Macrophages in streptozotocin-induced diabetic nephropathy: potential role in renal fibrosis

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Abstract

Background. Renal fibrosis is central to the progression of diabetic nephropathy; however, the mechanisms responsible for fibroblast and matrix accumulation in this disease are only partially understood. Macrophages accumulate in diabetic kidneys, but it is unknown whether macrophages contribute to renal fibrosis. Therefore, we examined whether macrophage accumulation is associated with the progression of renal injury and fibrosis in type 1 diabetic nephropathy and whether macrophages exposed to the diabetic milieu could promote fibroblast proliferation.

Methods. Kidney macrophages, renal injury and fibrosis were analysed in diabetic C57BL/6J mice at 2, 8, 12 and 18 weeks after streptozotocin injection. Isolated rat bone marrow macrophages were stimulated with diabetic rat serum or carboxymethyllysine (CML)-bovine serum albumin (BSA) to determine whether macrophage-conditioned medium could promote the proliferation of rat renal (NRK-49F) fibroblasts.

Results. Progressive injury and fibrosis in diabetic nephropathy was associated with increased numbers of kidney macrophages. Macrophage accumulation in diabetic mice correlated with hyperglycaemia (blood glucose, HbA1c levels), renal injury (albuminuria, plasma creatinine), histological damage and renal fibrosis (myofibroblasts, collagen IV). Culture supernatant derived from bone marrow macrophages incubated with diabetic rat serum or CML-BSA induced proliferation of fibroblasts, which was inhibited by pre-treating fibroblasts with interleukin-1 (IL-1) receptor antagonist or the platelet-derived growth factor (PDGF) receptor kinase inhibitor, STI-571.

Conclusion. Kidney macrophage accumulation is associated with the progression of renal injury and fibrosis in streptozotocin-induced mouse diabetic nephropathy. Elements of the diabetic milieu can stimulate macrophages to promote fibroblast proliferation via IL-1- and PDGF-dependent pathways which may enhance renal fibrosis.

Keywords: interleukin-1; macrophage; platelet-derived growth factor; streptozotocin-induced diabetic nephropathy; renal fibrosis

Introduction

Diabetic nephropathy is the largest single cause of end-stage renal failure worldwide. Despite the available modern therapies of glycaemic and blood pressure control, many patients continue to show progressive renal damage [1]. Therefore, it is extremely important to identify novel interventions to halt the progression of diabetic nephropathy.

Diabetic nephropathy has traditionally been considered to be a non-immune disease; however, an increased presence of glomerular and interstitial macrophages in diabetic kidneys has been reported in both human biopsies and animal models [2–5]. Macrophages mediate renal injury in experimental models of immune-mediated kidney disease [6], and correlate with renal impairment in human glomerulonephritis [7]. In comparison, little is known about the involvement of macrophages in diabetic nephropathy, since most studies identifying macrophages in diabetic kidneys have examined their presence at a single time point or prior to the development of proteinuria, and have not determined whether macrophage accumulation is associated with disease progression [2–4]. Therefore, it is difficult to ascertain whether macrophages appearing in diabetic kidneys are promoting disease or simply represent a response to injury.
Recent work has shown that macrophage depletion by irradiation treatment can inhibit the development of glomerular hypertrophy and production of collagen IV in the early stages of streptozotocin (STZ)-induced diabetic nephropathy [4]. This finding suggests that further investigation is warranted into determining the role of macrophages in diabetic nephropathy.

Progressive glomerular and interstitial accumulation of myofibroblasts and extracellular matrix is a critical factor in the progression of diabetic nephropathy. Myofibroblasts are thought to be the major source of interstitial matrix in human diabetic kidneys and their presence correlates significantly with progressive decline in renal function (1/creatinine slope), and is a better predictor of progressive diabetic nephropathy than either tubular atrophy or interstitial fibrosis [8]. Interstitial macrophages also appear during the development of interstitial fibrosis, but it is not known whether they participate in the accumulation of interstitial myofibroblasts and extracellular matrix in diabetic kidneys.

