Kidney International, Vol. 65 (2004), pp. 116–128

Macrophages in mouse type 2 diabetic nephropathy: Correlation with diabetic state and progressive renal injury

FIONA CHOW, ELYCE OZOLS, DAVID J. NIKOLIC-PATERSON, ROBERT C. ATKINS, and GREGORY H. TESCH

Department of Nephrology and Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia

Macrophages in mouse type 2 diabetic nephropathy: Correlation with diabetic state and progressive renal injury.

Background. Macrophage-mediated renal injury has been implicated in progressive forms of glomerulonephritis; however, a role for macrophages in type 2 diabetic nephropathy, the major cause of end-stage renal failure, has not been established. Therefore, we examined whether macrophages may promote the progression of type 2 diabetic nephropathy in db/db mice.

Methods. The incidence of renal injury was examined in db/db mice with varying blood sugar and lipid levels at 8 months of age. The association of renal injury with the accumulation of kidney macrophages was analyzed in normal db/+ and diabetic db/db mice at 2, 4, 6, and 8 months of age.

Results. In db/db mice, albuminuria and increased plasma creatinine correlated with elevated blood glucose and hemoglobin A_{1c} (HbA_{1c}) levels but not with obesity or hyperlipidemia. Progressive diabetic nephropathy in db/db mice was associated with increased kidney macrophages. Macrophage accumulation and macrophage activation in db/db mice correlated with hyperglycemia, HbA_{1c} levels, albuminuria, elevated plasma creatinine, glomerular and tubular damage, renal fibrosis, and kidney expression of macrophage chemokines [monocyte chemoattractant protein-1 (MCP-1), osteopontin, migration inhibitory factor (MIF), monocyte-colony-stimulating factor (M-CSF)]. The accrual and activation of glomerular macrophages also correlated with increased glomerular IgG and C3 deposition, which was itself dependent on hyperglycemia.

Conclusion. Kidney macrophage accumulation is associated with the progression of type 2 diabetic nephropathy in db/db mice. Macrophage accumulation and activation in diabetic db/db kidneys is associated with prolonged hyperglycemia, glomerular immune complex deposition, and increased kidney chemokine production, and raises the possibility of specific therapies for targeting macrophage-mediated injury in diabetic nephropathy.

Key words: db/db mice, chemokines, immune complex, glomerular injury, tubular injury, renal fibrosis.

Received for publication June 5, 2003
and in revised form July 24, 2003
Accepted for publication August 11, 2003

Diabetic nephropathy is the major cause of end-stage renal failure worldwide, with most cases due to the growing incidence of type 2 diabetes [1]. Current treatments, involving glycemic and blood pressure control, can slow the development of diabetic nephropathy, but do not stop the progression to end-stage renal failure [1, 2]. Therefore, it is important to identify new strategies and additional therapeutic targets for treating diabetic nephropathy.

Diabetic nephropathy is generally considered a non-immune disease; however, examination of human biopsies and animal models has shown the presence of macrophages in diabetic kidneys [3–8]. Macrophages are known to cause renal injury in experimental models of immune-mediated kidney disease and they correlate with renal impairment in human glomerulonephritis [9], but their role in diabetic nephropathy is poorly understood. It is unclear whether increased numbers of kidney macrophages in type 2 diabetes is simply a response to obesity per se, which itself can induce macrophage accrual [10, 11], or if hyperglycemia is the key factor driving macrophage accumulation. Furthermore, we do not know whether kidney macrophages correlate with renal impairment in type 2 diabetes and what mechanisms are involved in their accumulation.

In the current study, we address these questions in the db/db mouse model in which all mice become obese, but only a portion develop hyperglycemia. Because db/db mice have many similarities to the human disease [12], the findings of this study are likely to be important in understanding the role of macrophages in human type 2 diabetic nephropathy.

METHODS

Animal model

Type 2 diabetic mice (db/db) and normal heterozygote controls (db/+) were created from breeding pairs of C57BL/6 db/+ mice obtained from Jackson Laboratories (Bar Harbor, ME, USA) and identified by polymerase

chain reaction (PCR) tissue type analysis for the leptin receptor. No phenotypic differences were found between age-matched db/+ and C57BL/6 mice, which are homozygous (+/+) for the leptin receptor. Mice were maintained on a normal diet under standard animal house conditions and were examined for diabetes from 2 months of age. Mice were assessed every month for blood glucose (glucometer) and every 2 months for albuminuria. Hemoglobin A_{1c} (HbA_{1c}) (a marker of protein glycation) was determined from cardiac blood when animals were killed. An initial study (experiment 1) was performed on a group of 15 male and 12 female db/db mice, which were examined for the incidence of obesity, hyperlipidemia, hyperglycemia, and renal injury (albuminuria, increased plasma creatinine) and killed at 8 months of age. In a follow-up study (experiment 2), groups of male diabetic db/db mice ($N = 7$ to 10) and nondiabetic db/+ mice ($N = 5$ to 10) were killed at 2, 4, 6, or 8 months of age to evaluate kidney macrophage accumulation in relation to the progression of diabetic nephropathy.

Biochemical analysis

Urine was collected from mice housed in metabolic cages for 18 hours. Whole blood was collected in the presence of heparin via cardiac puncture of anesthetized mice. Triglycerides, cholesterol, HbA_{1c} levels, urine creatinine, and plasma creatinine were analyzed by the Department of Biochemistry at the Monash Medical Centre. Urine albumin was measure by enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX, USA).

Antibodies

Antibodies used in this study were rat antimouse CD45 (M1/9.3.4); rat antimouse CD68 (FA-11) (Serotec, Oxford, UK); rat antimouse CD4 (GK1.5); rat antimouse CD8 (YTS169.4); goat antimouse collagen IV (Santa Cruz Biotechnologies, Santa Cruz, CA, USA); rabbit anti-inducible nitric oxide synthase (iNOS) (Santa Cruz Biotechnologies); fluorescein-conjugated antiproliferating cell nuclear antigen (PCNA) (19A4) (Roche Biochemicals, Mannheim, Germany); antiactivated caspase-3 (Asp175) (Cell Signaling Technology, Beverly, MA, USA); fluorescein isothiocyanate (FITC)-conjugated sheep antimouse IgG $F(ab)_2$ fragments (Silenus, Melbourne, Australia); and FITC-conjugated goat antimouse C3 $F(ab)_2$ fragments (ICN Pharmaceuticals, Aurora, Ohio, USA). Isotype-matched irrelevant IgGs were used as negative controls. Antibodies not purchased from commercial sources were produced by cell culture of hybridomas obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA, USA).

Renal pathology

Formalin-fixed kidney sections (2 μm) were stained with periodic acid-Schiff (PAS) reagent to identify kidney structure and hematoxylin to distinguish cell nuclei. Digital images of glomeruli and interstitial areas were obtained from microscopy (magnification × 400). Glomerular cellularity was determined by counting the number of nuclei in 20 hilar glomerular tuft cross-sections (gcs) per animal. Glomerular volume was assessed by measuring the glomerular tuft area with computer image analysis. The mesangial matrix fraction was determined from the percentage area of PAS-stained material within the glomerular tuft. The percentage of atrophic tubules (dilatation, cell loss, necrosis) was assessed by scoring 400 renal cortical tubules per kidney in randomly selected microscopic fields. Interstitial volume was determined by point counting of at least 30% of the renal cortex area. All scoring was performed on blinded slides.

Probes

cDNA fragments of rat monocyte-colony-stimulating factor (M-CSF) (758 bp), rat migration inhibitory factor (MIF) (441 bp), rat monocyte chemoattractant protein-1 (MCP-1) (407 bp), and rat osteopontin (1083 bp) were amplified by reverse transcription (RT)-PCR and cloned into the pMOSBlue vector (Amersham Pharmacia Biotech, Sydney, Australia). Sense and antisense riboprobes for M-CSF, MIF, MCP-1, and osteopontin were labeled with digoxigenin (DIG)-uridine triphosphate (UTP) using a T7 RNA polymerase kit (Roche Biochemicals).

In situ hybridization

In situ hybridization to detect MCP-1, osteopontin, M-CSF, and MIF were performed on tissue sections fixed in 10% formalin or 2% paraformaldehyde-lysine-periodate (PLP) using DIG-labeled sense or antisense cRNA probes as previously described [11]. The hybridized probe was detected using alkaline phosphatase-conjugated sheep anti-DIG IgG and color development with NBT/BCIP (Roche Biochemicals).

Kidney deposition of IgG and C3

IgG and C3 deposits in the kidney were assessed in 4 μm snap-frozen tissue sections fixed in 100% ethanol for 10 minutes at 4°C. Tissue sections were rehydrated in phosphate-buffered saline (PBS) and incubated in 20% normal sheep serum for 30 minutes, followed by fluorescein-conjugated antibodies detecting mouse IgG (1:200) or C3 (1:50) for 30 minutes, washing, and mounting in antifade medium containing 5% DABCO (1,4-diazabicyclo[2.2.2] octane) (Sigma Chemical Co., St. Louis, MO, USA) and 70% glycerin in PBS (pH 8.5).

Immunofluorescence was assessed in 20 glomerular cross-sections per animal by semiquantitative scoring (0 to 5) of the area of glomerular tuft stained: 0 = <1%, 1 = 1% to 10%, 2 = 10% to 25%, 3 = 25% to 50%, 4 = 50% to 75%, and 5 = 75% to 100%.

