CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Reversing CXCL10 Deficiency Ameliorates Kidney Disease in Diabetic Mice

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The excessive accumulation of extracellular matrix material in the kidney is a histopathologic hallmark of diabetic kidney disease that correlates closely with declining function. Although considerable research has focused on the role of profibrotic factors, comparatively little attention has been paid to the possibility that a diminution in endogenous antifibrotic factors may also contribute. Among the latter, the ELR[−] CXC chemokines, CXCL9, CXCL10, and CXCL11, have been shown to provide a stop signal to prevent excessive fibrosis. Although the plasma concentrations of CXCL9 and CXCL11 were similar, those of CXCL10 were markedly lower in diabetic db/db mice compared with control db/m mice. In cell culture, CXCL10 inhibited kidney fibroblast collagen production in response to high glucose and the prosclerotic growth factor, transforming growth factor- β . *In vivo*, recombinant murine CXCL10 reduced mesangial and peritubular matrix expansion, albuminuria, and glomerular hypertrophy in db/db mice. In bone marrow, a major source of circulating chemokines, the concentration of CXCL10 was lower in cells derived from diabetic mice than from their nondiabetic counterparts. Silencing of CXCR3, the cognate receptor for CXCL10, abrogated the antifibrotic effects of bone marrow-derived secretions. In conclusion, experimental diabetes is a state of CXCL10 deficiency and that restoration of CXCL10 abundance prevented fibrosis and the development of diabetic kidney disease in mice. (*Am J Pathol* 2018; 188: 2763–2773; <https://doi.org/10.1016/j.ajpath.2018.08.017>)

Fibrotic diseases such as hepatic cirrhosis, pulmonary fibrosis, and most forms of chronic kidney disease account for >40% of all chronic disease in the developed world, and although they vary substantially in cause and clinical and structural manifestations, they share a range of common pathophysiological mechanisms.¹ Fibrosis is also a feature of long-term diabetes in which its extent and clinical impact range from asymptomatic muscle capillary basement membrane thickening, through minimally symptomatic cheiroarthropathy to the glomerulosclerosis and tubulointerstitial fibrosis of diabetic nephropathy that lead directly to progressive renal impairment and ultimately failure.^{2,3} Unsurprisingly, as a consequence of its increasingly recognized importance, numerous strategies that antagonize key fibrogenic pathways are being developed to target fibrotic disease in general⁴ and diabetic kidney disease specifically.^{4,5}

The physiological regulation of many biological systems is achieved by a balance of opposing forces, as with insulin versus glucagon, aldosterone versus the natriuretic peptides,

and the coagulation versus fibrinolytic cascades. However, despite knowledge of its existence, exploration of antifibrotic pathways with the possible exception of the matrix-degrading metalloproteinase and plasmin-dependent pathways^{6,7} has received comparatively little attention from either mechanistic or therapeutic viewpoints. First recognized for their ability to modulate leukocyte chemotaxis and immunity, more recent investigation has highlighted the pivotal role that chemokines play in the regulation of repair, regeneration, and remodeling after tissue injury.⁸ These small (8 to 14 kDa), closely related, protein molecules are classified into four families (C, CC, CXC, and CX3C) according to the number and arrangements of their N-terminal cysteine residues.⁹

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Among these, the CXC subfamily is further divided into those that contain an ELR (glutamic acid-leucine-arginine) motif preceding the first cysteine and those that do not.¹⁰ Notably, the differences between ELR⁺ and ELR⁻ CXC chemokines are functional as well as structural whereby the former are primarily proangiogenic whereas the latter, CXCL9, CXCL10, and CXCL11, exert antifibrotic activity in various organs, including the heart,¹¹ lung,¹² and kidney.¹³

Accordingly, we hypothesized that diabetes, as a profibrotic state, may be associated with a diminution in ELR⁻ CXC chemokines and that their restoration may ameliorate diabetic kidney disease to provide a new therapeutic approach to the disease.

