Monocyte chemoattractant protein-1 promotes the development of diabetic renal injury in streptozotocin-treated mice

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Diabetic nephropathy involves a renal inflammatory response induced by the diabetic milieu. Macrophages accumulate in diabetic kidneys in association with the local upregulation of monocyte chemoattractant protein-1 (MCP-1); however, the contribution of macrophages to renal injury and the importance of MCP-1 to their accrual are unclear. Therefore, we examined the progression of streptozotocin (STZ)-induced diabetic nephropathy in mice deficient in MCP-1 in order to explore the role of MCP-1-mediated macrophage accumulation in the development of diabetic kidney damage. Renal pathology was examined at 2, 8, 12 and 18 weeks after STZ treatment in MCP-1 intact (+/+) and deficient (−/−) mice with equivalent blood glucose and hemoglobin A1c levels. In MCP-1(+/+) mice, the development of diabetic nephropathy was associated with increased kidney MCP-1 production, which occurred mostly in tubules, consistent with our *in vitro* finding that elements of the diabetic milieu (high glucose and advanced glycation end products) directly stimulate tubular MCP-1 secretion. Diabetes of 18 weeks resulted in albuminuria and elevated plasma creatinine in MCP-1(+/+) mice, but these aspects of renal injury were largely suppressed in MCP-1(−/−) mice. Protection from nephropathy in diabetic MCP-1(−/−) mice was associated with marked reductions in glomerular and interstitial macrophage accumulation, histological damage and renal fibrosis. Diabetic MCP-1(−/−) mice also had a smaller proportion of kidney macrophages expressing markers of activation (inducible nitric oxide synthase or sialoadhesin) compared to diabetic MCP-1(+/+) mice. In conclusion, our study demonstrates that MCP-1-mediated macrophage accumulation and activation plays a critical role in the development of STZ-induced mouse diabetic nephropathy.

Kidney International (2006) **69,** 73–80. doi:10.1038/sj.ki.5000014

KEYWORDS: diabetic nephropathy; streptozotocin; macrophage; renal injury; monocyte chemoattractant protein-1; inducible nitric oxide synthase

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Received 12 May 2005; revised 4 July 2005; accepted 14 July 2005

Diabetic nephropathy is the most prevalent cause of end-stage renal failure worldwide. Despite established conventional therapy of glycemic and blood pressure control, many patients still develop progressive renal damage,[1] suggesting a need for additional treatments and the identification of appropriate therapeutic targets.

Macrophages are the major inflammatory cells found in diabetic kidneys and their accumulation is a recognized feature in both human biopsies and animal models.[2–5] Although macrophages are known to play a pivotal role in the pathogenesis of various experimental models of immune-mediated kidney disease,[6,7] their contribution to renal injury in diabetic nephropathy is unknown. It is not established whether macrophages are a major effector of diabetic renal damage, or merely recruited as a response to injury. Previous studies involving the use of irradiation[4] or administration of mycophenolate[8] suggest some renal protection by reducing macrophages in diabetic kidneys. However, both these treatments have immunological or renal effects in addition to macrophage depletion, and cannot be used for determining the long-term effects of macrophages on the progression of diabetic nephropathy.

The use of gene knockout mice to target the molecular mechanisms of macrophage accumulation and activation may provide a more specific, practicable means of determining the long-term effects of macrophages on the diabetic kidney. Macrophage infiltration into any injured tissue involves their extravascularization mediated by cell adhesion molecules and chemokines.[6] A number of cell adhesion molecules (ICAM-1, LFA-1, VCAM-1) and chemokines (OPN, monocyte chemoattractant protein-1 (MCP-1), migration inhibitory factor, macrophage inflammatory protein-1 α/β, RANTES) have been shown to be important in macrophage accumulation and renal injury in models of nondiabetic kidney damage,[6,9] and many of these molecules are also elevated in diabetic kidneys.[5,10] Therefore, the use of mice deficient in adhesion molecules or chemokines may allow us to explore the role of macrophages in the progression of diabetic nephropathy.

MCP-1 is a potent chemokine that is known to affect both macrophage accumulation and macrophage function.[11–13]

MCP-1 increases progressively in diabetic kidneys in animal models,[5,14] and urinary levels correlate with albuminuria in human diabetic nephropathy.[15] In diabetic kidneys, MCP-1 levels appear to be dependent on both the diabetic milieu[16] and activation of the renin–angiotensin system.[17,18]

In the current study, we examine the development of streptozotocin (STZ)-induced type I diabetic nephropathy in mice genetically deficient in MCP-1 in order to determine the role of MCP-1 in promoting macrophage accumulation and activation in this model, and to establish the importance of macrophages in the progression of diabetic renal injury.

RESULTS

Tubular MCP-1 production is increased by the diabetic milieu

Tubular cell (NRK52E) secretion of MCP-1 increased in the presence of high glucose (25 mmol/l) compared to cells cultured in serum-free media containing a normal physiological level of glucose (5 mmol/l) (Figure 1a). This effect was not due to osmotic stress because the use of L-glucose as an osmotic control did not increase MCP-1 levels. Stimulation of tubular cells with 200 μg/ml bovine serum albumin (BSA) did not alter MCP-1 secretion compared to media alone; however, an equivalent concentration of advanced glycation end product (AGE)–BSA induced a significant increase in tubular MCP-1 release (Figure 1b).