Immunohistochemistry staining

Immunoperoxidase staining for leukocytes (CD45, CD68, CD4, and CD8), macrophage proliferation (CD68/PCNA), macrophages expressing iNOS (CD68/iNOS), and collagen IV were performed on PLP-fixed kidney cryostat sections (5 μm). Immunostaining for apoptotic cells (activated caspase-3) were performed on formalin-fixed kidney paraffin sections (4 μm) [13]. Tissue sections were incubated for 20 minutes with 0.6% hydrogen peroxide followed by avidin and biotin block (Vector Laboratories, Burlingame, CA, USA) and 20% normal sheep serum to prevent nonspecific detection. Sections were then incubated overnight at 4°C with 5μg/mL of primary antibody in 1% bovine serum albumin (BSA). After washing in PBS, sections were incubated with biotinylated goat antibodies (antirat IgG, antimouse IgG or antirabbit IgG, 1:200) (Vector Laboratories) or biotinylated rabbit antibody (antigoat IgG, 1:200 Zymed, San Francisco, CA, USA) for 1 hour followed by ABC solution (ABC Kit) (Vector Laboratories) for 1 hour and developed with 3,3-diaminobenzidine (DAB) (Sigma Chemical Co.) to produce a brown color. For evaluating macrophages expressing PCNA or iNOS, tissue sections that were immunostained for CD68 were microwave-treated at 800W for 12 minutes in 10 mmol/L sodium citrate (pH 6) to retrieve nuclear antigens, prevent antibody cross-reactivity and inactivate endogenous alkaline phosphatase [14]. Following microwave treatment, sections were maintained at 4°C during incubations with 20% normal sheep serum/5% BSA (20 minutes) and then either fluorescein-conjugated anti-PCNA monoclonal antibodies (1:100) (Roche Biochemical) or rabbit anti-iNOS (1:100) (Santa Cruz Biotechnologies). After washing, sections were incubated at room temperature for 1 hour with alkaline phosphatase-conjugated sheep antibodies recognizing either fluorescein (1:300) (Roche Biochemical) or rabbit IgG (1:50) (Dako, Carpinteria, CA, USA) and developed with Fast Blue BB salt (Sigma Chemical Co.).

Quantitation of immunohistochemistry and in situ hybridization

Immunostained glomerular cells (leukocytes, PCNA, and iNOS) and glomerular cells expressing mRNA for chemokines (MCP-1, osteopontin, MIF, and M-CSF) were counted under high power (×400) in 20 hilar glomerular tuft cross-sections per animal. Immunostained interstitial cells (leukocytes and iNOS) were counted in 25 consecutive high power (×400) interstitial fields (representing 30% to 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. Expression of collagen IV was assessed as percent area stained within the glomerular tuft and cortical interstitium by computer image analysis. Tubular apoptosis was determined by counting the percentage of cortical tubules with cells expressing activated caspase-3 in 1000 tubular cross-sections (tcs) per animal. The percentage of tubules expressing mRNA for chemokines (MCP-1, osteopontin, MIF, and M-CSF) was assessed by scoring 400 renal cortical tubules per kidney cross-section in randomly selected microscopic fields. All scoring was performed on blinded slides.

Table 1. Characteristics of diabetic and nondiabetic db/db mice at 8 months (experiment 1)

	Total	Diabetic	Nondiabetic
Animals *number*	27	8	19
Blood glucose *mmol/L*	14.4 ± 10.2 (4.5–33)	28.8 ± 5.8[a] (16–33)	8.3 ± 2.5 (4.5–13.3)
Hemoglobin A_{1c} %	5.7 ± 2.4 (3.0–11.6)	8.6 ± 2.5[a] (4.7–11.6)	4.4 ± 0.8 (3.0–5.6)
Cholesterol *mmol/L*	4.6 ± 2.7 (1.8–12.5)	2.4 ± 0.7[b] (1.8–3.3)	5.6 ± 2.6 (3.4–12.5)
Triglycerides *mmol/L*	1.6 ± 1.6 (0.4–7.9)	2.8 ± 2.5[b] (0.6–7.9)	1.1 ± 0.6 (0.4–2.7)
Body Weight *g*	55.3 ± 11.4 (31.3–70.6)	40.7 ± 9.5[a] (31.3–58.4)	61.4 ± 4.1 (55.5–70.6)

Data are mean ± SD (range).
[a] $P < 0.0005$ vs. nondiabetic; [b] $P < 0.01$ vs. nondiabetic.

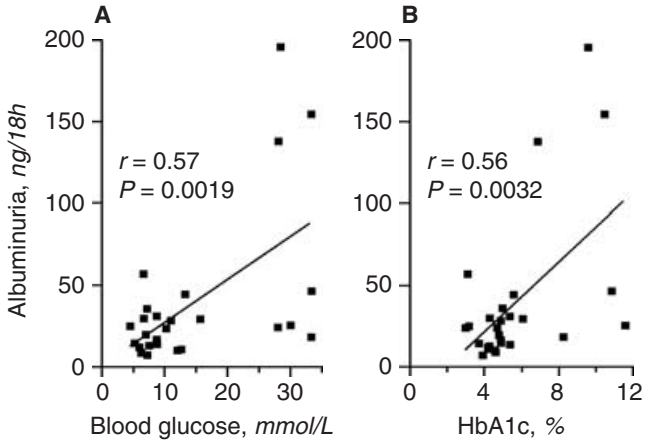


Fig. 1. Blood glucose and haemoglobin A_{1c} (HbA_{1c}) levels correlate with renal injury in db/db mice. A group of 15 male and 12 female db/db mice were assessed at 8 months of age for albuminuria, blood glucose, and HbA_{1c}levels. Albuminuria was found to correlate with (*A*) blood glucose and (*B*) HbA_{1c} levels. *r* = Pearson's coefficient.

Statistical analysis

Statistical differences between two groups were analyzed by the unpaired Student *t* test (parametric data) or

Table 2. Characteristics of normal db/+ and diabetic db/db mice with aging (experiment 2)

	db/+				db/db			
Age *months*	2	4	6	8	2	4	6	8
Animals *number*	5	10	10	9	7	7	9	7
Blood glucose *mmol/L*	6.1 ± 0.9	6.8 ± 0.6	6.9 ± 0.8	6.3 ± 1.0	11.7 ± 3.7[a]	18.5 ± 9.9[b]	19.8 ± 10[a]	28.9 ± 6.2[c]
Hemoglobin A_{1c} %	2.9 ± 0.7	2.6 ± 0.4	2.9 ± 0.3	2.9 ± 0.3	7.0 ± 1.2[c]	6.7 ± 0.9[c]	7.4 ± 2.0[c]	9.1 ± 2.1[c]
Cholesterol *mmol/L*	3.0 ± 0.7	3.1 ± 0.6	2.9 ± 0.7	2.8 ± 1.0	3.0 ± 0.7	2.5 ± 0.6	4.0 ± 1.5	2.4 ± 0.7
Triglycerides *mmol/L*	1.1 ± 0.4	1.1 ± 0.4	1.3 ± 0.5	1.1 ± 0.2	1.0 ± 0.2	0.7 ± 0.1	1.3 ± 0.8	3.1 ± 2.5
Body weight *g*	25.5 ± 1.2	34.9 ± 2.4	34 ± 3.6	37.5 ± 2.9	36.7 ± 2.6[c]	49.0 ± 4.8[c]	50.7 ± 7.8[c]	42.0 ± 9.5

Data are mean ± SD.
[a] $P < 0.01$; [b] $P < 0.05$; [c] $P < 0.0005$ vs. normal db/+ mice.

the Mann Whitney U test (nonparametric data), and differences between multiple groups of data were assessed by one-way analysis of variance (ANOVA) with Bonferroni'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 ± SD and values of $P < 0.05$ were considered significant. All analyses were accomplished using the software in GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Incidence of type 2 diabetes and renal injury in db/db mice (experiment 1)

By 2 months of age, all db/db mice became obese with a 50% increase in body weight (37 ± 2 g) compared to the lean db/+ control mice (24 ± 2 g). At 4 months, db/db mice displayed blood glucose levels ranging from normal to hyperglycemic (6 to 33 mmol/L). Of these animals, 47% of male (7/15) and 8% of female db/db (1/12) mice went on to develop overt diabetes (blood glucose ≥16 mmol/L at 8 months) (Table 1). Examination of db/db mice at 8 months demonstrated significant correlations between blood glucose levels and renal injury [albuminuria, $r = 0.57$; $P = 0.002$ (Fig. 1A); plasma creatinine, $r = 0.39$, $P = 0.04$]; and between HbA_{1c} levels and renal injury [albuminuria, $r = 0.55$, $P = 0.003$ (Fig. 1B); plasma creatinine, $r = 0.42$, $P = 0.03$]. In comparison, blood triglyceride levels were elevated in most (5/8) diabetic db/db mice (Table 1) but showed no correlation with renal injury (albuminuria, $r = 0.35$, $P = 0.07$; plasma creatinine, $r = 0.26$, $P = 0.19$). In contrast, cholesterol levels were elevated in nondiabetic db/db mice compared to diabetic db/db mice (Table 1), showing a correlation with body weight ($r = 0.50$, $P = 0.0078$) and an inverse correlation with hyperglycemia ($r = -0.61$, $P = 0.0008$). Furthermore, cholesterol levels did not correlate with renal injury (albuminuria, $r = -0.37$, $P = 0.06$; plasma creatinine, $r = -0.17$, $P = 0.41$). Most diabetic db/db mice showed either no gain or a loss of body weight between 6 and 8 months, which resulted in an inverse correlation between body weight and albuminuria ($r = -0.55$, $P = 0.002$), but no correlation between body weight and plasma creatinine ($r = 0.32$, $P = 0.10$).