Macrophages can secrete a number of cytokines including interleukin-1 (IL-1), platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGFB) which are capable of inducing profibrotic responses in kidney cells, including myofibroblast proliferation, extracellular matrix production and epithelial to myofibroblast transformation [9–11]. While there is evidence suggesting that elements of the diabetic milieu can stimulate the development of interstitial fibrosis, but it is not known whether they participate in the accumulation of interstitial myofibroblasts and extracellular matrix in diabetic kidneys.

In the current study, we examine the association of kidney macrophage accumulation with renal injury and fibrosis in the progression of diabetic nephropathy in STZ-treated mice. We also provide in vitro data that evaluate the mechanisms by which elements of the diabetic milieu can activate macrophages to promote renal fibroblast proliferation.

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Materials and methods

Animal model

Studies were performed on inbred male C57Bl/6J mice (8 weeks, 21–26 g) maintained on a normal diet under standard animal house conditions. Mice were given intraperitoneal injections of STZ (Sigma, St Louis, MO) in sodium citrate buffer (pH 4.5) on two consecutive days (125 mg/kg/day). Blood glucose was measured by tail vein sampling using the glucose oxidase enzymatic test (Medisense glucometer: Abbott Laboratories, Bedford, MA). Diabetes was defined as a morning blood glucose reading of >16 mM by 2 weeks after STZ. When blood glucose levels exceeded 30 mM, diabetic mice were given 0.4 U of insulin (Protaphane, Novo Nordisk, North Rocks, NSW, Australia) every second day to prevent weight loss while maintaining blood glucose levels within the hyperglycaemic range (16–30 mM). HbA1c (glycated haemoglobin) was determined from cardiac blood when animals were killed. Diabetic nephropathy was evaluated in groups of 10 mice killed at four time points (2, 8, 12 and 18 weeks) after STZ. A group of normal 26-week-old mice (n = 10) was used as controls.

Biochemical analysis

Urine was collected from mice housed in metabolic cages for 18 h. Whole blood was collected in the presence of heparin via cardiac puncture of anaesthetized mice. HbA1c levels, urine creatinine and plasma creatinine were analysed by the Department of Biochemistry at the Monash Medical Centre. Urine albumin was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX).

Antibodies

Antibodies used in this study were: rat anti-mouse CD45 (M1/9.3.4); rat anti-mouse CD68 (FA-11, Serotec, Oxford, UK); rat anti-mouse F4/80 (Serotec); fluorescein-conjugated anti-α-smooth muscle actin (1A4, Sigma); goat anti-mouse collagen IV (Santa Cruz Biotechnology, Santa Cruz, CA); anti-activated caspase-3 (Asp175, Cell Signaling Technology, Beverly, MA); fluorescein-conjugated anti-proliferating cell nuclear antigen (PCNA, Roche); goat anti-PDGF-B (R&D systems, Minneapolis, MN); and rabbit anti-PDGF-B (Genzyme, Cambridge, MA). Isootype-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).

Renal pathology

Formalin-fixed kidney sections (2 μm) were stained with periodic acid–Schiff’s (PAS) reagent to identify kidney structure and with haematoxylin 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. The percentage of atrophic tubules (as defined by dilatation, cell loss and necrosis) was scored by assessing 400 renal cortical tubules per kidney in randomly selected microscopic fields.

Immunohistochemistry analysis

Immunoperoxidase staining for leukocytes (CD45, CD68, CD4 and CD8) and collagen IV was performed on 2% paraformaldehyde–lysole–periodate (PLP)-fixed kidney cryostat sections (5 μm). Immunostaining for apoptotic cells (activated caspase-3) and α-smooth muscle actin (α-SMA) was performed on formalin-fixed kidney paraffin sections (4 μm). Tissue sections were incubated with 20% fetal calf serum (FCS) for 30 min and then overnight with 5 μg/ml of primary antibody in 1% bovine serum albumin (BSA) at 4°C. Sections labelled with non-conjugated antibodies were then incubated for 20 min each with 0.6% hydrogen peroxide followed by avidin and biotin block (Vector Laboratories, Burlingame, CA) and 20% FCS to prevent non-specific detection. After washing, these sections were
incubated with biotinylated goat antibodies (anti-rat IgG or anti-rabbit IgG, 1:200, Vector) or biotinylated rabbit antibody (anti-goat IgG, 1:200, Zymed, San Francisco, CA) for 1 h, followed by peroxidase-conjugated ABC solution (Vector) for 1 h, and developed with 3,3-diaminobenzidine (DAB, Sigma) to produce a brown colour. Sections labelled with fluorescein-conjugated antibodies were incubated for 20 min each with 0.6% hydrogen peroxide and 20% normal sheep serum. These sections were then incubated for 1 h with peroxidase-conjugated sheep anti-fluorescein Fab fragments (1:300, Roche) and developed with DAB (Sigma).