Induction of diabetes in STZ-treated mice

Administration of STZ resulted in 90% of MCP-1-intact (+/+) mice and 65% of MCP-1-deficient (−/−) mice developing overt diabetes (blood glucose >16 mmol/l) after 2 weeks. At 2 weeks, groups of MCP-1-intact and -deficient mice with equivalent blood glucose were selected for comparing the progression of diabetic nephropathy. Analysis of mice killed at 2, 8, 12 and 18 weeks after STZ demonstrated a similar development of diabetes between MCP-1-intact and -deficient strains based on equivalent levels of blood glucose and hemoglobin A1c (HbA1c) at each time point (Figure 2).

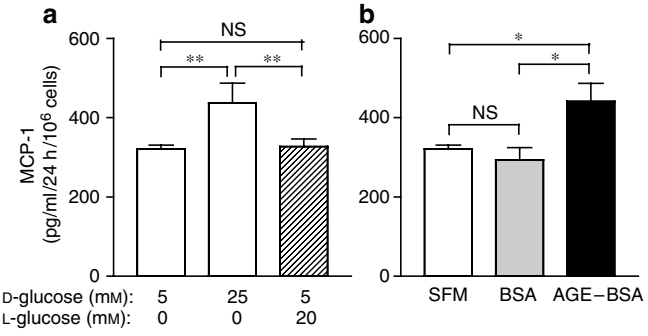

Figure 1 | Tubular MCP-1 secretion is increased by the diabetic milieu. (**a**) MCP-1 was secreted by NRK52E tubular cells cultured at normal physiological levels of D-glucose (5 mmol/l). Increasing the D-glucose to a concentration comparable with the blood glucose level seen in diabetic mice (25 mmol/l) resulted in increased tubular MCP-1 secretion. The use of L-glucose as an osmotic control demonstrated that the increase in tubular MCP-1 detected in the presence of high glucose was not due to elevated osmotic stress. (**b**) Stimulation with 200 μg/ml BSA did not increase tubular secretion of MCP-1 compared to serum-free media (SFM) alone. However, when cells were incubated in 200 μg/ml AGE–BSA, the tubular secretion of MCP-1 was significantly enhanced compared to BSA or SFM. Data are mean ± s.d.; $n = 3$; *$P < 0.05$, **$P < 0.01$, NS = not significant. Results were obtained from the means of three separate experiments.

Renal expression of MCP-1 increases with the duration of STZ diabetes

In situ analysis demonstrated that MCP-1 mRNA was not detectable in normal kidneys or MCP-1-deficient diabetic kidneys (Figure 3a–d). In comparison, MCP-1 was observed in some glomerular and tubular cells in MCP-1-intact mice after 2 weeks of STZ diabetes, but was not detected if these mice were prevented from becoming diabetic by daily insulin administration (Figure 3b and c). In diabetic MCP-1-intact mice, the number of glomerular and tubular cells expressing MCP-1 mRNA increased progressively with the duration of disease (Figure 4).

Deficiency of MCP-1 reduces kidney macrophage accumulation and activation in STZ-diabetic mice

Compared to normal mice, there was a three- to four-fold increase in total kidney leukocytes (CD45+) in diabetic MCP-1(+/+) mice at 18 weeks after STZ treatment. Further analysis of the diabetic kidneys indicated that almost all glomerular leukocytes (CD45: 2.83 ± 0.20 cells/glomerular tuft cross-sections (gcs), CD68: 2.58 ± 0.53 cells/gcs, CD4: 0.11 ± 0.02 cells/gcs, CD8: 0.017 ± 0.008 cells/gcs) and interstitial leukocytes (CD45: 492 ± 15 cells/mm^2, CD68: 475 ± 27 cells/mm^2, CD4: 16.7 ± 2.5 cells/mm^2, CD8: 0.67 ± 0.33 cells/mm^2) were macrophages. Following the onset of diabetes, increased numbers of kidney macrophages were noted as early as 2 weeks after STZ in both MCP-1-intact and -deficient mice (Figure 5a and b). Between 2 and 18 weeks, the recruitment of glomerular and interstitial macrophages was more pronounced in MCP-1(+/+) compared to MCP-1(−/−) diabetic mice such that macrophage accumulation was reduced by 50–90% in MCP-1(−/−) mice at week 18. In contrast, numbers of glomerular CD8 cells and interstitial CD4 and CD8 cells did not change with the induction of diabetes in either MCP-1(+/+) or (−/−) mice. However, glomerular CD4 cells, which were rare in normal mice (0.025 ± 0.035 cells/gcs), did increase fourfold in diabetic kidneys, but were unaffected by the absence of MCP-1 at any time point (data not shown).

Levels of urine MCP-1 obtained from normal and diabetic MCP-1-intact mice correlated with number of glomerular macrophages ($r = 0.442$, $P = 0.0054$) and interstitial macrophages ($r = 0.334$, $P = 0.040$) detected by CD68 immunostaining.