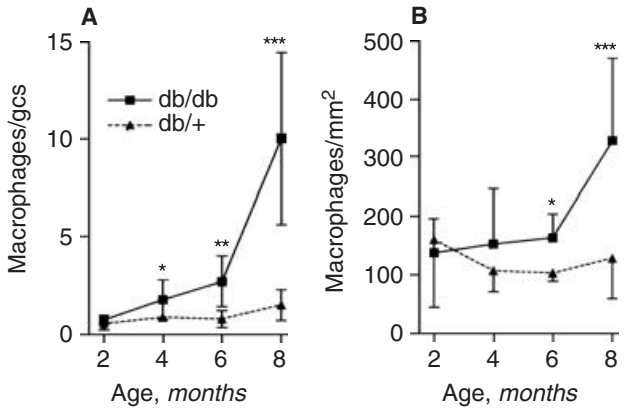


Fig. 2. Kidney macrophage accumulation in db/db and db/+ mice. Immunostaining analysis identified increases in (*A*) glomerular macrophages and (*B*) interstitial macrophages during the progression of type 2 diabetes in db/db mice. In comparison, age-matched, normal db/+ control mice have no significant increase in glomerular or interstitial macrophages. Data = mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0005$.

Kidney macrophage accumulation is associated with hyperglycaemia in diabetic mice (experiment 2)

Following the onset of diabetes in male db/db mice, there was a progressive increase in blood glucose and HbA_{1c} levels between 2 and 8 months of age, which was significantly greater than normal male db/+ mice at all time points (Table 2). Similarly, a progressive increase in kidney leukocytes was identified in diabetic male db/db mice, which was not seen in normal db/+ controls (Fig. 2).

Comparison of total kidney leukocytes (CD45+) with the numbers of macrophages (CD68+) and T cells (CD4+, CD8+) at 8 months of age indicated that almost all glomerular leukocytes (CD45, 11.0 ± 4.3 cells/gcs; CD68, 10.0 ± 4.4 cell/gcs; CD4, 0.19 ± 0.08 cells/gcs; CD8, 0.014 ± 0.006 cells/gcs) and interstitial leukocytes (CD45, 350 ± 66 cells/mm^2; CD68, 330 ± 65 cells/mm^2; CD4, 8.9 ± 0.9 cells/mm^2; CD8, 1.4 ± 0.5 cells/mm^2) in diabetic db/db kidneys were macrophages. During the progression of diabetes, increased numbers of kidney macrophages

Table 3. Correlation of body characteristics with kidney macrophages in normal db/+ and diabetic db/db mice

	Glomerular macrophages		Interstitial macrophages	
	db/+	db/db	db/+	db/db
Blood glucose	−0.27 (NS)	0.52 (0.0032)	−0.42 (0.013)	0.48 (0.0067)
Hemoglobin A_{1c}	0.059 (NS)	0.55 (0.0019)	−0.068 (NS)	0.38 (0.041)
Triglycerides	−0.15 (NS)	0.27 (NS)	−0.17 (NS)	0.23 (NS)
Cholesterol	0.14 (NS)	−0.31 (NS)	−0.022 (NS)	−0.29 (NS)
Body weight	0.39 (0.027)	−0.18 (NS)	−0.19 (NS)	−0.22 (NS)

Analysis was performed on combined groups of either db/+or db/db mice from 2, 4, 6 and 8 months.
Data are *r* value based on Pearson's correlation coefficient; (*P* value); NS is not significant.

Table 4. Renal analysis of normal db/+ and diabetic db/db mice

	db/+				db/db			
Age *months*	2	4	6	8	2	4	6	8
Animals *number*	5	10	10	9	7	7	9	7
Renal dysfunction								
Urine *mL*	0.4 ± 0.1	1.4 ± 0.5	1.0 ± 0.3	1.3 ± 0.6	0.7 ± 0.5	1.6 ± 0.7	2.1 ± 0.8[a]	3.9 ± 1.2[a]
Albuminuria *ng/18 hours*	3.9 ± 1.6	15.3 ± 6.7	6.4 ± 4.8	8.0 ± 3.7	31 ± 19[b]	31 ± 15[b]	33 ± 16[a]	87 ± 74[b]
Plasma creatinine μ*mol/L*	33 ± 4	33 ± 7	36 ± 10	30 ± 7	31 ± 8	29 ± 9	35 ± 7	63 ± 63[b]
Glomerular								
Volume $\mu m^3 \times 10^4$	26.5 ± 5.2	33.0 ± 6.3	24.1 ± 4.9	26.1 ± 6.7	26.3 ± 5.0	25.1 ± 3.9	40.8 ± 7.7[c]	47.9 ± 7.8[c]
Cellularity *cells/gcs*	35.9 ± 2.3	30.6 ± 4.4	31.8 ± 3.7	36.5 ± 2.3	31.3 ± 3.2	32.8 ± 4.1	44.8 ± 4.1[c]	53.1 ± 4.4[c]
IgG deposits (0–5)	1.2 ± 0.4	1.6 ± 0.5	1.8 ± 0.4	1.8 ± 0.4	1.7 ± 0.5	2.3 ± 0.5[b]	3.2 ± 0.8[c]	4.4 ± 0.5[c]
C3 deposits (0–5)	0.2 ± 0.5	0.7 ± 0.5	0.9 ± 0.6	1.0 ± 0.0	0.6 ± 0.5	1.4 ± 0.5[b]	1.7 ± 0.5[c]	2.9 ± 0.7[c]
Matrix fraction %	26.8 ± 2.0	27.0 ± 1.2	27.2 ± 1.2	29.3 ± 2.9	30.6 ± 4.1	33.2 ± 0.8[a]	43.7 ± 4.0[c]	46.6 ± 4.4[c]
Collagen IV % *area*	19.1 ± 0.7	19.5 ± 1.0	18.3 ± 0.9	18.7 ± 1.3	20.4 ± 0.1	21.2 ± 1.5[a]	23.8 ± 1.8[c]	27.8 ± 0.7[c]
Tubules								
Atrophic %	0.16 ± 0.06	0.16 ± 0.06	0.17 ± 0.07	0.17 ± 0.04	0.28 ± 0.14	0.39 ± 0.39	1.0 ± 0.69[a]	3.3 ± 1.3[a]
Apoptotic %	0.11 ± 0.1	0.07 ± 0.09	0.13 ± 0.13	0.10 ± 0.13	0.15 ± 0.12	0.30 ± 0.26	0.30 ± 0.30	0.74 ± 0.5[b]
Interstitial								
Volume%	1.6 ± 0.5	1.8 ± 0.7	1.6 ± 0.5	2.6 ± 1.3	3.1 ± 0.5[a]	3.4 ± 0.8[c]	3.9 ± 0.5[c]	6.0 ± 0.3[c]
Collagen IV% *area*	18.3 ± 1.6	19.0 ± 1.0	19.5 ± 0.9	20.5 ± 1.1	18.8 ± 1.7	19.3 ± 1.4	21.3 ± 1.7[a]	24.7 ± 2.3[c]
Macrophages (MØ)								
MØ/gcs	0.53 ± 0.33	0.88 ± 0.16	0.78 ± 0.44	1.5 ± 0.8	0.73 ± 0.24	1.8 ± 1.0	2.7 ± 1.3[a]	10.0 ± 4.4[a]
% $iNOS^+$ glom MØ	6.2 ± 8.5	5.3 ± 3.2	6.5 ± 6.6	5.2 ± 4.8	21 ± 13	20 ± 6[c]	20 ± 11[b]	16 ± 14
% $PCNA^+$ glom MØ	2.9 ± 6.0	ND	ND	4.6 ± 5.8	7.9 ± 6.0	ND	ND	10 ± 15
int MØ/mm^3	160 ± 116	107 ± 36	103 ± 13	129 ± 69	138 ± 58	153 ± 94	163 ± 41[a]	330 ± 141[a]
% $iNOS^+$ int MØ	1.2 ± 1.1	2.1 ± 3.4	2.3 ± 2.3	2.1 ± 2.4	2.3 ± 1.9	5.3 ± 4.1	4.6 ± 1.9[b]	4.8 ± 3.9
% $PCNA^+$ int MØ	0.13 ± 0.35	ND	ND	0.39 ± 0.91	0.62 ± 0.9	ND	ND	0.91 ± 1.0

Abbreviations are: MØ, macrophage; gcs, glomerular cross-section; iNOS, inducible nitric oxide synthase; glom, glomerular; int, interstitial; PCNA, proliferating cell nuclear antigen; ND, not determined.
Data are mean ± SD.
[a] $P < 0.01$; [b] $P < 0.05$; [c] $P < 0.0005$ vs. normal db/+ mice.

first appeared in glomeruli at 4 to 6 months and then later at 6 to 8 months in the interstitium (Fig. 2). At 8 months, there was approximately a sevenfold increase in glomerular macrophages and a threefold increase in interstitial macrophages in diabetic db/db mice compared to db+ controls. In diabetic db/db mice, both glomerular and interstitial macrophages correlated with blood glucose and HbA_{1c} levels, but not with obesity, triglycerides or cholesterol levels (Table 3).