Immunostained glomerular leukocytes were counted under high power (>400) in 20 hilar gcs per animal. Immunostained interstitial leukocytes 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 mm² graticule fitted in the eyepiece of the microscope and expressed as cells/mm². Glomerular and interstitial collagen IV and interstitial α-SMA were assessed from the percentage area of immunostaining within the glomerular tuft or 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. All scoring was performed on blinded slides.

Double immunostaining for the simultaneous detection of PCNA⁺ proliferating cells and α-SMA⁺ interstitial myofibroblasts or F4/80⁺ interstitial macrophages was performed on methacarn-fixed paraffin sections (4 μm). Immunoperoxidase staining for F4/80 or α-SMA was performed as described for α-SMA in formalin-fixed sections. After development with DAB, slides were placed in 400 ml of 10 mM sodium citrate buffer pH 6.0 and microwave treated for 12 min at 800 W. Slides were then incubated sequentially with 20% normal sheep serum for 20 min followed by fluorescein-conjugated PCNA monoclonal antibody (Roche, 1:100 in 1% BSA) overnight at 4°C and alkaline phosphatase-conjugated sheep anti-fluorescein Fab fragments (1:300 in 1% BSA) for 1 h. PCNA⁺ cells were then observed by development with fast blue BB salt (Sigma) to produce a blue colour.

**Preparation of macrophage-conditioned medium**

Rat bone marrow macrophages were isolated from dissected femurs and tibias of Sprague-Dawley rats and maintained in culture as previously described [6]. After 8 days of growth in 20% L-cell-conditioned medium (LCM) and 10% FCS in low glucose Dulbecco’s modified Eagle’s medium (DMEM), macrophages were seeded into 24-well tissue culture plates at a density such that the final concentration of rat serum was diluted in serum-free medium and added at 15% stock concentration such that the final concentration of rat serum was diluted in serum-free medium and added at 15% stock concentration such that the final concentration of rat serum was diluted in serum-free medium and added at 15% stock concentration such that the final concentration of rat serum was diluted in serum-free medium and added at 15% stock concentration such that the final concentration of rat serum was diluted in serum-free medium and added at 15% stock concentration such that the final concentration of rat serum was diluted in serum-free medium and added at 15% stock concentration such that the final concentration of rat serum was diluted in serum-free medium and added at 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lose viability at concentrations of serum <0.1%. Fibroblast proliferation was determined 48h later by the uptake of [3H]thymidine (0.5μCi/well, Amersham Biosciences, UK) during the last 6h of culture and quantitated using a β-counter (Wallace Rack-beta, WallacOy, Turku, Finland). In some experiments, cell number was determined by adding a tetrazolium compound (MTS) to media and measuring its conversion to formazan during the last 4h of culture by colorimetric assessment (Celltiter, Promega, Madison, WI) and comparing values against a standard curve. Replicates of six wells were used in each experiment. All experiments were performed at least three times.

Cytotoxicity assay

The cytotoxic effects of diabetic serum and CML-BSA on macrophages, and of macrophage-conditioned media, IL-1ra and STI-571 on fibroblasts were determined by measurement of released lactate dehydrogenase (LDH) activity. Briefly, macrophages were incubated for 72h and fibroblasts for 48h as described above; however, 8h before completion, the medium was removed and cells were washed and incubated with serum-free medium (200μl for spontaneous release, 100μl for total release). At 30min before the end of culture, 100μl of medium containing 2% Triton X-100 was added to selected cells to facilitate total release of cellular LDH. After centrifugation, the cell supernatant was assessed for LDH activity by colorimetric assay (Cytotoxicity Detection Kit, Roche) and cytotoxicity was determined as the percentage spontaneous LDH release by cells under the applied conditions.