Materials and Methods

Animal Studies

All animal studies were approved by the St. Michael's Hospital Animal Ethics Committee in accordance with the NIH's *Guide for the Care and Use of Laboratory Animals*.¹⁴ In the first set of animal studies (animal study 1), plasma concentrations of putative antifibrotic factors were compared in diabetic and nondiabetic animals. Male diabetic db/db (*BKS.Cg-Dock7m* *+/+ Leprdb/J*) and nondiabetic db/m (*Dock7m* *+/+ Leprdb*, heterozygote from the colony) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were housed at the St. Michael's Hospital Animal Research Vivarium in a temperature-controlled room (22°C) with a 12 hours:12 hours light/dark cycle and free access to food and water. Blood samples were taken at termination at 12 weeks of age.

In the second set of experiments (animal study 2), 24 6-week-old male diabetic db/db and 24 age-matched db/m mice were housed for the next 6 weeks, as described above. Two weeks later, at 8 weeks of age, both db/db and db/m mice were randomized to receive either CXCL10 or vehicle. Recombinant murine CXCL10 (PeproTech Inc., Rocky Hill, NJ) dissolved in 20 µL of 0.25% mouse albumin/phosphate-buffered saline (PBS) was given to mice intramuscularly daily with a dose of 1 µg/day (0.03 mg/kg per day) for nondiabetic db/m mice for 4 weeks, as previously reported.¹² Diabetic db/db mice received 3 µg/day (0.06 mg/kg per day) for 4 weeks. The higher dose in this animal group was based not only on their larger body mass but also on previous studies showing that CXCL10 is degraded by di-peptidyl peptidase 4 (DPP4)¹⁵ and that the activity of this enzyme is increased two- to threefold in diabetes.¹⁶ For the control groups, 20 µL of 0.25% mouse albumin/PBS was given by daily intramuscular injection.

A third set of 28 animals with 7 mice per group (animal study 3) were treated as in study 2 above to obtain sufficient blood for automated hematologic analysis that included assessments of hemoglobin, differential white cell, and platelet counts.

Kidney Structure and Function

Body weight and blood glucose (Accu-Chek Advantage; Roche, Mississauga, ON, Canada) were determined every 2 weeks. Albuminuria was quantified after 24 hours of metabolic caging at baseline (8 weeks of age) and study end (12 weeks of age) by enzyme-linked immunosorbent assay (ELISA; AssayMax mouse albumin; Assaypro, St. Charles, MO). Systolic blood pressure was measured noninvasively in conscious mice by using an occlusive tail-cuff plethysmograph attached to a pneumatic pulse transducer (Powerlab; ADInstruments, Colorado Springs, CO), at the end of the study, as previously described.¹⁷ At the end of the 4-week study period mice were terminated by lethal anesthesia after which time their kidneys were removed, decapsulated, and sliced sagittally before immersion fixation in 10% neutral buffered formalin, embedding in cryostat matrix (Tissue-Tek, Sakura, Kobe, Japan), or snap-freezing in liquid nitrogen. Sections (4 µm) were used for histochemical and immunohistochemical staining as described below. Mesangial expansion was assessed in a masked fashion by using a semiquantitative technique as previously described.¹⁸

Immunohistochemistry

Immunohistochemistry for type IV collagen was performed as previously described¹⁷ with type IV collagen deposition detected with the use of a polyclonal goat anti-mouse collagen type IV antibody (Southern Biotech, Birmingham, AL), diluted 1:100 in PBS incubation. Normal goat serum instead of primary antibody served as the negative control. To quantify its abundance, stained sections were scanned with the Aperio Ultra-Resolution Digital Scanning System (Aperio Technologies Inc., Vista, CA) with images analyzed by using Aperio ImageScope software version 12.1.5029 to assess the extent of type IV collagen deposition in 30 randomly selected glomeruli from 12 random nonoverlapping ×20 fields for each animal in a masked fashion.

Immunostaining was also undertaken to localize the site-specific expression of CXCR3 within the kidney by using a polyclonal anti-mouse CXCR3 antibody diluted 1:200 (NBP2-12239; Novus Biological, Littleton, CO) with further processing undertaken as for type IV collagen above.