Kidney macrophages increase with diabetic renal injury and fibrosis

Increased glomerular macrophage accumulation in diabetic db/db mice coincided with the onset and progression of glomerular damage (hypertrophy, hypercellularity, sclerosis) and increased glomerular deposition of IgG and C3 at 6 to 8 months (Table 4) (Figs. 3 and 4). Similarly, increased numbers of interstitial macrophages surrounding cortical tubules, vessels and Bowman's capsule, were identified at 6 to 8 months, coinciding with a marked increase in albuminuria and plasma creatinine, detection of tubular injury (tubular dilatation, atrophy, apoptosis) and the start of interstitial fibrosis (increased interstitial volume, collagen IV deposition) (Table 4) (Fig. 3).

In diabetic db/db mice, glomerular macrophage accumulation correlated with albuminuria, glomerular deposition of IgG and C3, glomerular hypertrophy, glomerular hypercellularity, glomerular matrix fraction, and glomerular collagen IV, but not with plasma creatinine (Table 5). Interstitial macrophages correlated with albuminuria, plasma creatinine, tubular atrophy, tubular

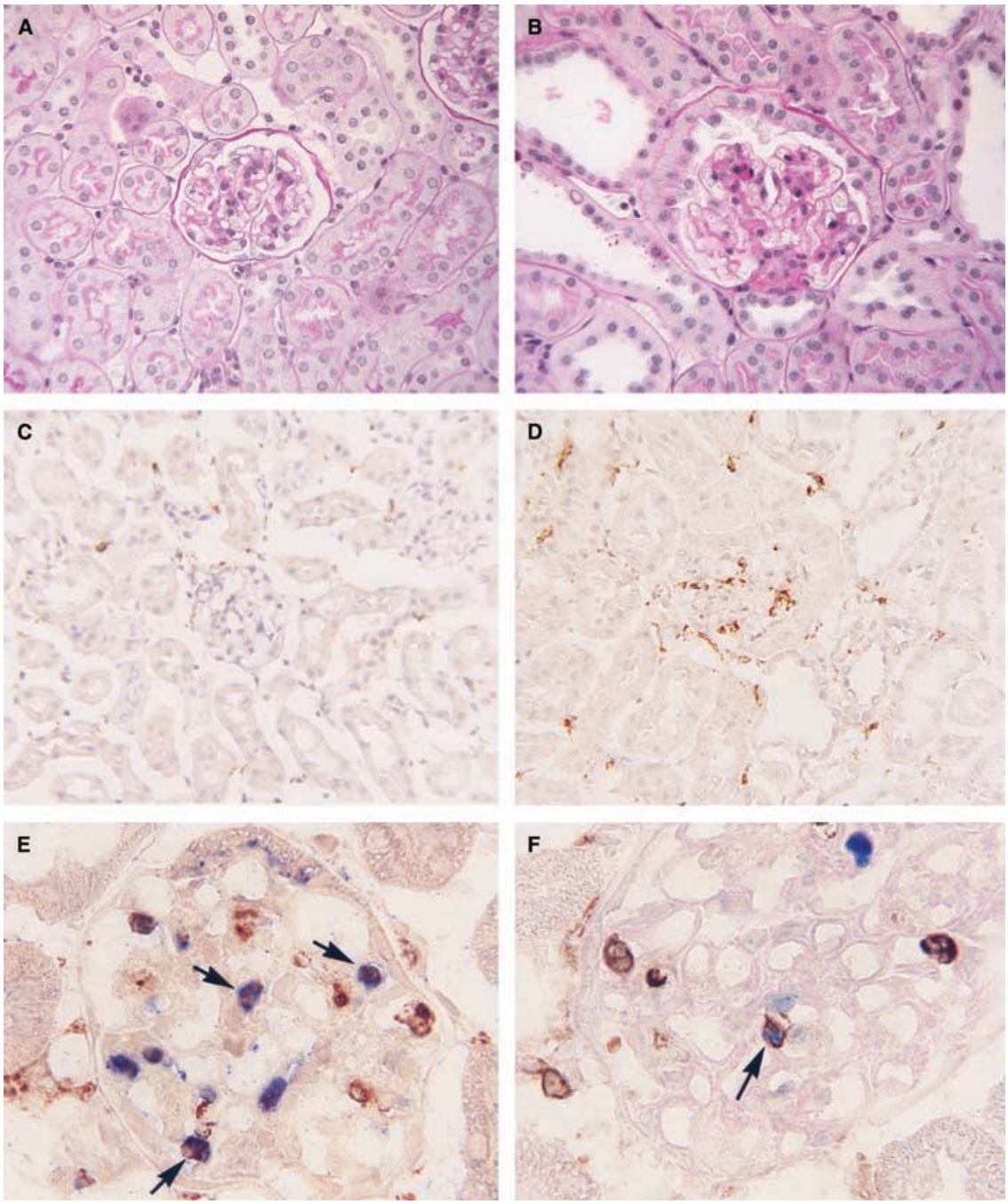


Fig. 3. Macrophage association with renal damage in diabetic db/db kidneys. Histologic staining with periodic acid-Schiff (PAS) reagent and hematoxylin shows the normal structure of (*A*) a db/+ kidney at 8 months of age. In comparison, there is significant glomerular damage (hypertrophy, hypercellularity and glomerulosclerosis), tubular injury (dilatation, atrophy), and interstitial infiltrate in (*B*) a diabetic db/db kidney at the same age. Immunostaining shows only a few macrophages (brown) in a db/+ kidney at 8 months of age (*C*), and many glomerular and interstitial macrophages in a diabetic db/db kidney at 8 months (*D*). Two-color immunostaining shows that some macrophages (brown) in diabetic kidneys (*E*) express inducible nitric oxide synthase (iNOS) (blue), indicating their potential for causing renal injury (arrows). In addition, there are macrophages (brown) in diabetic kidneys (*F*), which express proliferating cell nuclear antigen (PCNA) (blue), demonstrating that there is local macrophage proliferation (arrow) (magnification A to D × 250; E and F × 1000).

apoptosis, interstitial volume, and interstitial collagen IV (Table 5).

Glomerular and interstitial macrophages in diabetic kidneys were frequently found to be proliferating (PCNA+) or activated (iNOS+) (Fig. 3), and their numbers increased with progressive macrophage accumulation. The proportion of glomerular and interstitial macrophages expressing iNOS was two- to fourfold