Statistical analysis

Statistical differences between two groups were analysed by the unpaired Student’s t-test, and differences between multiple groups were assessed by one-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test. Correlation analyses were performed using Pearson’s coefficient. 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).

Results

Kidney macrophages increase with hyperglycaemia in STZ-diabetic nephropathy

Compared with normal mice, blood glucose and HbA1c levels were significantly elevated at 2 weeks after STZ injection and continued to increase until week 12 (Table 1). Diabetic mice developed the histological changes of early diabetic nephropathy, including glomerular hypertrophy and hypercellularity, glomerular and interstitial fibrosis, tubular atrophy and interstitial myofibroblast accumulation (Table 2, Figure 1), in association with a progressive increase in kidney leukocytes (Table 3).

Assessment of kidney immunostaining for total CD45+ leukocytes and CD68+ macrophages demonstrated that most glomerular and interstitial leukocytes accumulating in diabetic kidneys were macrophages (Figures 1 and 2). Increased numbers of kidney macrophages were noted as early as 2 weeks post-STZ and, by 18 weeks, there was a 3-fold increase in both glomerular and interstitial macrophages in diabetic compared with control mice (Figure 2). The progressive increase in kidney macrophages correlated with blood glucose (glomerular CD68, r = 0.64, P < 0.0001; interstitial CD68, r = 0.78, P < 0.0001) and HbA1c levels (glomerular CD68, r = 0.72, P < 0.0001; interstitial CD68, r = 0.73, P < 0.0001).

Macrophage accumulation correlates with progressive renal injury and fibrosis in STZ-diabetic nephropathy

Increased glomerular macrophage accumulation in diabetic mice (Figure 2) preceded or coincided with the development of renal injury (albuminuria and elevated plasma creatinine) and preceded the onset of glomerular damage (hypertrophy, hypercellularity and sclerosis) (Tables 1 and 2). Similarly, increased numbers of interstitial macrophages surrounding cortical tubules, vessels and Bowman’s capsule (Figures 1 and 2) coincided with, or preceded, renal injury.

Table 1. Baseline characteristics of mice

<table>
<thead>
<tr>
<th>Normal Duration of STZ diabetes (weeks)</th>
<th>2</th>
<th>8</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.3±2.0</td>
<td>20.9±2.9</td>
<td>22.2±2.3</td>
<td>20.2±2.1</td>
</tr>
<tr>
<td>KBWR (%)</td>
<td>0.68±0.08</td>
<td>0.76±0.12</td>
<td>0.84±0.16</td>
<td>1.03±0.16</td>
</tr>
<tr>
<td>BG (mmol/l)</td>
<td>10.1±1.9</td>
<td>24.6±5.9</td>
<td>29.7±3.2</td>
<td>30.9±5.3</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.2±0.4</td>
<td>5.3±0.5</td>
<td>6.4±2.2</td>
<td>9.0±1.4</td>
</tr>
<tr>
<td>PCr (μmol/l)</td>
<td>25.5±3.6</td>
<td>43.3±12.0</td>
<td>42.0±5.0</td>
<td>46.3±13.2</td>
</tr>
<tr>
<td>UAER (µg/18h)</td>
<td>7.8±2.7</td>
<td>9.6±3.2</td>
<td>10.5±4.6</td>
<td>23.3±14.9</td>
</tr>
<tr>
<td>Urine (ml/18h)</td>
<td>1.6±0.5</td>
<td>0.94±0.15</td>
<td>1.2±0.4</td>
<td>1.3±0.7</td>
</tr>
</tbody>
</table>

STZ = streptozotocin; KBWR = kidney to body weight ratio; BG = blood glucose; PCr = plasma creatinine, UAER = urine albumin excretion rate.

Data are the mean ± SD.

