04-27.70)</td>
<td>26.23 (20.6-35.83)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM or median (25-75% percentile). *p<0.001 diabetic vs non-diabetic mice; † p<0.01 diabetic MCP-1⁺⁺⁺⁺ mice vs non-diabetic mice and vs diabetic MCP-1⁻⁻⁻⁻.
Figure 1. The CCR2 receptor is expressed by human podocytes. CCR2 protein expression was studied in human cultured podocytes by immunoblotting as described in the 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.

Figure 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 hours. Results were corrected for the expression of the housekeeping gene GAPDH and expressed as percentage decrease as compared to control (n=3, *p<0.01 rh-MCP-1 at 2 hours vs control). (B) Podocytes were exposed to rh-MCP-1 (10 ng/ml) for 2, 4, 6, 12, and 24 hours and (C) to rh-MCP-1 (0.1-10-100ng/ml) for 4 hours. Nephrin expression, assessed by immunofluorescence, was expressed as percentage change in relative fluorescence intensity (RFI) as compared to control [n=3 *p<0.01 rh-MCP-1 at 2, 12 and 24 hours over control (white bar); †p<0.001 rh-MCP-1 at 4 and 6 hours over control; ‡p<0.05 rh-MCP-1 at 1ng/ml over control; §p<0.001 rh-MCP-1 at 10ng/ml over control]. Representative immunofluorescence images are shown in panel D (vehicle) and E (rh-MCP-1 at 10ng/ml for 4 hours) (Magnification 400 X). (F) Podocytes were exposed to MCP-1 (10 ng/ml) for 0, 10, 30, 60, 120, 180 minutes (upper panel) and 10 minutes in the absence and/or in the presence Y27632 (Y27 10μM), a specific Rho kinase inhibitor (lower panel). Both total and phosphorylated myosin phosphatase target subunit 1 (MYPT1) were assessed by immunoblotting on total protein extracts. Representative blottings are shown. (G) Podocytes were exposed to rh-MCP-1 (10ng/ml) in the presence and in the absence of RS102895 (RS 6μM), a CCR2 receptor antagonist, and Y27632 (Y27 10μM), a specific Rho kinase inhibitor, added 60 minutes prior to rh-MCP-1. After 2 hour incubation nephrin mRNA levels were measured by real-time PCR, corrected for the expression of the housekeeping gene GAPDH, and expressed as percentage change over control (n=3, *p <0.05 rh-MCP-1 vs others). (H) At 4 hours nephrin protein expression was assessed by indirect immunofluorescence using a low-light video camera and expressed as percentage change in relative fluorescence intensity (RFI) as compared to control (n=3; *p<0.05 rh-MCP-1 vs others).

Figure 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 hours, then synaptopodin expression assessed by immunofluorescence. Representative immunofluorescence images are shown (Magnification 800X). (C) Results were expressed as percentage change in relative fluorescence intensity (RFI) as compared to control (n=3 *p=ns rh-MCP-1 vs control).

Figure 4. CCR2 staining of human glomeruli from controls and patients with diabetic nephropathy. CCR2 protein expression was evaluated in human glomeruli from controls (A,D) and diabetic patients with overt nephropathy (B,E) by immunohistochemistry as described in the Methods. Non-specific staining was determined by pre-absorbing the anti-CCR2 antibody with a 10-fold excess of control peptide (C). Double immunofluorescence for CCR2 (F) and the podocyte marker synaptopodin (G) performed on the diabetic glomeruli showed colocalisation of the positive staining, as demonstrated by merging (H). Magnification 400X, (80X D,E). Arrows and arrowhead indicate podocytes and mesangial cells, respectively.
Figure 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 non-diabetic MCP-1+/+ and MCP-1−/− mice were stained for nephrin, synaptopodin, and ZO-1 by immunofluorescence as described in the Methods. (A,C,E) Quantification of glomerular staining for nephrin (*p<0.01 diabetic MCP-1+/+ vs non-diabetic 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 non-diabetic MCP-1+/+ mice), and ZO-1 (p=ns) (B,D,F) Representative figures of nephrin, synaptopodin, and ZO-1 glomerular staining. Magnification 400 X.

Figure 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 non-diabetic MCP-1+/+ and MCP-1−/− mice by immunoblotting as described in the Methods. Densitometry analysis and representative immunoblots are shown. * p<0.05 diabetic vs others

Figure 7. Morphology of podocyte foot process (transmission electron microscopy, x 7,000) in non-diabetic MCP-1+/+ (A), diabetic MCP-1+/+ (B), diabetic MCP-1−/− (C), and non-diabetic MCP-1−/− (D) mice 10 weeks after the onset of streptozocin-induced diabetes.
Figure 2
Figure 3

A

B

C

Synaptotagmin Protein Expression

(control) mcp-1 (10 ng/ml)

RFI (percentage change over control)
Figure 4
Figure 5

A
Non-diabetic

Diabetic

C
Non-diabetic

Diabetic

E
Non-diabetic

Diabetic

MCP-1++; MCP-1−
Figure 6

A  NEPHRIN

\[ \beta\text{-TUBULIN} \]

![Graph showing NEPHRIN protein expression](image)

B  SYNAPTO

\[ \beta\text{-TUBULIN} \]

![Graph showing SYNAPTO protein expression](image)

C  ZO-1

\[ \beta\text{-TUBULIN} \]

![Graph showing ZO-1 protein expression](image)