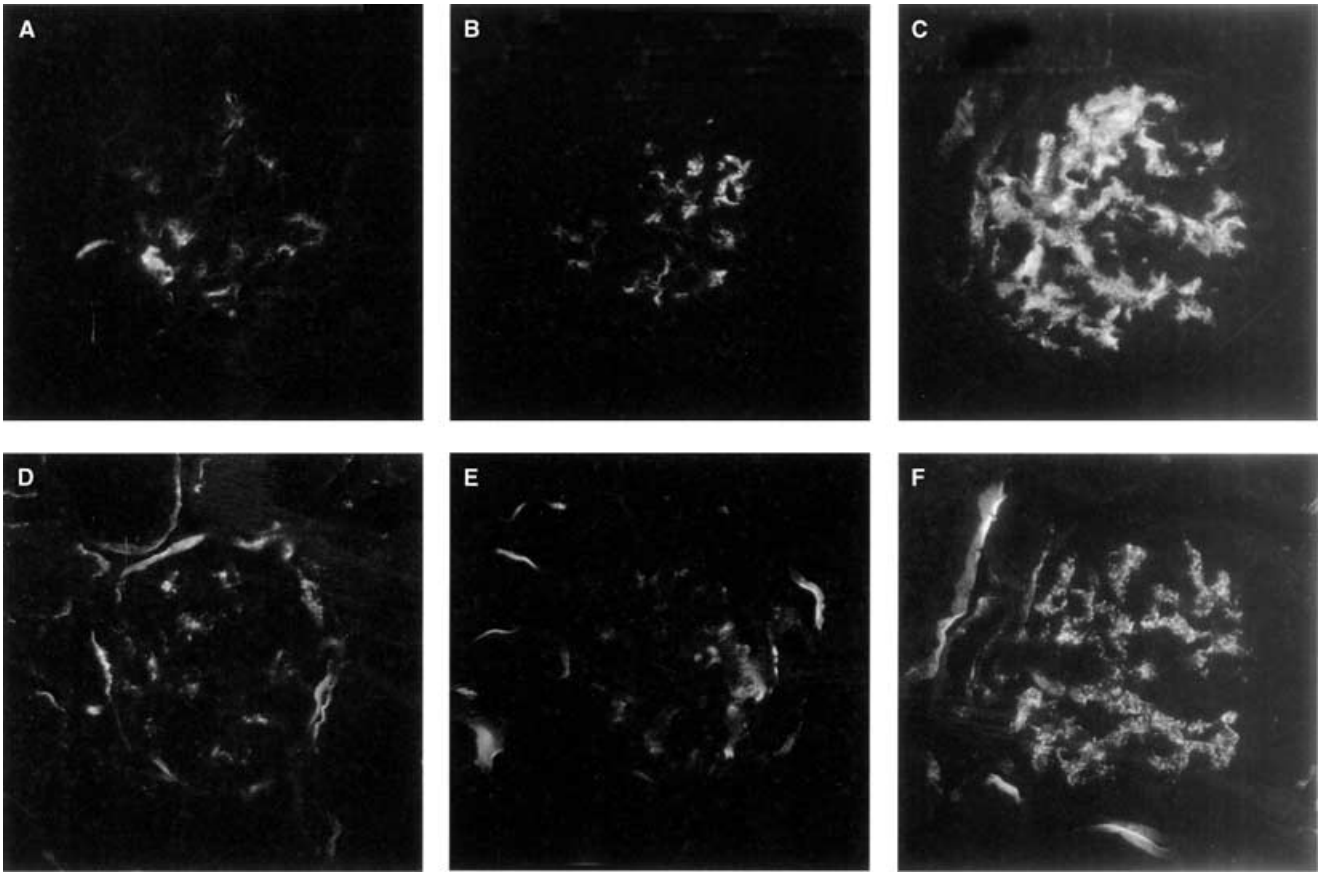


Fig. 4. Glomerular deposition of IgG and C3 in diabetic db/db kidneys. Immunofluorescence staining of 8-month-old mice identified weak glomerular staining for IgG in a normal db/+ kidney (*A*) and a nondiabetic db/db kidney (*B*), which increased markedly in a diabetic db/db kidney (*C*). Similarly, the glomerular deposition of C3 was weakly detectable in a normal db/+ kidney (*D*) and a nondiabetic db/db kidney (*E*), but was significantly greater in a db/db kidney (*F*) (magnification × 400).

greater in diabetic db/db compared to db/+ kidneys (Table 4). An increase in the percentage of macrophages expressing iNOS was first detected in glomeruli at the onset of diabetes (2 months) and then later in the interstitium (4 months). In contrast, the proportion of macrophages expressing PCNA was smaller and did not significantly change in diabetic compared to normal kidneys.

Increased expression of macrophage chemokines in type 2 diabetic nephropathy

At 8 months of age, diabetic db/db mice have increased numbers of glomerular cells and cortical tubules expressing mRNA for MCP-1, osteopontin, MIF, and M-CSF compared to age-matched, normal db/+ mice and 2-month-old diabetic db/db mice (Fig. 5), indicating that the up-regulation of these chemokines within the kidney is dependent on the presence and duration of type 2 diabetes. A strong association between kidney chemokine expression and prolonged hyperglycaemia was confirmed by correlation analysis (Table 6). Glomerular chemokine expression also correlated with glomerular deposition of IgG and C3 (Table 6). Chemokine expression appeared in both damaged tubules and glomeruli in diabetic kidneys (Figs. 6 and 7). Within glomeruli, expression appeared to be predominantly localized in podocytes (Fig. 7) and occasionally seen in mesangial cells (not shown). Although a similar staining pattern was seen for each chemokine, the mRNA detection proved to be specific in each case since there was no staining detected in diabetic glomeruli or damaged tubules with our sense control probes, which is consistent with previous studies [15]. Glomerular and interstitial macrophage accumulation correlated with the expression of chemokines seen in diabetic glomeruli and cortical tubules, respectively (Table 6). However, only tubular osteopontin correlated with the increase in the proportion of activated (iNOS+) interstitial macrophages in diabetic kidneys ($r = 0.60$, $P = 0.0054$). In addition, proliferating glomerular macrophages correlated with glomerular M-CSF expression ($r = 0.66$, $P = 0.0013$); however, the small number of interstitial macrophages proliferating in diabetic kidneys did not correlate with tubular M-CSF expression ($r = 0.12$, $P = 0.61$).

Table 5. Correlation of renal injury with kidney accumulation of macrophages and the percentage of activated [inducible nitric oxide synthase (iNOS+)] macrophages

	Glomerular macrophages		Interstitial macrophages	
	Total	% iNOS+	Total	% iNOS+
Renal Dysfunction				
Albuminuria	0.54 (0.002)	0.51 (0.004)	0.62 (0.0003)	0.49 (0.0056)
Plasma Creatinine	0.28 (NS)	0.86 (<0.0001)	0.41 (0.023)	0.91 (<0.0001)
Glomerular				
Volume	0.71 (<0.0001)	0.48 (0.0072)	—	—
Cellularity	0.73 (<0.0001)	0.60 (0.0004)	—	—
IgG	0.76 (<0.0001)	0.63 (<0.0001)	—	—
C3	0.73 (<0.0001)	0.53 (0.0014)	—	—
Matrix fraction	0.63 (0.0002)	0.56 (0.0013)	—	—
Collagen IV	0.80 (<0.0001)	0.65 (0.0001)	—	—
Tubular				
Atrophy	—	—	0.76 (<0.0001)	0.59 (0.0005)
Apoptosis	—	—	0.75 (<0.0001)	0.33 (NS)
Interstitial				
Volume	—	—	0.52 (0.0030)	0.43 (0.018)
Collagen IV	—	—	0.78 (<0.0001)	0.47 (0.011)

Analysis was performed on a combined group of db/db mice from 2, 4, 6, and 8 months. Data are *r* value based on Spearman's coefficient for IgG and C3 and Pearson's coefficient for all other correlations; (*P* value); NS is not significant.

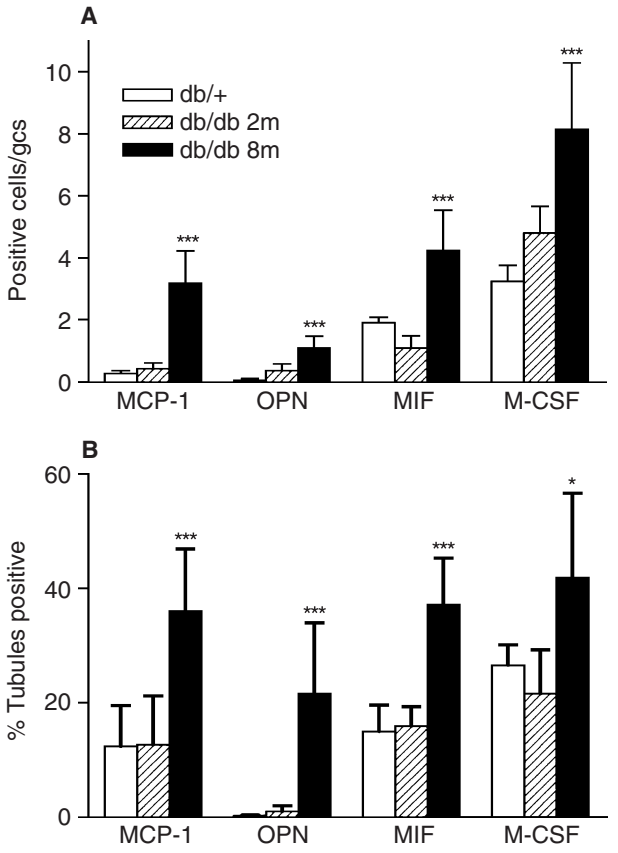


Fig. 5. Quantitation of glomerular and tubular expression of macrophage chemokines in diabetic db/db mice. In situ hybridization was used to detect mRNA for monocyte chemoattractant protein-1 (MCP-1), migration inhibitory factor (MIF), osteopontin (OPN) and monocyte-colony-stimulating factor (M-CSF) in (*A*) glomerular cells and (*B*) cortical tubules in diabetic db/db mice at 2 and 8 months of age and in normal db/+ mice at 8 months of age. Data are mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.0005$.

DISCUSSION

Our study suggests that kidney macrophage accumulation plays a pathologic role in the progression of type 2 diabetic nephropathy in db/db mice. Kidney macrophages were found to increase with the duration of diabetes, the severity of renal injury and loss of renal function, and their accumulation in glomeruli and the interstitium correlated with progressive glomerular and tubular injury, respectively.

Our population of db/db mice included animals ranging from moderately obese mice with normal blood lipids and hyperglycemia to severely obese mice with hyperlipidemia and normal blood glucose levels. Examination of these mice allowed us to distinguish the importance of hyperglycemia and hyperlipidemia in the accumulation of kidney macrophages and the development of renal injury in this model. We found that kidney macrophages and renal injury correlated significantly with blood glucose and HbA_{1c} levels, but not with obesity or blood lipids, suggesting that prolonged hyperglycemia may be a critical factor driving kidney macrophage accumulation and renal damage in type 2 diabetes.