Macrophage activation was examined at week 18 by two-color immunostaining on the basis of expression of inducible nitric oxide synthase (iNOS) and sialoadhesin (CD169). In MCP-1(+/+) mice, the development of diabetes not only increased the number of kidney macrophages but also the percentage of glomerular and interstitial macrophages expressing CD169 and iNOS (Figure 5c and d). In MCP-1(+/+)

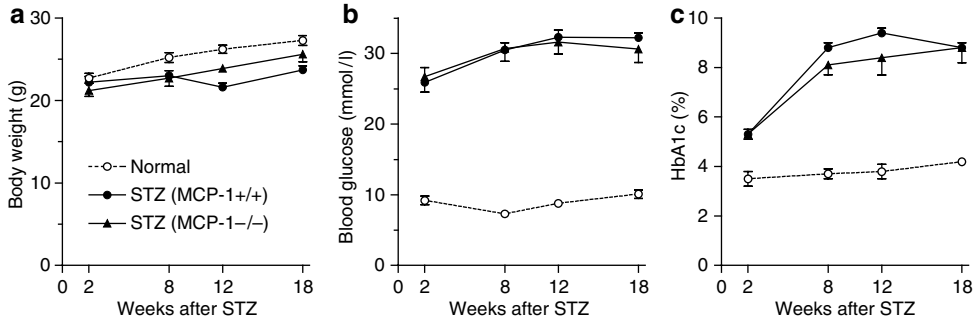

Figure 2 | Development of diabetes in STZ-treated mice. Groups of MCP-1 intact (+/+) and deficient (−/−) mice were chosen at 2 weeks after STZ treatment for equivalent diabetes and were monitored for the continuing development of diabetes at weeks 8, 12 and 18. These groups were examined for (**a**) body weight, (**b**) blood glucose and (**c**) HbA1c levels and results were compared with age-matched normal MCP-1(+/+) mice. Data are mean ± s.e.m.; $n = 8$–10 for each group.

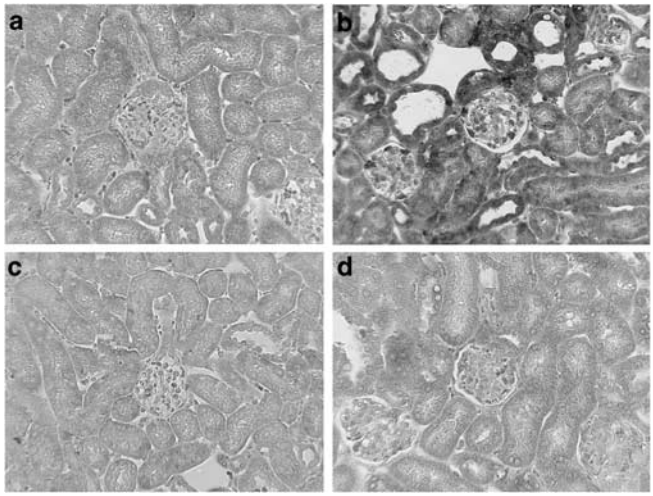

Figure 3 | Diabetes induces kidney MCP-1 expression. *In situ* hybridization indicated that MCP-1 mRNA was absent in (**a**) a normal MCP-1(+/+) mouse kidney but was present in the dilated tubules and some glomerular cells of (**b**) a diabetic MCP-1(+/+) mouse kidney at 2 weeks after STZ treatment. In comparison, no MCP-1 mRNA was detected in the kidney of (**c**) a normoglycemic STZ-treated MCP-1(+/+) mouse, which had been given insulin to prevent the onset of diabetes. As expected, MCP-1 mRNA was also absent from the kidney of (**d**) a diabetic MCP-1(−/−) mouse at 2 weeks after STZ treatment. Original magnification: **a**–**d**, ×400.

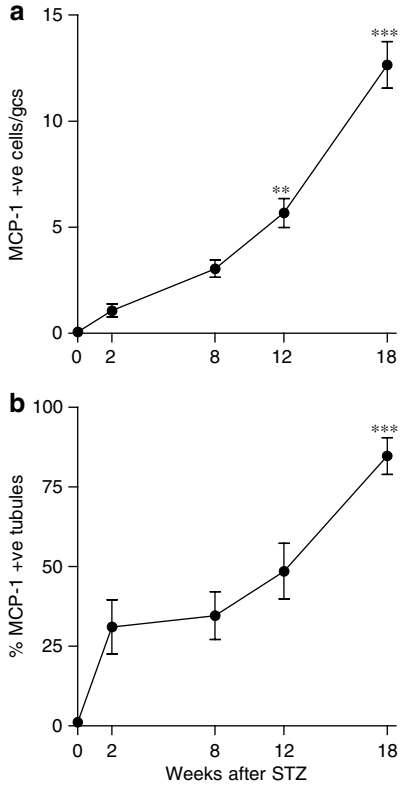

Figure 4 | Kidney MCP-1 expression increases with the progression of diabetes in STZ-treated mice. *In situ* hybridization analysis of diabetic kidneys from STZ-treated MCP-1(+/+) mice identified increasing numbers of (**a**) glomerular cells and (**b**) tubules expressing MCP-1 mRNA as the duration of diabetes progressed. Data are mean ± s.e.m.; $n = 8$–10; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs week 2 after STZ treatment.

diabetic kidneys, most glomerular and interstitial macrophages expressed CD169 and almost 20% of glomerular macrophages expressed iNOS; however, very few interstitial macrophages displayed iNOS expression. In comparison, MCP-1(−/−) diabetic kidneys had a reduced percentage of CD169+ macrophages and their percentage of iNOS+ macrophages was equal to nondiabetic mice.

Renal injury is attenuated in STZ-diabetic mice deficient in MCP-1

Urine albumin excretion was normal at the onset of STZ diabetes (2 weeks) but increased progressively in MCP-1(+/+) diabetic mice and was threefold greater than normal at 18 weeks after STZ (Figure 6a). In comparison, diabetic MCP-1(−/−) mice exhibited no increase in urine albumin excretion throughout disease, with levels remaining the same as nondiabetic mice at all time points (Figure 6a).