For CD68 staining, slides were deparaffinized through xylenes and an alcohol gradient and taken to water. Endogenous peroxidase activity was blocked in H₂O₂/methanol for 12 minutes. Antigen retrieval was undertaken by using heat-induced epitope retrieval with Tris-EDTA buffer (pH 9) for 7 minutes. Nonspecific antibody binding was blocked by using Vector ImmPRESS blocking reagent (catalog number MP-7401; Vector Laboratories, Burlingame, CA) for 20 minutes at room temperature. Sections were then incubated in rabbit anti-CD68 primary antibody (catalog number ab125212; Abcam, Cambridge, UK) diluted 1:600 and incubated for 1 hour at room temperature. After washes, sections

were incubated in ImmPRESS peroxidase Polymer Anti-Rabbit IgG reagent for 30 minutes at room temperature. Positive immunoreactive staining was visualized by using Steady DAB/Plus (catalog number ab103723; Abcam). Mayer's hematoxylin was used as a counterstain before dehydrating and mounting on coverslips.

Chemokine Assays

The concentration of CXCL10 was measured by solid-phase sandwich ELISA kits for murine chemokines (Quantikine ELISA Kits; R&D Systems, Minneapolis, MN) in stored heparinized mouse plasma and cell conditioned medium, according to the manufacturer's instructions.

Antifibrotic Activity

Antifibrotic activity was determined by examining fibroblast collagen synthesis. In brief, rat kidney fibroblasts (NRK49F) were serum-starved before incubation with 5 ng/mL transforming growth factor (TGF)- β (R&D Biosystems, Minneapolis, MN) and/or 25 mmol/L glucose, before incubation with [3 H]-proline (1 μ Ci/well; Amersham Biosciences, Little Chalfont, UK) for 44 hours. Incorporation of [3 H]-proline was measured by using a liquid scintillation counter (LS 6000 Beckman Instruments; Beckman Coulter, Mississauga, ON, Canada) that provides an indirect measurement of collagen synthesis.¹⁹ The effects of recombinant mouse CXCL10 (rmCXCL10; PeproTech Inc.) were also examined as was bone marrow-conditioned medium before and after transfecting cells with either scramble of the CXCR3 shRNA (number 336311; Qiagen, Hilden, Germany).

Reporter Assay

Smad3-dependent transcriptional activity was followed by a Smad3-responsive reporter construct that contains four consecutive Smad-binding elements driving the expression of firefly luciferase (SBE4-Luc, provided by A.B. Roberts), as previously reported.²⁰ In brief, NRK49F rat kidney fibroblasts were plated (0.15×10^6 cells/well) onto 24-well plates and after 24 hours were cotransfected with SBE4-Luc and the normalizing vector RL-TK renilla luciferase by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA), according to the manufacturer's recommendation and as previously reported.²¹ After 16 hours, cells were serum-starved for 3 hours, then incubated with 5 ng/mL TGF- β 1 with or without 0.1 to 100 ng/mL rmCXCL10 for 24 hours. Then the cells were lysed, and luciferase activity was determined by using the Dual Luciferase Reporter Assay System kit (Promega, Madison, WI) and a luminometer (Lumat 9507; Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's instructions. For each condition, treatments were performed in duplicates, and experiments were repeated at least three times. From each sample, firefly luciferase activity was normalized to the renilla luciferase activity of the

same sample. Results were then expressed as fold changes compared with the mean firefly/renilla ratio of untreated controls.

Real-Time Quantitative PCR

Gene expression was quantified in kidney tissue by real-time PCR as previously reported.²² In brief, SYBR green-based measurement of gene expression was quantified by using a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA) with predesigned sequence-specific primers for CD68 and CD206 obtained from Integrated DNA Technologies (Coralville, IA). Transcript abundance was assessed relative to the housekeeping gene, *RPL13A*, with forward and reverse primers of 5'-GATGAACACCAACCCGTCCTC-3' and 5'-CACCATCCGCTTTTCTTGT-3', respectively. Data were then analyzed by using Applied Biosystems Comparative CT method.