Macrophages in diabetic kidneys were found in the glomerular tuft and around dilated tubules, which were sites shown to express macrophage chemokines, indicating a potential mechanism by which prolonged hyperglycemia can promote kidney macrophage accumulation. In situ hybridisation identified marked increases in glomerular and tubular expression of MCP-1, MIF, osteopontin, and M-CSF with the duration of diabetes in db/db mice, which correlated with glomerular and interstitial macrophage accumulation, respectively. Tubular expression of MCP-1 has previously been demonstrated in human diabetic kidneys [16] and tubular osteopontin levels have been shown to correlate with interstitial macrophages in streptozotocin-induced type 1 diabetic nephropathy in rats [17]. Both MCP-1 and osteopontin are known to be required for kidney macrophage accumulation and renal injury in acute and chronic models of kidney disease [18–21]. In this study, we demonstrated that tubular expression of MIF and M-CSF are

Table 6. Correlation of renal chemokine expression with blood glucose, hemoglobin A_{1c} (HbA_{1c}), glomerular IgG and C3 deposition, kidney accumulation of macrophages, and the percentage of activated [inducible nitric oxide synthase (iNOS+)] macrophages

	Glomerular cells				Cortical tubules			
	MCP-1	MIF	OPN	M-CSF	MCP-1	MIF	OPN	M-CSF
Blood glucose	0.75 (0.0001)	0.76 (0.0001)	0.86 (<0.0001)	0.75 (0.0001)	0.69 (0.0009)	0.85 (<0.0001)	0.86 (<0.0001)	0.59 (0.006)
HbA_{1c}	0.63 (0.0030)	0.63 (0.0031)	0.63 (0.0029)	0.74 (0.0002)	0.58 (0.0074)	0.61 (0.0046)	0.58 (0.0072)	0.21 (NS)
IgG	0.86 (<0.0001)	0.85 (<0.0001)	0.75 (0.0002)	0.83 (<0.0001)	—	—	—	—
C3	0.73 (0.0003)	0.74 (0.0002)	0.79 (<0.0001)	0.72 (0.0004)	—	—	—	—
Glom MØ	0.82 (<0.0001)	0.87 (<0.0001)	0.66 (0.0014)	0.83 (<0.0001)	—	—	—	—
% $iNOS^+$ glom MØ	0.036 (NS)	0.014 (NS)	0.29 (NS)	−0.046 (NS)	—	—	—	—
int MØ	—	—	—	—	0.52 (0.018)	0.74 (0.0002)	0.65 (0.0020)	0.55 (0.012)
% $iNOS^+$ int MØ	—	—	—	—	0.34 (NS)	0.25 (NS)	0.60 (0.0054)	0.094 (NS)

Abbreviations are: MCP-1, monocyte chemoattractant protein-1; MIF, migration inhibitory factor; OPN, osteopontin; M-CSF, monocyte-colony-stimulating factor; MØ, macrophage; int, interstial; glom, glomerular; NS, not significant.

Analysis was performed on a combined group of animals, including normal db/+ mice at 8 months and db/db mice at 2 and 8 months. Data are *r* value based on Spearman's coefficient for IgG and C3 and Pearson's coefficient for all other correlations; (*P* value).

also associated with kidney macrophage accumulation in diabetic nephropathy. Although MIF and M-CSF share the ability to influence macrophage migration and activation, they have distinct differences of action on macrophages compared to MCP-1 and osteopontin. MIF inhibits macrophages migrating away from its source and counter-regulates the immunosuppressive effects of glucocorticoids [22, 23]. M-CSF, in addition to being a macrophage chemoattractant, is the major factor responsible for local macrophage proliferation at sites of renal inflammation [24]. Kidney expression of MIF and M-CSF are associated with the progression of human proliferative glomerulonephritis and have been shown to play a pathologic role in animal models of renal disease [24–27]. Hence, this study has identified renal production of MIF and M-CSF as two potential additional mechanisms of macrophage accumulation in diabetic kidneys resulting from prolonged hyperglycemia.

Immune complex formation in diabetic nephropathy may also play a role in kidney macrophage accumulation. Several studies examining renal biopsies have noted glomerular immune complex deposition in human diabetic nephropathy; however, it is unsure whether these findings are directly related to the diabetes or an additional insult [28, 29]. Previous work has also shown that glomerular levels of IgG and C3 increase with the duration of diabetes in db/db mice [30]. In the current study, we demonstrated that glomerular macrophages in diabetic db/db kidneys correlated with increased glomerular deposition of IgG and C3, suggesting that glomerular immune complexes may promote glomerular macrophage accrual. Notably, age-matched db/db mice that did not develop hyperglycemia showed no increase in glomerular IgG and C3 deposition compared to db/+ controls, indicating that their glomerular levels are associated with hyperglycemia rather than obesity or the genetic make-up of these animals. This finding is consistent with studies in streptozotocin-treated rats in which insulin treatment or islet transplantation reduced glomerular immunocomplex deposition [31, 32]. Mesangial cells cultured in the presence of IgG complexes produce increased amounts of MCP-1 and M-CSF, a process that is dependent on Fc receptor occupancy [33]. Furthermore, experimental immune complex nephritis shows increased glomerular chemokine expression in association with glomerular macrophage accumulation [34]. Therefore, it is possible that glomerular macrophage accumulation in diabetic db/db mice may be, in part, promoted by immune complex–induced glomerular chemokine expression.

Most immunolocalization studies indicate that tubular epithelial cells are the major source of macrophage chemokines during renal inflammation. Indeed, proinflammatory stimuli are very effective in promoting tubular epithelial cell production of macrophage chemokines [35]. In contrast to glomeruli, we found no immune complex deposition in the tubules of diabetic db/db kidneys. Therefore, to understand whether chemokines promote interstitial macrophage accumulation in diabetic kidneys, it is important to determine how the diabetic milieu can stimulate macrophage chemokine production by tubular epithelial cells. Previous work has shown that glycated albumin can induce MCP-1 production by cultured mesangial cells [36], suggesting a role for MCP-1 in macrophage recruitment in diabetic glomeruli. Since tubular epithelial cells also possess receptors for advanced glycation end products (AGEs) [37], it is feasible that AGEs, such

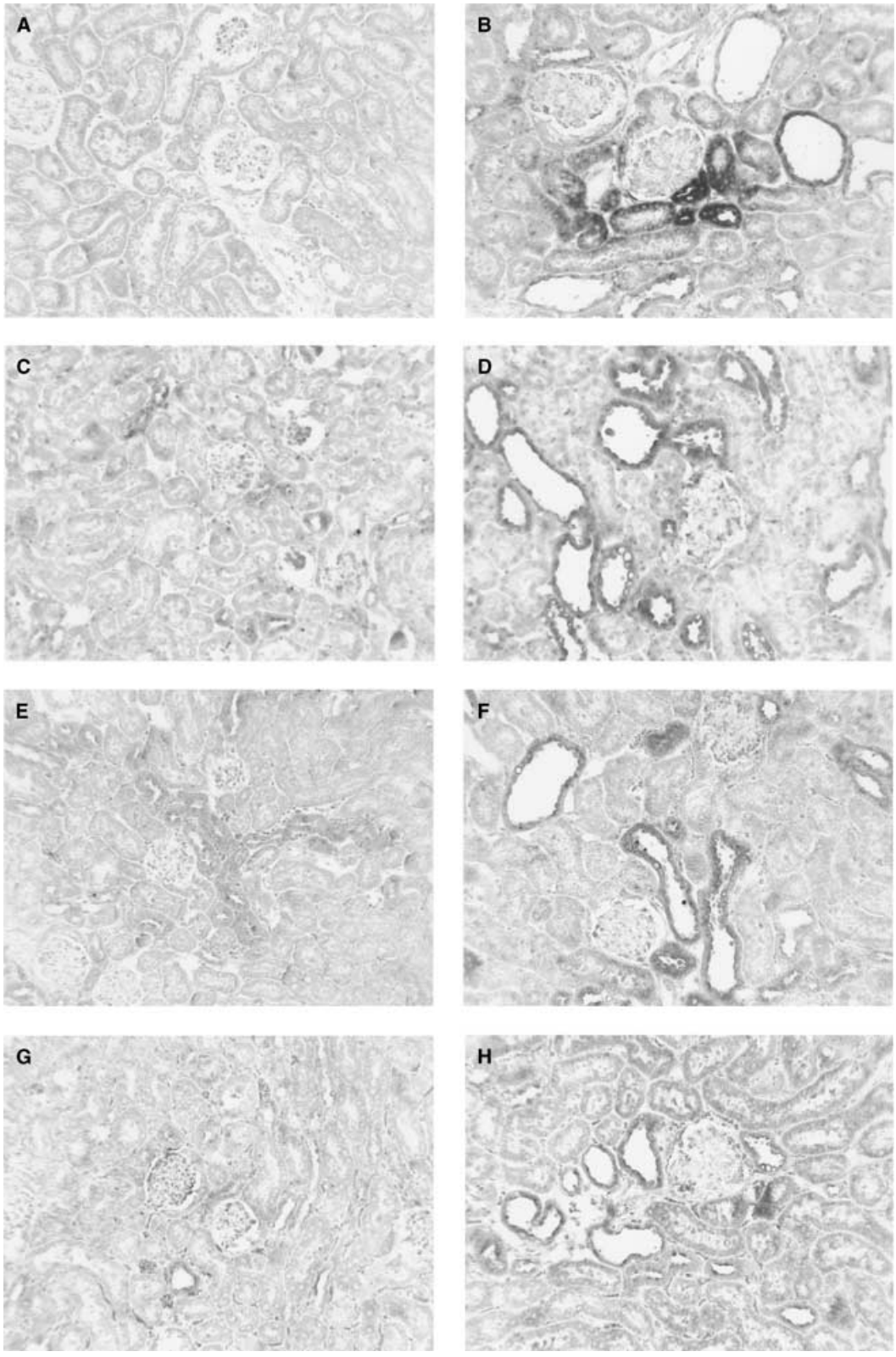
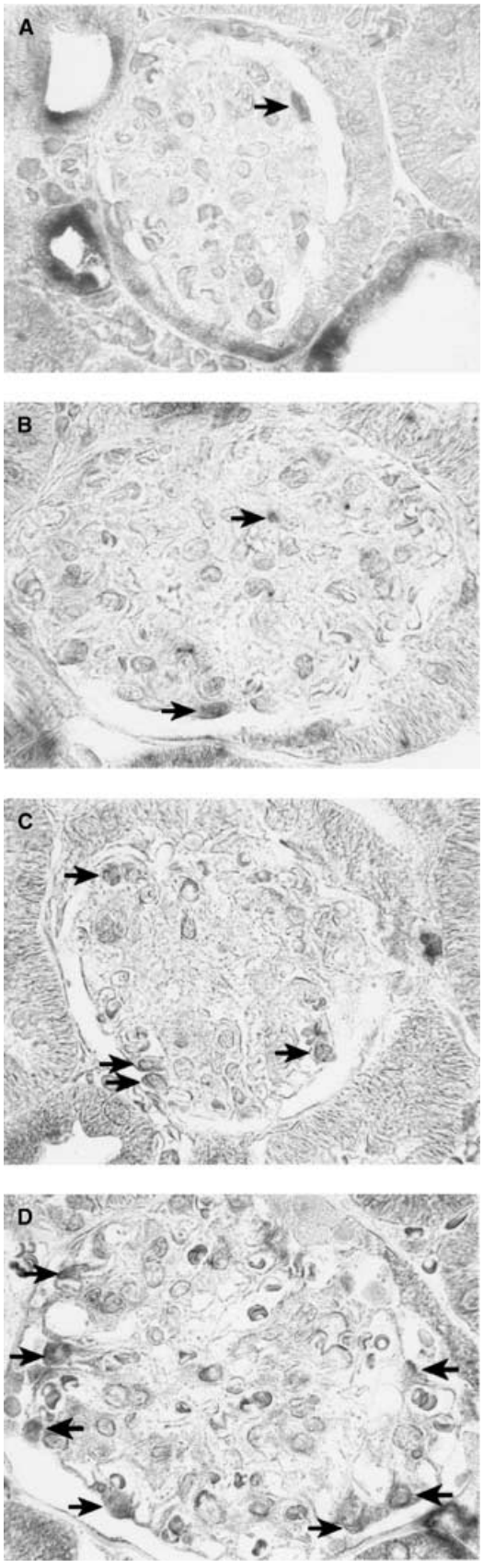