*P < 0.05, **P < 0.01, ***P < 0.001 vs normal by ANOVA.
Macrophages in diabetic nephropathy

### Table 2. Summary of histological renal injury in diabetic vs normal mice

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Duration of STZ diabetes (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Glomerular volume (μm³ × 10⁴)</td>
<td>24.7 ± 5.6</td>
<td>21.4 ± 4.5</td>
</tr>
<tr>
<td>Glomerular cellularity (cells/gcs)</td>
<td>33.4 ± 0.5</td>
<td>33.6 ± 3.8</td>
</tr>
<tr>
<td>Glomerular collagen IV (% area)</td>
<td>18.4 ± 0.6</td>
<td>17.8 ± 0.2</td>
</tr>
<tr>
<td>Atrophic tubules (%)</td>
<td>0.12 ± 0.06</td>
<td>0.30 ± 0.10b</td>
</tr>
<tr>
<td>Apoptotic tubules (%)</td>
<td>0.09 ± 0.09</td>
<td>0.39 ± 0.18c</td>
</tr>
<tr>
<td>Intestinal collagen IV (% area)</td>
<td>19.4 ± 1.7</td>
<td>20.4 ± 1.3</td>
</tr>
<tr>
<td>Intestinal α-SMA (% area)</td>
<td>1.4 ± 0.5</td>
<td>3.7 ± 0.2c</td>
</tr>
</tbody>
</table>

Data are the mean ± SD. *P < 0.05, bP < 0.01, cP < 0.001 vs normal by ANOVA.

(albuminuria and elevated plasma creatinine), tubular damage (tubular dilatation, atrophy and apoptosis) and interstitial fibrosis (increased interstitial α-SMA and collagen IV deposition) (Tables 1 and 2).

In diabetic mice, glomerular macrophage accumulation correlated with renal dysfunction, albuminuria, glomerular hypertrophy and hypercellularity, and glomerular collagen IV deposition (Table 3). Interstitial macrophages correlated with renal dysfunction, albuminuria, tubular atrophy and apoptosis, and interstitial collagen IV and α-SMA (Table 3). Double immunostaining showed the presence of proliferating interstitial myofibroblasts in a diabetic kidney, which were in a similar location to interstitial macrophages in a serial section (Figure 3).

### Macrophages exposed to a diabetic milieu promote fibroblast proliferation

Given the strong correlation between macrophage accumulation and α-SMA expression, we explored whether elements of the diabetic milieu could promote cultured macrophages to release factors that would stimulate renal fibroblast proliferation.

For control purposes, we initially verified that NRK-49F fibroblasts proliferated in response to PDGF-αβ and IL-1β, and that these effects could be abolished by pre-incubation of fibroblasts with STI-571 and IL-1ra, respectively (Figure 4a). In addition, we noted an interdependency of PDGF and IL-1 responses, since pre-incubation with STI-571 reduced the proliferative effect of IL-1β, and pre-incubation with IL-1ra reduced the proliferative effect of PDGF-αβ. The inhibitory effects of IL-1ra and STI-571 on fibroblast proliferation were not due to cytotoxicity, since the doses used did not increase cellular LDH release (Table 4).

Conditioned medium from rat bone marrow-derived macrophages exposed to diabetic rat serum induced a greater proliferation of renal fibroblasts than medium from macrophages exposed to control serum (Figure 4b). This proliferative response was inhibited by pre-treating fibroblasts with either IL-1ra or STI-571. Because the macrophage-conditioned medium used was diluted extensively, the glucose levels of the media added to fibroblasts were similar (control = 5.3 mM, diabetic = 5.5 mM), suggesting that the observed increase in fibroblast proliferation was not related to direct glucose exposure. Furthermore, the addition of 0.45% control or diabetic rat serum directly onto fibroblasts for 48 h caused an equally small increase in proliferation, suggesting that any other serum factors created by the induction of diabetes were not directly promoting fibroblast proliferation.