Cell Culture

Bone marrow cells were cultured as previously described.^{19,23} Briefly, bone marrow was flushed from the femurs and tibias of 4- and 12-week-old male db/m and db/db mice by using sterile Dulbecco's PBS. The collected material was then centrifuged at 1400 rpm for 7 minutes at 22°C, and the supernatant was discarded after completion. Cells were then resuspended and plated on culture flasks coated with human fibronectin (10 μ g/mL; BD Biosciences, San Jose, CA) and maintained in endothelial cell growth medium-2 (Lonza, Walkersville, MD) at 37°C with 5% CO₂ for 7 to 10 days. As previously described, this culture process gives rise to a mixed population of nonerythroid, predominantly mononuclear cells.^{19,23}

To determine whether CXCL10 was also secreted by other cell types, its abundance was examined in the culture medium of murine monocytes (WEHI-274.1, ATCC CRL-1679; ATCC, Manassas, VA), fibroblasts (NIH/3T3, ATCC CRL-1658; ATCC), and glomerular endothelial cells (C57-6014G; Cell Biologics, Chicago, IL). Monocytes and fibroblasts were plated (0.15×10^6 cells/well) onto 24-well plates and cultured in 10% fetal calf serum supplemented with Dulbecco's modified Eagle's medium until reaching 80% confluency. Cells were then washed with PBS and cultured in serum-free medium for a further 24 hours before harvesting the conditioned medium. Glomerular endothelial cells underwent the same procedure apart from being cultured in endothelial cell medium (M1168; Cell Biologics) rather than Dulbecco's modified Eagle's medium.

Immunoblot Analysis

Bone marrow-conditioned medium was concentrated 10-fold by using a >10-kDa cutoff centrifuge filtration column (Millipore, Billerica, MA). Specimens were then filtered by using a 0.45- μ m filter (Millipore) and were examined by

protein array (Proteome Profiler Cytokine Array kit), according to the manufacturer's instructions.

The abundance of CXCL10 and CXCR3 was assessed by immunoblot analysis, as previously described,²⁴ using specific anti-CXCR3 (Novus Biological) and anti-CXCL10 (R&D Systems) antibodies. In brief, kidney tissue that had been rapidly collected, snap-frozen in liquid nitrogen, and stored at -80°C was used. Lysates were prepared by homogenizing frozen tissue in ice-cold lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5% Triton X-100 that contained 1 mmol/L Na_3VO_4 , 50 mmol/L NaF, 25 mmol/L β -glycerophosphate, 10 mmol/L Na pyrophosphate, 10 $\mu\text{g/mL}$ aprotinin, 10 $\mu\text{g/mL}$ leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol). After 30 minutes of incubation on ice, lysates were centrifuged to remove cell debris. Protein concentration was determined by using Bradford reagent (Bio-Rad, Hercules, CA) and standardized with known amounts of bovine serum albumin. Western blot analysis was performed by resolving 50 μg of total protein on a 4% to 20% Tris-Glycine gel by denaturing discontinuous gel electrophoresis according to the Laemmli method and transferring to nitrocellulose membranes (GE Healthcare Life Sciences, Mississauga, ON, Canada). After incubating in blocking solution [5% nonfat dry milk, Tris-buffered saline (pH 7.5), 0.1% Tween 20,] membranes were immunoblotted with primary antibodies at 1:1000 dilution overnight at 4°C . Membranes were then washed three times (5 minutes each) with Tris-buffered saline with Tween 20, and blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) for 1 hour at room temperature. The membranes were washed three times, and proteins were detected by the enhanced chemiluminescence system (ThermoFisher, Waltham, MA). Signals were digitized then analyzed with ImageJ software version 1.50i (<https://imagej.nih.gov/ij>; NIH, Bethesda, MD). Results were calculated as the mean of at least three experiments relative to glyceraldehyde-3-phosphate dehydrogenase immunolabeling.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Between-group differences were analyzed by one-way analysis of variance with Fisher's protected least significant difference test post hoc. All statistics were performed with GraphPad Prism software version 5 for Mac OS X (GraphPad Software Inc., San Diego, CA). A P value of <0.05 was considered statistically significant.