In the absence of diabetes, renal function assessed as the plasma creatinine level was similar in MCP-1(+/+) mice (31 ± 1.8 μM) and MCP-1(−/−) mice (31.3 ± 1.9 μM) at 26 weeks of age. Compared to nondiabetic animals, plasma

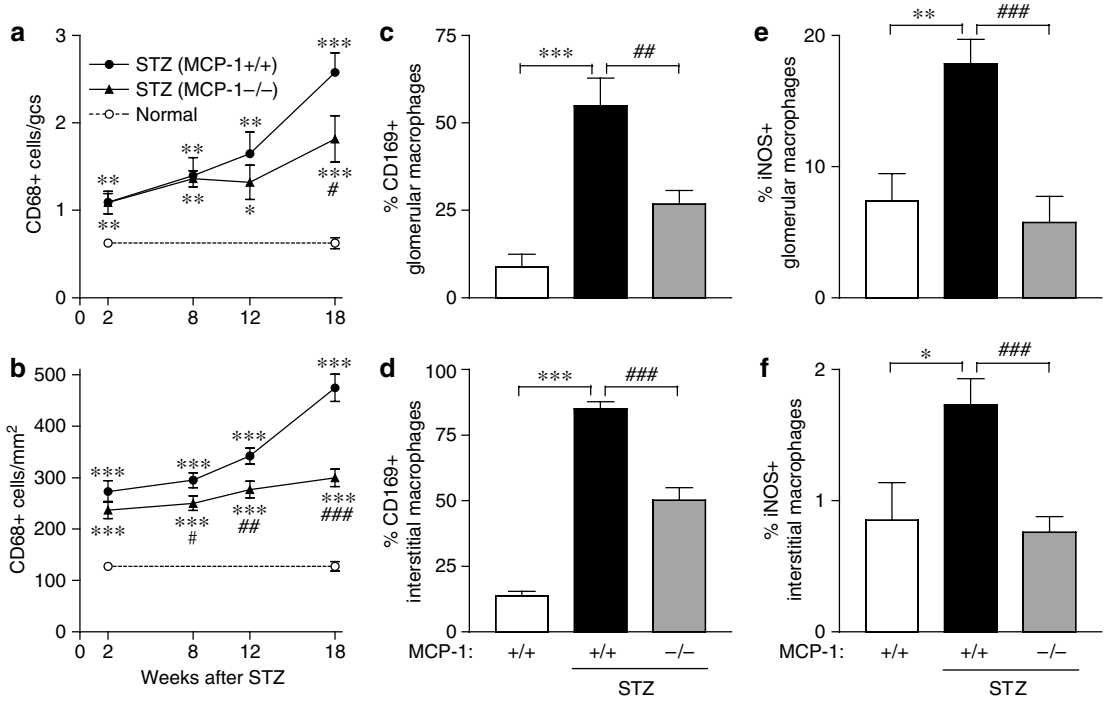

Figure 5 | Macrophage accrual and activation in STZ-treated diabetic kidneys. In comparison to normal nondiabetic mice, immunostaining identified a significant increase in CD68+ macrophage recruitment in (**a**) the glomeruli and (**b**) the interstitium of diabetic kidneys in STZ-treated MCP-1 (+/+) mice, which progressed with disease duration. At 12 and 18 weeks after STZ, MCP-1(−/−) mice had markedly less kidney macrophages than MCP-1(+/+) mice. An increased proportion of kidney macrophages in STZ-treated MCP-1(+/+) mice were found to express CD169 in (**c**) the glomeruli and (**d**) the interstitium compared to normal mice; however, this was significantly reduced in STZ-treated MCP-1(−/−) mice. Similarly, a greater percentage of (**e**) glomerular and (**f**) interstitial macrophages were found to express iNOS in STZ-treated MCP-1(+/+) mice compared to normal mice, whereas the percentage of macrophages expressing iNOS in STZ-treated MCP-1(−/−) mice was similar to normal. Data are mean±s.e.m.; $n=8$–10; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs normal; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs STZ MCP-1(+/+) mice. gcs= glomerular cross-section.

creatinine was elevated in diabetic MCP-1(+/+) mice at 2 weeks of disease and was not different from levels in diabetic MCP-1(−/−) mice (Figure 6b). This increase in plasma creatinine at 2 weeks was due to the development of diabetes and its impact on the kidney (e.g. hyperfiltration, tubular dysfunction), because prevention of hyperglycemia with insulin treatment for 2 weeks prevented this rise in plasma creatinine ($30.7\pm1.7\,\mu$M) in MCP-1(+/+) mice. Between 2 and 18 weeks of diabetes, MCP-1(+/+) mice showed a progressive increase in plasma creatinine, whereas the plasma creatinine in the MCP-1(−/−) diabetic mice remained unchanged and was similar to age-matched nondiabetic mice at 18 weeks after STZ.

The kidney-to-body weight ratio was increased in MCP-1(+/+) diabetic mice at week 12 ($1.10\pm0.05\%$) and week 18 ($0.91\pm0.02\%$) compared to nondiabetic mice ($0.80\pm0.04\%$, $P<0.05$), whereas the ratio in diabetic MCP-1(−/−) mice remained at normal levels at week 12 ($0.78\pm0.02\%$) and week 18 ($0.80\pm0.04\%$).