Fig. 6. Increased tubular expression of macrophages chemokines in diabetic db/db mice. In situ hybridization detected little mRNA expression (purple) of (*A*) osteopontin, (*C*) monocyte chemoattractant protein-1 (MCP-1), (*E*) migration inhibitory factor (MIF), and (*G*) monocyte-colony-stimulating factor (M-CSF) in normal db/+ kidneys at 8 months of age. In comparison, dilated tubules in diabetic db/db kidneys at 8 months frequently expressed mRNA for (*B*) osteopontin, (*D*) MCP-1, (*F*) MIF, and (*H*) M-CSF. No staining was detected in diabetic kidneys when sense control probes were used (data not shown) (magnification A to H $\times$ 250).



as glycated albumin, may also induce chemokine production by tubular epithelial cells. However, in the complex environment of diabetic kidneys it is difficult to ascertain whether tubular chemokine production is the result of direct or indirect action of the diabetic milieu on tubules.

The correlation of kidney macrophages with increased albuminuria, plasma creatinine, and tissue damage suggests a role for macrophage-mediated renal injury in diabetic nephropathy. Recent work, involving the adoptive transfer of macrophages into leukocyte-depleted animals has established that macrophages can induce glomerular injury, promoting proteinuria and mesangial proliferation [38]. Furthermore, the ability of macrophages to induce glomerular injury is enhanced if their activation status is increased by pretreatment with interferon-gamma (IFN-γ) [39]. This finding is supported by in vitro studies indicating that activated macrophages can promote renal cell death. Lipopolysaccharide stimulation of bone marrow–derived macrophages results in the secretion of substances known to induce apoptosis in cultured mesangial and tubular epithelial cells [20, 40]. Indeed, macrophages can produce a variety of substances which can promote renal injury [nitric oxide, reactive oxygen species, interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), metalloproteinases] [9], and inhibiting the action of some of these molecules in models of acute renal injury reduces kidney damage [41–43]. In vitro studies have demonstrated that AGEs can induce macrophage secretion of nitric oxide, reactive oxygen species, and TNF-α [44–46], indicating that the diabetic milieu may promote macrophage-mediated kidney damage. In addition, glomerular expression of iNOS is associated with macrophage infiltration in rat immune complex glomerulonephritis [47] and in vitro studies have shown that immune complexes can increase nitric oxide production by macrophages in the presence of IFN-γ [48], indicating that glomerular immune complexes in diabetic nephropathy may also promote macrophage activation. In diabetic db/db kidneys, we noted an increase in the numbers and proportion of macrophages expressing iNOS, suggesting that these kidney macrophages were producing nitric oxide and potentially causing tissue injury. Indeed, the percentage of iNOS+ macrophages was threefold greater in glomeruli compared to the interstitium, indicating that glomerular immune complex deposition may be responsible

Fig. 7. Glomerular expression of macrophages chemokines in 8-month-old diabetic db/db mice. In situ hybridization detected podocytic-like cells expressing mRNA (arrows) for (*A*) osteopontin, (*B*) monocyte chemoattractant protein-1 (MCP-1), (*C*) migration inhibitory factor (MIF), and (D) monocyte-colony-stimulating factor (M-CSF) in the diabetic glomeruli of db/db mice at 8 months (magnification A to D $\times$ 1000).

for the increased activation of macrophages in diabetic glomeruli.

In addition to causing injury, macrophage activation may also promote renal fibrosis through the secretion of fibroblast growth factor (FGF) and PDGF [49], profibrotic factors [IL-1, transforming growth factor-β (TGF-β)] [50–52], and molecules that activate TGF-β (plasmin, thrombospondin-1) [52, 53]. Macrophage accumulation in diabetic db/db kidneys correlated with increases in glomerular and interstitial volume and glomerular and interstitial collagen IV deposition, showing an association between macrophages and the progression of renal fibrosis. Therefore, macrophages have multiple potential mechanisms by which they can mediate the progression of diabetic nephropathy.

CONCLUSION

This study suggests that prolonged hyperglycemia promotes the accumulation of kidney macrophages, which are associated with the progression of type 2 diabetic nephropathy. However, proof of a pathologic role will require further experiments that selectively deplete macrophages during the progression of diabetic nephropathy. If a pathologic role for macrophages is proven, the selective targeting of macrophages may become an additional therapeutic strategy for halting the advancement of diabetic nephropathy.

ACKNOWLEDGMENTS

We thank Yingjie Han for his technical support. This work was supported by a Ph.D. scholarship (Fiona Chow) and a grant (#198712) from the National Health and Medical Research Council of Australia.

Reprint requests to Dr. Greg Tesch, Department of Nephrology, Monash Medical Centre, 246 Clayton Road, Clayton, Victoria 3168, Australia.
E-mail: gtesch@hotmail.com