Results

Plasma CXCL10 Is Reduced in Experimental Diabetes

To determine whether diabetes was associated with alteration of antifibrotic chemokines, the plasma concentrations of the three ELR⁺ CXC chemokines, CXCL9, CXCL10, and CXCL11, were examined in animal study 1 with eight,

Figure 1 Plasma CXCL10. The concentration of CXCL10 in the plasma of diabetic db/db and nondiabetic db/m mice was determined by enzyme-linked immunosorbent assay. Compared with db/m mice the plasma concentration of CXCL10 was lower in db/db mice. Data are expressed as means $\pm$ SEM. * $P < 0.05$.

12-week-old diabetic db/db mice (plasma glucose, 17.2 ± 3.1 mmol/L) and eight, age-matched nondiabetic db/m controls (blood glucose, 5.3 ± 0.5 mmol/L). Compared with normoglycemic db/m mice, diabetic db/db mice had lower plasma concentrations of CXCL10 (Figure 1). Circulating concentrations of other ELR⁺ CXC chemokines, CXCL9 and CXCL11, were below the limit of detection by ELISA in both groups.

CXCL10 Attenuates Stimulated Collagen Synthesis and TGF- β Signaling

High glucose and the prototypical fibrogenic growth factor, TGF- β , contribute to the development of fibrosis in diabetes. Consistent with this, high glucose and to a greater extent TGF- β increased ^3H -proline incorporation, a marker of collagen synthesis in cultured kidney fibroblasts. Coincubating these fibroblasts with rmCXCL10 attenuated both high glucose and TGF- β -induced collagen synthesis (Figure 2).

Because the actions of TGF- β were mediated by a downstream signaling pathway that pivots on the activity of the regulatory protein, Smad3, a Smad3-responsive reporter construct was used to assess the effects of rmCXCL10 activation. In both the unstimulated and TGF- β -stimulated

Figure 2 Antifibrotic activity. Cultured fibroblasts were stimulated with 5 ng/mL transforming growth factor (TGF)- β or high glucose (HG; 25 mmol/L). Co-incubation with 10 ng/mL CXCL10 attenuated collagen production in response to both conditions as measured by the incorporation of ^3H -proline. Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus control; † $P < 0.05$ versus high glucose; ‡ $P < 0.05$ versus TGF- β . AU, arbitrary unit.

Figure 3 Smad3 reporter activity. Cells transfected with Smad3 reporter construct, SBE4-Luc/pRL-TK, were examined with and without stimulation by transforming growth factor (TGF)-β1. Recombinant murine CXCL10 dose dependently inhibited SBE4-Luc activity in the basal state and in response to TGF-β1. Data are expressed as means ± SEM. *n* = 3 per group. **P* < 0.05 versus control; †*P* < 0.05 versus TGF-β. AU, arbitrary unit.

setting, rmCXCL10 attenuated Smad3 reporter activity in a dose-dependent manner (Figure 3).

rmCXCL10 Administration Attenuates Nephropathy in the db/db Mouse

Having found a diminution in the circulating concentration of CXCL10 in diabetic animals, it was determined whether its restoration might ameliorate the functional and structural manifestations of experimental diabetic nephropathy. Accordingly, a second animal study (animal study 2) was performed whereby rmCXCL10 was administered to db/db and db/m mice, elevating the plasma CXCL10 concentrations in db/db mice and to a greater extent in db/m mice (Table 1).

Without affecting glycemic control or blood pressure, diabetic mice that received rmCXCL10 daily for 4 weeks had less albuminuria compared with diabetic mice that received vehicle alone (Table 1). Consistent with this functional manifestation of reduced injury, db/db mice that had received rmCXCL10 also displayed an attenuation in the typical histopathologic changes in this animal model of

diabetic nephropathy. Notably, the extent of glomerular enlargement, mesangial expansion, and type IV collagen accumulation were all attenuated by administering rmCXCL10 to db/db mice (Figures 4 and 5). Similarly, in the tubulointerstitium, the magnitude of peritubular collagen deposition was also reduced in db/db mice that had received rmCXCL10 (Figure 5). rmCXCL10 was without effect in nondiabetic db/m mice.