STZ-diabetic mice deficient in MCP-1 have reduced histological renal injury

In view of the impressive reduction in renal macrophage counts and albuminuria in the MCP-1(−/−) diabetic mice, we further assessed our experimental model for histological renal damage. Assessment of histological staining at 18 weeks of disease revealed that glomerular hypertrophy, glomerular hypercellularity and the percentage of damaged tubules were reduced in diabetic MCP-1(−/−) compared to diabetic MCP-1(+/+) mice (Table 1). Protection from tubular injury in mice lacking MCP-1 was detected early in the development of diabetes. At 2 weeks after STZ, there was a significant increase in the detection of atrophic tubules in diabetic MCP-1(+/+) mice ($0.39\pm0.08\%$ in STZ-treated vs normal $0.08\pm0.02\%$, $P<0.001$), which was reduced in diabetic MCP-1(−/−) mice ($0.15\pm0.04\%$, $P<0.01$) (Figure 7).

Immunostaining assessment of renal fibrosis found a significant increase in the expression of glomerular and interstitial collagen IV and interstitial accumulation of α–smooth muscle actin+ myofibroblasts in MCP-1(+/+) diabetic mice at 18 weeks, which was largely prevented in MCP-1(−/−) diabetic mice (Table 1).

DISCUSSION

Our study demonstrates that induction of kidney MCP-1 production by the development of diabetes is a critical factor in the progression of kidney macrophage accumulation and early diabetic renal injury in mice treated with STZ. Kidney macrophage accrual in MCP-1(+/+) mice increased

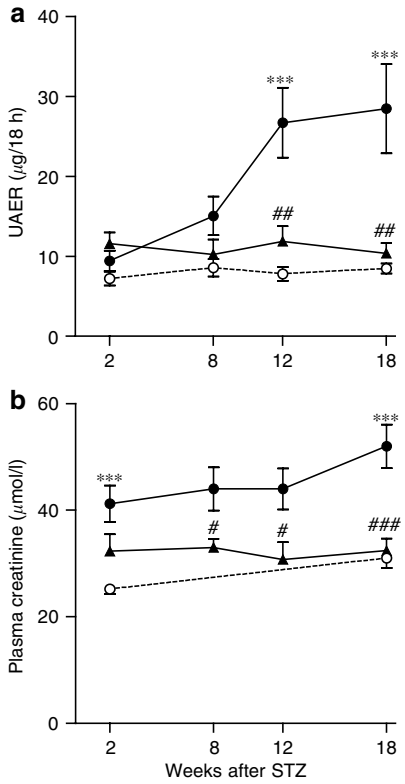

Figure 6 | Renal injury in STZ-treated diabetic mice. (**a**) The urine albumin excretion rate (UAER) was similar in normal and diabetic mice at 2 weeks after STZ treatment. At 12 and 18 weeks after STZ, the UAER was increased two- to three-fold in diabetic MCP-1(+/+) mice; however, the UAER in diabetic MCP-1(−/−) mice remained near normal at these time points. (**b**) The onset of diabetes resulted in increased plasma creatinine in STZ-treated MCP-1(+/+) mice at 2 weeks, which was further increased at week 18. In comparison, the plasma creatinine level in STZ-treated MCP-1(−/−) mice remained significantly lower than STZ-treated MCP-1(+/+) mice throughout the analysis period and was not different from normal animals. Data are mean ± s.e.m.; $n = 8$–10; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs normal; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs STZ MCP-1(+/+) mice.

Table 1 | Histopathology and renal fibrosis (at week 18 after STZ)

	Normal MCP-1(+/+)	Diabetic MCP-1(+/+)	Diabetic MCP-1(−/−)
Glomerular volume ($\mu m^3 \times 10^4$)	24.5 ± 1.3	30.6 ± 1.7[a]	25.9 ± 1.0[b]
Glomerular cellularity (cells/gcs)	31.7 ± 1.1	44.1 ± 1.5[c]	38.0 ± 1.3[a,b]
Glomerular collagen IV (% area)	15.3 ± 0.44	19.2 ± 0.58[c]	15.4 ± 0.63[d]
Atrophic tubules (%)	0.11 ± 0.02	8.85 ± 0.46[c]	3.98 ± 0.38[c,d]
Interstitial collagen IV (% area)	20.7 ± 0.61	25.3 ± 1.2[c]	20.6 ± 0.27[d]
Interstitial α-SMA (% area)	1.2 ± 0.09	7.82 ± 1.09[c]	2.3 ± 0.24[d]

Data are mean ± s.e.m.
[a] $P < 0.01$.
[b] $P < 0.05$.
[c] $P < 0.001$ vs normal.
[d] $P < 0.001$ vs diabetic MCP-1(+/+).
gcs, glomerular cross-section; SMA, smooth muscle actin.

progressively with the duration of diabetes and correlated with renal injury, renal expression of MCP-1 mRNA and urine MCP-1 levels. Examination of MCP-1(+/+) and (−/−) mice with equivalent diabetes found that MCP-1 deficiency