REFERENCES

1. Ritz E, Tarng DC: Renal disease in type 2 diabetes. *Nephrol Dial Transplant* 16 (Suppl 5):11–18, 2001
2. Keane WF, Lyle PA: Recent advances in the management of type 2 diabetes and nephropathy: Lessons from the RENAAL study. *Am J Kidney Dis* 41(3 Suppl 2):S22–S25, 2003
3. Furuta T, Saito T, Ootaka T, *et al*: The role of macrophages in diabetic glomerulosclerosis. *Am J Kidney Dis* 21:480–485, 1993
4. Lavaud S, Michel O, Sassy-Prigent C, *et al*: Early influx of glomerular macrophages precedes glomerulosclerosis in the obese Zucker rat model. *J Am Soc Nephrol* 7:2604–2615, 1996
5. Coimbra TM, Janssen U, Grone HJ, *et al*: Early events leading to renal injury in obese Zucker (fatty) rats with type II diabetes. *Kidney Int* 57:167–182, 2000
6. Phillips AO, Baboolal K, Riley S, *et al*: Association of prolonged hyperglycemia with glomerular hypertrophy and renal basement membrane thickening in the Goto Kakizaki model of non–insulin-dependent diabetes mellitus. *Am J Kidney Dis* 37:400–410, 2001
7. Young BA, Johnson RJ, Alpers CE, *et al*: Cellular events in the evolution of experimental diabetic nephropathy. *Kidney Int* 47:935–944, 1995
8. Sassy-Prigent C, Heudes D, Mandet C, *et al*: Early glomerular macrophage recruitment in streptozotocin-induced diabetic rats. *Diabetes* 49:466–475, 2000
9. Nikolic-Paterson DJ, Atkins RC: The role of macrophages in glomerulonephritis. *Nephrol Dial Transplant* 16 (Suppl 5):3–7, 2001
10. Hattori M, Ito K, Kawaguchi H, Yamaguchi Y: Probucol reduces renal injury in the ExHC rat. *Nephron* 67:459–468, 1994
11. Miyazaki K, Isbel NM, Lan HY, *et al*: Up-regulation of macrophage colony-stimulating factor (M-CSF) and migration inhibitory factor (MIF) expression and monocyte recruitment during lipid-induced glomerular injury in the exogenous hypercholesterolaemic (ExHC) rat. *Clin Exp Immunol* 108:318–323, 1997
12. Cohen MP, Clements RS, Hud E, *et al*: Evolution of renal function abnormalities in the db/db mouse that parallels the development of human diabetic nephropathy. *Exp Nephrol* 4:166–171, 1996
13. Duan WR, Garner DS, Williams SD, *et al*: Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin18 with the TUNEL method for quantitation of apoptosis in histological sections of PC-3 subcutaneous xenografts. *J Pathol* 199:221–228, 2003
14. Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC: A novel, simple, reliable, and sensitive method for multiple immunoenzyme labeling: Use of microwave oven heating to block antibody crossreactivity and retrieve antigens *J Histochem Cytochem* 43:97–102, 1995
15. Rice EK, Tesch GH, Cao Z, *et al*: Induction of MIF synthesis and secretion by tubular epithelial cells: A novel action of angiotensin II. *Kidney Int* 63:1265–1275, 2003
16. Wada T, Furuichi K, Sakai N, *et al*: Up-regulation of monocyte chemoattractant protein-1 in tubulointerstitial lesions of human diabetic nephropathy. *Kidney Int* 58:1492–1499, 2000
17. Kelley DJ, Wilkinson-Berka JL, Ricardo SD, *et al*: Progression of tubulointerstitial injury by osteopontin-induced macrophage recruitment in advanced diabetic nephropathy of transgenic (mRen-2)27 rats. *Nephrol Dial Transplant* 17:985–991, 2002
18. Yu XQ, Nikolic-Paterson DJ, Mu W, *et al*: A functional role for osteopontin in experimental crescentic glomerulonephritis in the rat. *Proc Assoc Am Physicians* 110:50–64, 1998
19. Tesch GH, Maifert S, Schwarting A, *et al*: MCP-1 dependent leukocytic infiltrates are responsible for autoimmune disease in MRL-*Fas*^{lpr} mice. *J Exp Med* 190:1813–1824, 1999
20. Tesch GH, Schwarting A, Kinoshita K, *et al*: Monocyte chemoattractant protein-1 promotes macrophage-mediated tubular, but not glomerular injury, in nephrotoxic serum nephritis. *J Clin Invest* 103:73–80, 1999
21. Ophascharoensuk V, Giachelli CM, Gordon K, *et al*: Obstructive uropathy in the mouse: role of osteopontin in interstitial fibrosis and apotosis. *Kidney Int* 56:571–580, 1999
22. Calandra T, Bernhagen J, Metz CN, *et al*: MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* 377:68–71, 1995
23. Yang N, Nikolic-Paterson DJ, Ng YY, *et al*: Reversal of established rat crescentic glomerulonephritis by blockade of macrophage migration inhibitory factor (MIF): Potential role of MIF in regulating glucocorticoid production. *Mol Med* 4:413–424, 1998
24. Le Meur Y, Tesch GH, Hill PA, *et al*: Macrophage accumulation at a site of renal inflammation is dependent on the M-CSF/c-fms pathway. *J Leuk Biol* 72:530–537, 2002
25. Lan HY, Yang N, Nikolic-Paterson DJ, *et al*: Expression of macrophage migration inhibitory factor in human glomerulonephritis. *Kidney Int* 57:499–509, 2000
26. Isbel NM, Nikolic-Paterson DJ, Hill PA, *et al*: Local macrophage proliferation correlates with increased renal M-CSF expression in human glomerulonephritis. *Nephrol Dial Transplant* 16:1638–1647, 2001
27. Lan HY, Bacher M, Yang N, *et al*: The pathogenic role of macrophage migration inhibitory factor in immunologically induced kidney disease in the rat. *J Exp Med* 185:1455–1465, 1997
28. Sabnis SG, Antonovych TT: Diabetic nephropathy with superimposed immune complex glomerulonephritis. *South Med J* 76:456–461, 1983
29. Cavallo T, Pinto JA, Rajaraman S: Immune complex disease complicating diabetic glomerulosclerosis. *Am J Nephrol* 4:347–354, 1984

30. Meade CJ, Brandon DR, Smith W, *et al*: The relationship between hyperglycemia and renal immune complex deposition in mice with inherited diabetes. *Clin Exp Immunol* 43:109–120, 1981
31. Abrass CK: Evaluation of the presence of circulating immune complexes and their relationship to glomerular IgG deposits in streptozotocin-induced diabetic rats. *Clin Exp Immunol* 57:17–24, 1984
32. Wehner H, Kosters W, Strauch M, Staudenmeir M: Effect of islet transplantation on the glomerular changes in streptozotocin-diabetic rats. *Virchows Arch A Pathol Anat Histol* 388:137–154, 1980
33. Hora K, Satriano JA, Santiago A, *et al*: Receptors for IgG complexes activate synthesis of monocyte chemoattractant peptide 1 and colony-stimulating factor 1. *Proc Natl Acad Sci USA* 89:1745–1749, 1992
34. Anders HJ, Vielhauer V, Kretzler M, *et al*: Chemokine and chemokine receptor expression during initiation and resolution of immune complex glomerulonephritis. *J Am Soc Nephrol* 12:919–931, 2001
35. van Kooten C, Daha MR, van Es LA: Tubular epithelial cells: A critical cell type in the regulation of inflammatory processes. *Exp Nephrol* 7:429–437, 1999
36. Banba N, Nakamura T, Matsumura M, *et al*: Possible relationship of monocyte chemoattractant protein-1 with diabetic nephropathy. *Kidney Int* 58:684–690, 2000
37. Oldfield MD, Bach LA, Forbes JM, *et al*: Advanced glycation end products cause epithelial-myofibroblast transdifferentiation via the receptor for advanced glycation end products (RAGE). *J Clin Invest* 108:1853–1863, 2001
38. Ikezumi Y, Hurst LA, Masaki T, *et al*: Adoptive transfer studies demonstrate that macrophages can induce proteinuria and mesangial proliferation. *Kidney Int* 63:83–95, 2003
39. Ikezumi Y, Atkins RC, Nikolic-Paterson DJ: Interferon-gamma augments acute macrophage-mediated renal injury via a glucocorticoid-sensitive mechanism. *J Am Soc Nephrol* 14:888–898, 2003
40. Duffield JS, Erwig LP, Wei X, *et al*: Activated macrophages direct apoptosis and suppress mitosis of mesangial cells. *J Immunol* 164:2110–2119, 2000
41. Rangan GK, Wang Y, Tay YC, Harris DC: Early administration of PDTC in Adriamycin nephropathy: Effect on proteinuria, cortical tubulointerstitial injury, and NF-kappaB activation. *Renal Failure* 23:773–780, 2001
42. Lan HY, Nikolic-Paterson DJ, Zarama M, *et al*: Suppression of experimental crescentic glomerulonephritis by the interleukin-1 receptor antagonist. *Kidney Int* 43:479–485, 1993
43. Lan HY, Yang N, Metz C, *et al*: TNF-alpha up-regulates renal MIF expression in rat crescentic glomerulonephritis. *Mol Med* 3:136–144, 1997
44. Wu CH, Huang CM, Lin CH, *et al*: Advanced glycation end-products induce NF-kappaB dependent iNOS expression in RAW 264.7 cells. *Mol Cell Endocrinol* 194:9–17, 2002
45. Bassi AM, Ledda S, Valentini S, *et al*: Damaging effects of advanced glycation end-products in the murine macrophage cell line J774A.1. *Toxicol In Vitro* 16:339–347, 2002
46. Rashid G, Luzon Amid-Ad, Korzets Z, *et al*: The effects of advanced glycation end-products and aminoguanidine on TNF-α production by rat peritoneal macrophages. *Perit Dial Int* 21:122–129, 2001
47. Jansen A, Cook T, Taylor GM, *et al*: Induction of nitric oxide synthase in rat immune complex glomerulonephritis. *Kidney Int* 45:1215–1219, 1994
48. Mozaffarian N, Berman JW, Casadevall A: Immune complexes increase nitric oxide production by interferon-gamma-stimulated murine macrophage-like J774.16 cells. *J Leukoc Biol* 57:657–662, 1995
49. Yamamoto T, Katayama I, Nishioka K: Fibroblast proliferation by bleomycin stimulated peripheral blood mononuclear cell factors. *J Rheumatol* 26:609–615, 1999
50. Mampaso F, Bricio T, Martin A, Molina A: Production of interleukin-1-like cytokine by cultured rat glomerular macrophages. *Immunology* 76:408–412, 1992
51. Versey DA, Cheung CW, Cuttle L, *et al*: Interleukin-1beta stimulates human renal fibroblast proliferation and matrix production by means of a transforming growth factor-beta-dependent mechanism. *J Lab Clin Med* 140:342–350, 2002
52. Khalil N, Corne S, Whitman C, Yacyshyn H: Plasmin regulates the activation of cell-associated latent TGF-beta 1 secreted by rat alveolar macrophages after in vivo bleomycin injury. *Am J Respir Cell Mol Biol* 15:252–259, 1996
53. Yehualaeshet T, O'Connor R, Green-Johnson J, *et al*: Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. *Am J Pathol* 155:841–851, 1999