Because CXCL10 has been associated with proinflammatory and autoimmune states, circulating leukocytes and kidney macrophages were assessed. Of the 28 animals in study 3 (*n* = 7 per group), specimens from three mice were clotted and not suitable for analysis (1 db/m + rmCXCL10, 1 db/db + vehicle, 1 db/db + rmCXCL10), specimen size was too small to be analyzed in another two (1 db/m + vehicle, 1 db/db + rmCXCL10), and one animal from the db/db + vehicle group showed evidence of sepsis with a neutrophil count of $39 \times 10^9/L$ and 6% band forms and was excluded (Table 2). Hemoglobin concentration and monocyte and platelet counts were similar among all four groups. Neutrophil numbers were slightly higher in db/db mice, and lymphocyte counts were slightly lower compared with db/m mice. rmCXCL10 was without effect on circulating cell counts in either db/m or db/db mice (Table 1). In the kidney, no differences in the pan-macrophage marker or *CD68* mRNA were observed between groups, contrasting the increased expression of the M2 macrophage marker *CD206* in rmCXCL10-treated db/db mice (Figure 5).

Bone Marrow CXCL10 Secretion Is Reduced in Experimental Diabetes and Contributes to Its Antifibrotic Activity

Because nonerythroid cells of hematopoietic origin are the predominant source of chemokines, their concentrations were compared in the secretory output of bone marrow cells derived from both db/db and db/m mice. Although the concentrations of CXCL9 and CXCL11 were below the limits of detection by immunoblot analysis, CXCL10 was readily detected in the conditioned medium derived from

Table 1 Summary of the Animal Characteristics

Characteristic	db/m + Vehicle	db/m + CXCL10	db/db + Vehicle	db/db + CXCL10
No.	8	8	8	8
CXCL10, pg/mL	119 ± 18	594 ± 146*†	59 ± 12	344 ± 106†
HbA _{1c} , %	4.07 ± 0.07	4.10 ± 0.06	9.30 ± 0.44*	9.66 ± 0.11*
HbA _{1c} , mmol/mol	20.7 ± 0.7	21.0 ± 0.6	78.3 ± 4.7*	82.2 ± 1.7*
SBP, mmHg	108 ± 5	108 ± 3	105 ± 4	111 ± 7
Body weight, g	30.8 ± 0.7	29.5 ± 0.6	44.6 ± 1.2*	45.4 ± 0.5*
Kidney weight, g	0.21 ± 0.01	0.20 ± 0.00	0.23 ± 0.01	0.23 ± 0.01*
Kidney weight/body weight, %	0.68 ± 0.03	0.67 ± 0.02	0.51 ± 0.03*	0.51 ± 0.01*
Urine albumin, μg/24 hours	41.6 ± 5.4	39.6 ± 2.0	393.8 ± 51.5*	296.2 ± 36.9*†

Values are expressed as means ± SEM.

**P* < 0.05 versus db/m + vehicle group.

†*P* < 0.05 versus db/db + vehicle group.

HbA_{1c}, glycated hemoglobin; SBP, systolic blood pressure.

Figure 4 Glomerular histopathology. **A–F:** Kidney sections were stained with periodic acid-Schiff. Representative photomicrographs from nondiabetic db/m or diabetic db/db mice treated with vehicle (**A** and **C**) or recombinant murine (rm)CXCL10 (**B** and **D**) are shown along with graphs displaying the magnitude of glomerular volume (**E**) and mesangial expansion (**F**). Compared with vehicle-treated db/db mice those that received rmCXCL10 showed reduced glomerular hypertrophy and mesangial expansion. Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus db/m mice; † $P < 0.05$ versus vehicle-treated db/db mice. Scale bars: 50 μm . Original magnification, $\times 400$.

bone marrow of young db/m mice but to a far lesser extent in cells from diabetic db/db mice of the same age when assessed by immunoblot analysis (Figure 6). When quantified by ELISA, the concentration of CXCL10 in conditioned medium from the bone marrow–derived cells of db/m mice (1613 ± 179 pg/mL) was numerically higher than in age-matched db/db mice (736 ± 139 pg/mL).