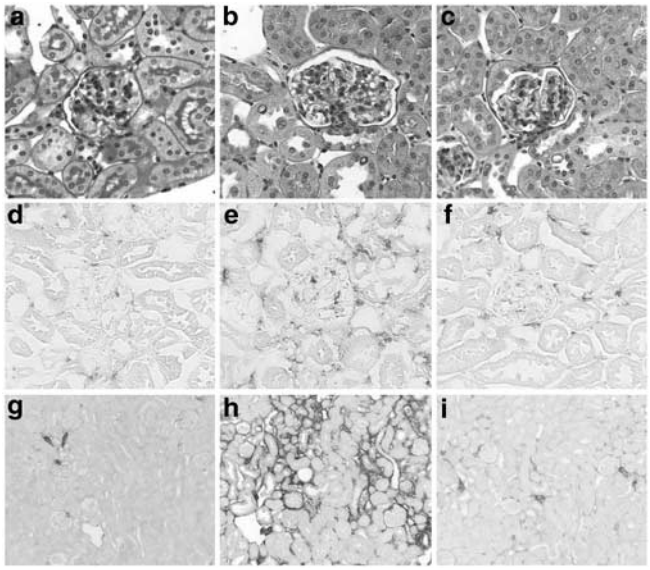

Figure 7 | Renal histopathology in STZ-treated diabetic mice. Histological staining with PAS and hematoxylin shows the normal kidney structure of (**a**) a nondiabetic mouse. In comparison, there is significant damage to the glomeruli (hypertrophy, hypercellularity, mesangial PAS deposits) and tubules (dilatation, atrophy) in (**b**) a diabetic MCP-1(+/+) mouse at 18 weeks after STZ, which is attenuated in (**c**) an MCP-1(−/−) mouse with equivalent diabetes. Immunostaining for CD68 shows very few macrophages in (**d**) a normal mouse kidney, which is markedly increased in (**e**) the glomeruli and interstitium of an MCP-1(+/+) mouse kidney at 18 weeks after STZ. In comparison, reduced numbers of kidney macrophages were detected in (**f**) an MCP-1(−/−) mouse with equivalent STZ-induced diabetes. Immunostaining of α-smooth muscle actin was detected in kidney vessels in (**g**) a normal mouse. Many interstitial cells expressing α-smooth muscle actin were identified in the diabetic kidney of (**h**) an MCP-1(+/+) mouse at 18 weeks after STZ, and these cells were markedly reduced in (**i**) an MCP-1(−/−) mouse with the same level of diabetes. Original magnifications: **a**–**f**, × 400; **g**, × 250.

markedly reduced kidney macrophage accumulation and prevented the development of diabetic nephropathy.

In vitro experiments demonstrated that a high glucose concentration (25 mmol/l), similar to blood glucose levels observed in diabetic mice, could induce MCP-1 secretion in cultured tubular cells, providing evidence that tubular MCP-1 production in diabetic kidneys could be due to a direct response to high blood glucose. This is supported by our *in situ* hybridization results showing that normalization of blood glucose with insulin prevents induction of kidney MCP-1 mRNA in STZ-treated mice. In addition, our *in vitro* studies indicate that the formation of advanced glycation end products due to hyperglycemia can also stimulate tubular MCP-1 secretion. Therefore, hyperglycemia can directly and indirectly promote kidney MCP-1 production during the development of diabetes.

The attenuation of renal injury and fibrosis in diabetic mice deficient in MCP-1 is associated with a reduction in kidney macrophage recruitment, which is consistent with the known function of MCP-1 *in vitro* and in other animal models.[9] Although macrophage recruitment may be the

major function of MCP-1 in diabetic nephropathy, it is also possible that MCP-1 could directly promote damage in intrinsic kidney cells or that MCP-1 may induce additional responses such as macrophage activation or the recruitment and activation of T cells. Previously, we have shown that MCP-1 does not directly injure tubular cells *in vitro*,[19] suggesting that the pathological actions of MCP-1 in the kidney are more likely to be indirect. In our current study, we found evidence that MCP-1 may be contributing to macrophage activation in diabetic mice. MCP-1(−/−) mice had reduced tubular injury compared to MCP-1(+/+) mice at 2 weeks after STZ when macrophage accumulation was similarly elevated in both strains. Therefore, we hypothesized that if MCP-1-dependent tubular injury is due to macrophages, there must be a difference in macrophage activity in the presence of MCP-1 to mediate this effect. Further examination found that MCP-1(+/+) diabetic kidneys contained a greater proportion of macrophages expressing CD169 and iNOS, supporting the concept of increased macrophage activation in the presence of MCP-1. CD169 promotes macrophage adhesion and is a macrophage marker of chronic inflammation.[20] Its expression on monocytes is known to increase with monocyte MCP-1 production,[21] indicating that MCP-1 may play a functional role in promoting macrophage expression of CD169 during diabetic nephropathy. Similarly, MCP-1 is known to induce iNOS and its product NO in cultured mouse macrophages, and these molecules are considered to be markers of macrophage activation.[12,22] Macrophage production of NO is thought to contribute to oxidative stress and renal injury during diabetes by promoting protein nitrosylation; however, this remains controversial.[23–25] Whether or not T cells play a role in diabetic nephropathy remains to be determined. Most forms of renal injury that are T-cell dependent exhibit similar increases in the kidney accumulation of macrophages and T-cells;[6] however, in diabetic nephropathy, the resident numbers of kidney T cells are virtually unchanged, whereas kidney macrophages increase progressively with disease. In our study, we found that MCP-1 deficiency had no impact on the recruitment of CD4+ and CD8+ T cells. However, their presence in diabetic kidneys, although small, does not rule out that MCP-1 may have an effect on T-cell activity. Indeed, *in vitro* studies have shown that MCP-1 can regulate T-cell differentiation,[26] which may contribute to renal injury directly or indirectly by enhancing macrophage activation.