To identify whether AGEs were responsible for the macrophage responses to diabetic rat serum, we cultured macrophages in serum-free medium containing either control BSA or CML-BSA, and examined the fibroblast response to the new conditioned medium. Fibroblasts exposed to conditioned medium from CML-BSA-treated macrophages showed increased proliferation compared with control medium, which was similar to the responses of diabetic over normal rat serum (Figure 4c). This effect of growth factors on macrophages was dose dependent (Figure 5a and b). We also observed a reduction in fibroblast proliferation to the conditioned medium from CML-BSA-treated macrophages when fibroblasts were pre-treated with IL-1ra or STI-571 (Figure 4c). We did not observe any significant proliferation when control BSA or CML-BSA was added directly to fibroblasts for 48 h.

To determine if macrophages stimulated with CML-BSA were producing greater levels of growth factors, we analysed and compared the conditioned media added to fibroblasts were similar (control = 5.3 mM, diabetic = 5.5 mM), suggesting that the observed increase in fibroblast proliferation was not related to direct glucose exposure. Furthermore, the addition of 0.45% control or diabetic rat serum directly onto fibroblasts for 48 h caused an equally small increase in proliferation, suggesting that any other serum factors created by the induction of diabetes were not directly promoting fibroblast proliferation.

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To determine if macrophages stimulated with CML-BSA were producing greater levels of growth factors, we analysed and compared the conditioned medium from cells treated with CML-BSA and control BSA. Macrophages incubated with CML-BSA were found to secrete more IL-1β and PDGF-β than an equivalent number of macrophages treated with control BSA (Figure 6). The increased macrophage release of IL-1β and PDGF-β by CML-BSA was not due to cytotoxicity, since cellular LDH release was the same for macrophages cultured with control BSA, CML-BSA and serum-free medium alone (Table 4).

### Discussion

Our study suggests that kidney macrophage accumulation may be involved in the progression of type 1 diabetic nephropathy. Kidney macrophages were
Fig. 1. Histopathology in diabetic mouse kidneys at 18 weeks after STZ. Histological staining with periodic acid-Schiff’s (PAS) reagent and haematoxylin shows the glomerular and tubulointerstitial structure of (A) a normal mouse kidney, which is significantly changed in (B) a diabetic kidney. Diabetic mice developed the pathological characteristics of early diabetic nephropathy, including glomerular hypertrophy, hypercellularity and mesangial expansion (arrows), tubules which were dilated and atrophic (arrowheads), and interstitial volume expansion. Immunostaining detected few CD68⁺ macrophages in (C) a normal mouse kidney, which was markedly increased in the glomeruli and interstitium of (D) a diabetic kidney. Immunodetection of α-smooth muscle actin appeared only in vessels of (E) a normal mouse kidney. In comparison, many interstitial myofibroblasts expressing α-smooth muscle actin were found in (F) a diabetic mouse kidney. Immunostaining identified collagen IV expression in glomeruli, vessels and basement membrane in (G) a normal mouse kidney, which increased in (H) a diabetic mouse kidney. Collagen IV also appeared within the expanding interstitium of diabetic kidneys. Magnification: (A–D) ×400, (E–H) ×250.
found to increase with the duration of diabetes, and their numbers correlated with severity of renal injury and dysfunction, and the progression of glomerular and interstitial fibrosis.

In this mouse model of type 1 diabetic nephropathy, kidney macrophage accumulation correlated with the progression of renal injury and glomerular and tubular damage, which is similar to our findings in type 2 diabetic db/db mice [5]. The influx of kidney macrophages occurs more rapidly in the type 1 model where the onset of diabetes is immediate compared with the db/db type 2 model where diabetes develops spontaneously over 2–4 months. This observation is consistent with our data that macrophage accumulation is closely associated with hyperglycaemia in both models and previous work demonstrating that insulin treatment after induction of diabetes prevents accrual of kidney macrophages [4,5]. Macrophages are known to cause early glomerular injury in STZ-induced diabetes and also possess the mechanisms to cause progressive

Table 3. Correlation of kidney macrophage accumulation with renal injury

<table>
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<tr>
<th>Renal injury</th>
<th>Glomerular macrophages</th>
<th>Interstitial macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albuminuria</td>
<td>0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma creatinine</td>
<td>0.46&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.45&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Renal pathology</td>
<td></td>
<td></td>
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<tr>
<td>Glomerular volume</td>
<td>0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Glomerular cellularity</td>
<td>0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Glomerular collagen IV</td>
<td>0.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Atrophic tubules</td>
<td>–</td>
<td>0.51&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Apoptotic tubules</td>
<td>–</td>
<td>0.28&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Interstitial collagen IV</td>
<td>–</td>
<td>0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interstitial α-SMA</td>
<td>–</td>
<td>0.56&lt;sup&gt;c&lt;/sup&gt;</td>
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Data are r-values from all normal and STZ-diabetic mice using Pearson’s coefficient.