In this study, we showed that MCP-1 is a major contributor to macrophage recruitment in mouse diabetic kidneys; however, its absence did not totally prevent macrophage accumulation. Other macrophage chemokines, such as osteopontin, migration inhibitory factor and RANTES, are also expressed by renal cells during diabetes[5,10] and may be involved in macrophage recruitment. In addition, diabetic kidneys produce increased levels of macrophage colony stimulating factor, which can increase macrophage numbers by inducing local proliferation.[5,27] Although MCP-1 deficiency did not entirely abrogate macrophage accrual, its effect was sufficient to prevent the development of diabetic renal injury. This suggests that total macrophage blockade may not be necessary for preventing nephropathy and it may be the state of macrophage activation, which is most important for progressive renal injury. Indeed, maintaining some kidney macrophages may be beneficial to normal renal health by preserving immune surveillance and phagocytic clearance.[6]

This study supports the growing evidence that macrophages are key mediators of renal damage in diabetic nephropathy. Elements of the diabetic milieu can promote macrophage recruitment by inducing renal expression of MCP-1 and cell adhesion molecules, and can stimulate macrophage responses that cause renal injury. *In vitro* studies have demonstrated that AGEs induce macrophage secretion of nitric oxide, reactive oxygen species, tumor necrosis factor-α and interleukin-1,[28–31] which promote inflammation and tissue damage. In addition, macrophages stimulated with high glucose or AGEs can promote renal fibrosis through the secretion of fibroblast growth factors (interleukin-1, platelet derived growth factor)[28,32] and active transforming growth factor-β1.[33] Therefore, macrophages can contribute to the progression of diabetic nephropathy through multiple mechanisms.

In summary, this study has demonstrated that MCP-1-mediated macrophage accumulation and activation is a critical mechanism in the development of early diabetic nephropathy in STZ-treated mice. As upregulation of kidney MCP-1 is a feature of human diabetic renal injury associated with macrophage recruitment and disease progression, neutralizing MCP-1 activity should be viewed as an important therapeutic goal in the treatment of diabetic nephropathy.

MATERIALS AND METHODS

Animal model

Mice genetically deficient in MCP-1[34] were backcrossed 8 times onto the C57BL/6J strain and compared with MCP-1-intact C57BL/6J mice. Both strains were maintained on a normal diet under standard animal house conditions. To induce diabetes, MCP-1(+/+) and (−/−) male mice at 8 weeks of age were given intraperitoneal injections of STZ (Sigma-Aldrich, St Louis, MO) at 125 mg/kg/day for 2 consecutive days.[28] This was the minimum dose of STZ required to induce overt diabetes (blood glucose >16 mmol/l) in at least 50% of MCP-1(−/−) mice at 2 weeks after administration. Blood glucose was measured via tail vein sampling at specified times (between 0900 and 1000 hours) on alert, nonfasted animals (Medisense glucometer, Abbott Laboratories, Bedford, MA). Groups of MCP-1(+/+) and (−/−) diabetic mice with equivalent blood glucose (25 ± 3 mmol/l) were selected at week 2 ($n=8$–10) and monitored until being killed at 2, 8, 12 or 18 weeks after STZ in order to evaluate kidney macrophage accumulation and renal injury. HbA1c was determined from cardiac blood when animals were killed. Groups of nondiabetic MCP-1(+/+) mice ($n=10$) were assessed for blood glucose and albuminuria and then killed when age-matched to week 2 STZ-treated mice (10 weeks of age) and week 18 STZ-treated mice (26 weeks of age) to obtain control tissue and blood. Analysis was also performed on nondiabetic MCP-1(−/−) mice ($n=6$) killed at 26 weeks of age. In addition, tissue and blood samples were also collected from MCP-1(+/+) mice ($n=5$) at 2

weeks after STZ, which were prevented from developing hyperglycemia by twice daily subcutaneous administration of 0.5 U isophane insulin (Protophane, Novo Nordisk, Sydney).

At 12–18 weeks, a minority of mice in each diabetic group (10–15%) began showing a progressive weight loss associated with extreme hyperglycemia (blood glucose >30 mmol/l) and were given isophane insulin to prevent further weight loss while maintaining diabetes.[28]

Biochemical analysis

Urine was collected from mice housed in metabolic cages for 18 h (between 1600 and 1000). Whole blood was collected in the presence of heparin via cardiac puncture of anaesthetized mice. HbA1c levels and plasma creatinine were analyzed by the Department of Biochemistry at the Monash Medical Centre. Urine albumin was measured by the ELISA kit (Bethyl Laboratories, Montgomery, TX).

Antibodies

Antibodies used in this study were as follows: rat anti-mouse CD45 (M1/9.3.4); rat anti-mouse CD68 (FA-11, Serotec, Oxford, UK); rat anti-mouse CD4 (GK1.5); rat anti-mouse CD8 (YTS169.4); rat anti-CD169 (Serotec); goat anti-mouse collagen IV (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-iNOS (Santa Cruz Biotechnology) and fluorescein-conjugated anti-α–smooth muscle actin (1A4, Sigma). Isotype-matched irrelevant IgGs were used as negative controls. Noncommercial antibodies were produced by cell culture of hybridomas obtained from the American Tissue Culture Collection (ATCC, Manassas, VA).

Renal pathology

Formalin-fixed kidney sections (2 μm) were stained with periodic acid Schiff's (PAS) reagent and hematoxylin. Digital images of glomeruli and interstitial areas were obtained from microscopy (magnification ×400). Glomerular cellularity was determined by counting the cell nuclei in 20 hilar gcs per animal. Glomerular volume was calculated by measuring the glomerular tuft area with computer image analysis. The percentage of atrophic tubules (dilatation, cell loss, necrosis) was assessed by scoring 400 renal cortical tubules per kidney in randomly selected microscopic fields. All scoring was performed on blinded slides.