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01, <sup>c</sup>P < 0.001 vs normal.

Fig. 2. Progressive macrophage accumulation in STZ-induced diabetic nephropathy. Immunostaining for CD45<sup>+</sup> total leukocytes and CD68<sup>+</sup> macrophages shows a progressive increase in (a) glomerular leukocytes and (b) interstitial leukocytes in diabetic mouse kidneys which consist of mostly macrophages. Data are the mean ± SD, n = 10, P < 0.0001 by ANOVA at all time points (2, 8, 12 and 18 weeks) compared with normal animals at an equivalent age to mice after 18 weeks of STZ.

Fig. 3. Macrophages surround proliferating interstitial fibroblasts in diabetic kidneys. (A) Double immunostaining demonstrates that some interstitial myofibroblasts expressing α-smooth muscle actin are proliferating (PCNA<sup>+</sup> nuclei, arrows). Immunostaining of a serial section (B) shows that there are many F4/80<sup>+</sup> macrophages surrounding the interstitial proliferating cells (PCNA<sup>+</sup> nuclei, arrows) which are in the same proximity to the proliferating myofibroblasts.
glomerular and tubular damage [4,6,7]. In vitro studies have demonstrated that AGEs can induce macrophage secretion of nitric oxide, reactive oxygen species and tumour necrosis factor-α (TNF-α) [16,17], mediators by which macrophages may cause renal injury [7]. Renal deposition of AGEs and increased levels of kidney oxidative stress and TNF-α are all prominent features of diabetic nephropathy [18,19]. In addition, we have shown previously that many glomerular and interstitial macrophages express nitric oxide in mouse diabetic kidneys [5]. Therefore, it is feasible that the accumulating macrophages in diabetic kidneys contribute to the progression of renal injury.

In our current study of STZ-induced type 1 diabetic nephropathy, and in our previous analysis of type 2 diabetic db/db mice, we found that kidney macrophages were also associated with increased expression of interstitial α-SMA, and glomerular and interstitial collagen IV, suggesting a possible causal relationship. In the STZ model, we also showed that proliferating interstitial myofibroblasts were co-localized with kidney macrophages, indicating that macrophages may be involved in promoting the proliferation of these fibroblasts. Macrophages cultured in diabetic rat serum or CML-BSA produced conditioned medium that enhanced fibroblast proliferation above that of macrophages cultured in control rat serum or control BSA, respectively, and was not cytotoxic to fibroblasts. Control rat serum and control BSA did not appear to stimulate macrophages since their presence or absence in macrophage-conditioned media resulted in a similar fibroblast proliferative response. Diabetic rat serum caused a significant but much smaller induction of fibroblast proliferation when directly applied to fibroblasts, which was not different from the effects of

<table>
<thead>
<tr>
<th>Table 4. Cytotoxicity of diabetic sera, CML-BSA and cytokine inhibitors</th>
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<tr>
<td>A. Percentage spontaneous LDH release by bone marrow macrophages</td>
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<tr>
<td>SFM CRS DRS Control CML-BSA</td>
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<tr>
<td>Macrophages</td>
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<tr>
<td>B. Percentage spontaneous LDH release by NRK-49F fibroblasts</td>
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<tr>
<td>SFM Macrophage-conditioned medium</td>
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<tr>
<td>Alone +CRS +DRS +Control +CML-BSA</td>
</tr>
<tr>
<td>NRK-49F</td>
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<tr>
<td>NRK-49F + 6.6±1.9</td>
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<tr>
<td>IL-1ra</td>
</tr>
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Data are the mean±SD, n=6. SFM=serum-free medium; CRS=control rat serum; BSA=control BSA.