In situ hybridization

cDNA fragments of rat MCP-1 (407 bp) were amplified by reverse transcription polymerase chain reaction and cloned into the pMOSBlue vector (Amersham Pharmacia Biotech, Sydney, Australia). Sense and antisense riboprobes for MCP-1 were labelled with digoxigenin (DIG)-UTP using a T7 RNA polymerase kit (Roche Biochemicals, Mannheim, Germany). DIG-labelled antisense cRNA probes were used to detect MCP-1 in formalin-fixed tissue sections by *in situ* hybridization.[5]

Immunohistochemistry staining

Immunoperoxidase staining for leukocytes (CD45, CD68, CD4, CD8), macrophages expressing iNOS (iNOS+/CD68+) or CD169 (CD169+/CD68+), and collagen IV was performed on paraformaldehyde-lysine-periodate fixed kidney sections (5 μm). Sections were incubated for 20 min each with 0.6% hydrogen peroxide followed by avidin and biotin block (Vector Laboratories, Burlingame, CA) and 20% normal sheep serum. Sections were then incubated overnight at 4°C with 5 μg/ml of primary antibody in 1% BSA. Thereafter, sections were incubated sequentially with biotinylated goat anti-rat IgG (1:200, Vector Laboratories) or biotinylated rabbit anti-goat IgG (1:200, Zymed, San Francisco, CA) for 45 min followed by avidin–biotin–peroxidase complex (ABC Kit, Vector Laboratories) for 45 min and developed with diaminobenzidine (Sigma). For evaluating macrophages expressing iNOS or CD169, tissue sections that were immunostained for CD68 were microwave-treated to retrieve antigens, prevent antibody crossreactivity and inactivate endogenous alkaline phosphatase.[35] Following microwave treatment, sections were incubated with 20% normal sheep serum/5% BSA (20 min) and then with rabbit anti-iNOS overnight at 4°C (1:100, Santa Cruz) or rat anti-mouse CD169 (1:200, Serotec) in 1% BSA. Sections labelled with iNOS antibody were then incubated at room temperature for 1 h with alkaline phosphatase-conjugated sheep anti-rabbit IgG (1:50, Dako) and developed with Fast Blue BB salt (Sigma).[5] Sections labelled with CD169 antibody were incubated sequentially with avidin, biotin, biotinylated goat anti-rat IgG, avidin–biotin–alkaline phosphatase complex (Vector Laboratories) and were then developed with Fast Blue BB salt.

Quantitation of immunohistochemistry and *in situ* hybridization

Immunostained glomerular cells (leukocytes, iNOS, CD169) and glomerular cells expressing MCP-1 mRNA were counted at high magnification (×400) in 20 gcs per animal. Immunostained interstitial cells (leukocytes, iNOS, CD169) were counted in 25 consecutive high-power (×400) interstitial fields (representing 30–40% of kidney cortex in the cross-section) by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscope and expressed as cells/mm^2. Collagen IV and α-smooth muscle actin expressions were assessed as percent area stained within the glomerular tuft or the cortical interstitium by image analysis software (Image Pro Plus, Media Cybernetics, CA). The percentage of tubules expressing MCP-1 mRNA was assessed by scoring 400 renal cortical tubules per kidney cross-section in randomly selected microscopic fields. All scoring was performed on blinded slides.

Analysis of MCP-1 production by tubular epithelial cells

Proximal tubular epithelial cells (NRK52E) were seeded into 24-well tissue culture plates at 1×10^5cells/well in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and allowed to adhere overnight. The next day, medium was replaced with 1% FCS/DMEM and cells were cultured for 24 h. The following day, when cells were confluent, media were replaced with serum-free media containing insulin-transferrin-selenium supplement (ITS, Invitrogen) and either 5 mmol/l D-glucose (low glucose), 25 mmol/l D-glucose (high glucose), 5 mmol/l D-glucose and 20 mmol/l L-glucose (osmotic control), control–BSA (200 μg/ml) or AGE–BSA (200 μg/ml). After incubation for 24 h, the cell supernatant was collected and assessed for MCP-1 content by the ELISA kit (Pharmingen, San Diego, CA). The cell number in each well was counted using a hemacytometer (Reichert, Buffalo, NY). Data were collected from three separate experiments.

To prepare AGE–BSA, 4 mM BSA was mixed with an equal volume of 1 M D-glucose and filtered (0.2 μm). This solution was then incubated in the dark at 37°C for 12 weeks under sterile anaerobic conditions. Control BSA was made by the same procedure without D-glucose. These preparations were purified to remove endotoxin as previously described.[28]

Statistical analysis

Statistical differences between two groups were analyzed by the unpaired Student's *t*-test (parametric data) or the Mann–Whitney *U*-test (nonparametric data), and differences between multiple groups of data were assessed by one-way analysis of variance using either Bonferroni's or Tukey's multiple comparison test. Correlation analyses were performed using Pearson's coefficient (parametric data) or Spearman's coefficient (nonparametric data). Data were recorded as the mean $\pm$ s.e. and values of $P<0.05$ were considered significant. All analyses were accomplished using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

ACKNOWLEDGMENTS

This work was supported by a PhD scholarship (Fiona Chow) and a grant from the National Health and Medical Research Council of Australia.

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