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Fig. 4. Diabetes promotes macrophage-induced fibroblast proliferation via production of IL-1 and PDGF. (a) IL-1β and PDGF induced proliferation of NRK-49F fibroblasts during a 48 h incubation period, and these responses were abolished by preincubation with IL-1ra and STI-571, respectively. In addition, the proliferative responses to IL-1β and PDGF appear to be interdependent as indicated by the partial inhibition of IL-1-induced proliferation with STI-571 and PDGF-induced proliferation with IL-1ra. (b) NRK-49F cells have small and equivalent proliferative responses to 0.45% control and 0.45% diabetic rat sera. In comparison, conditioned medium from bone marrow macrophages stimulated with diabetic rat serum induced a much greater fibroblast proliferative response compared with conditioned medium from macrophages stimulated with control rat serum. The proliferative responses from these macrophage-conditioned media were reduced by pre-treatment with either IL-1ra or STI-571. (c) Control BSA or CML-BSA did not enhance proliferation of NRK-49F cells. In contrast, conditioned medium from bone marrow macrophages stimulated with control BSA or CML-BSA induced a marked increase in fibroblast proliferation which was significantly greater in conditioned medium from macrophages exposed to CML-BSA. The proliferative responses from these macrophage-conditioned media were also reduced by pre-treatment with either IL-1ra or STI-571. Data are the mean±SD, n=6, *P<0.05, **P<0.01, ***P<0.001, NS=not significant.
normal rat serum. CML-BSA and control BSA had no direct effect on fibroblast proliferation. These results suggest that the actions of the diabetic milieu on macrophages may indirectly promote fibroblast proliferation to a greater extent than any direct effects of the diabetic milieu on fibroblasts.

Using specific inhibitors of cytokine signalling, we found that diabetic rat serum and CML-BSA induce a macrophage response which promotes fibroblast proliferation via production of IL-1 and PDGF. Furthermore, we showed that the effects of IL-1 and PDGF on fibroblast proliferation are interdependent, since specific receptor signalling blockade of either cytokine could inhibit the proliferative response induced by the other. Although macrophages can produce these cytokines, this study did not identify whether the IL-1 and PDGF causing fibroblast proliferation were derived from macrophages or were produced by fibroblasts in response to a macrophage-secreted stimulant. However, we were able to detect IL-1β and PDGF-β in the macrophage-conditioned medium, which was significantly greater in medium derived from CML-BSA-treated macrophages. The amounts of IL-1β in macrophage-conditioned medium were equal to or higher than those used in the preliminary cytokine-induced proliferation assays. Therefore, it is not surprising that the macrophage-conditioned medium, containing PDGF in addition to high levels of IL-1β, was able to promote fibroblast proliferation to a greater extent than moderate levels of either cytokine alone.

Our results suggesting that macrophage-derived IL-1 and PDGF may be contributing to kidney fibroblast proliferation during diabetes is consistent with other studies. Rodent models show increased expression of IL-1 in diabetic nephropathy [19], and a striking upregulation of PDGF (up to 22-fold) has been described in human diabetic kidneys [20]. β2-microglobulin modified with AGEs is known to induce IL-1β and PDGF secretion by human peripheral blood monocytes [12,13], suggesting that infiltrating kidney macrophages may be a source of these cytokines in diabetic nephropathy. Previous in vitro experiments have demonstrated that conditioned medium derived from alveolar macrophages activated by exposure to crocidolite fibres induces fibroblast proliferation via production of IL-1β [21] and PDGF [22], indicating that macrophages can promote fibroblast proliferation by release of these cytokines.
In summary, this study demonstrates that kidney macrophage accumulation is associated with progression of renal injury and fibrosis in mouse type 1 diabetic nephropathy. Our in vitro findings suggest that the formation of AGEs during diabetes may induce kidney macrophages to promote fibroblast proliferation via the production of IL-1 and PDGF. Since interstitial myofibroblast accumulation appears critical for the progression of fibrosis in diabetic nephropathy, our data indicate that therapies targeting macrophage accumulation or activation in diabetic nephropathy may have significant benefit.

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Conflict of interest statement. None declared.

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