The secretory output of bone marrow–derived cells displays potent antifibrotic activity.¹⁹ To determine the contribution of CXCL10 to the antifibrotic activity of the bone marrow secretions, the ability of its conditioned medium to inhibit high glucose and TGF- β –induced collagen production in the presence or absence of its cognate receptor CXCR3 was assessed. Although the bone marrow–conditioned medium inhibited both high glucose and TGF- β –induced collagen production, these effects were abrogated by CXCR3 knockdown (Figure 7).

Although CXCL10 was below the limit of detection by ELISA in conditioned medium from monocytes and endothelial cells, it was readily detectable in conditioned medium from fibroblasts (30.5 ± 2.2 pg/mL) albeit to a lesser extent than in the conditioned medium of cultured bone marrow cells.

Kidney CXCL10 and CXCR3 Expression

The expression of CXCL10 and CXCR3 in the kidneys of db/m and db/db mice was examined by immunoblot analysis.

Although this confirmed their presence in the kidneys of all animal groups, no differences were evident between groups (Figure 8). Immunohistochemistry localized expression of CXCR3 predominantly to the parietal epithelium of glomeruli in both db/m and db/db mice (Figure 8).

Discussion

Recognized histopathologically as glomerulosclerosis and tubulointerstitial fibrosis, the expanding extracellular matrix that develops in the diabetic kidney obliterates capillary networks to cause glomerular filtration rate decline and, ultimately, kidney failure.²⁵ Although a wealth of research, performed over the past two decades, has documented the excessive production of profibrotic factors in diabetes,²⁶ comparatively little is known about endogenous antifibrotic factors. These data show that not only the plasma concentration of the antifibrotic cytokine CXCL10 is reduced in experimental diabetes but also the administration of recombinant CXCL10 attenuates both the functional and structural manifestations of kidney disease in the db/db mouse model of diabetic nephropathy.

The response to injury, be it physical, chemical, or biological, involves an ordered sequence of responses that in mammals mostly leads to repair by connective tissue rather than regeneration. Of importance, this adaptive repair process shuts down with predictable precision, allowing organ

Figure 5 Kidney collagen type IV deposition and macrophage marker expression. Kidney sections were immunolabeled with anti-collagen type IV antibody. **A–L:** Representative photomicrographs showing glomerular (**A–D**) or tubulointerstitial (**E–H**) collagen type IV deposition from db/m or db/db mice treated with vehicle (**A, C, E, and G**) or recombinant murine (rm)CXCL10 (**B, D, F, and H**) are shown along with graphs displaying the proportional area that was immunostained (**I and J**) and the expression of CD68 and CD206 mRNA (**K and L**). Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus db/m animals; † $P < 0.05$ versus vehicle-treated db/db animals. Scale bars: 50 μ m (**A–D**); 100 μ m (**E–H**). Original magnification: $\times 400$ (**A–D**); $\times 100$ (**E–H**). AU, arbitrary unit.

integrity to be restored. However, the response to chronic or repeated injury may be maladaptive with the continuing elaboration of large quantities of disorganized extracellular matrix. The pathologic scarring that then results interferes with parenchymal function, ultimately leading to organ

failure as seen in cirrhotic liver disease, pulmonary fibrosis, and the glomerulosclerosis/tubulointerstitial fibrosis in chronic kidney disease. Accordingly, reducing the activity of profibrotic factors has been a focus of therapeutic initiatives whereby for more than two decades research has

Table 2 Summary of the Complete Blood Count

Variable	db/m + Vehicle	db/m + CXCL10	db/db + Vehicle	db/db + CXCL10
No.	7	7	7	7
Hemoglobin, g/dL	17.8 ± 1.3	15.2 ± 1.3	16.1 ± 0.1	16.1 ± 0.4
WBCs, ×10 ⁹ /L	12.8 ± 0.9	10.6 ± 0.8	10.7 ± 0.1*	10.5 ± 0.3*
Neutrophils, ×10 ⁹ /L	0.72 ± 0.06	0.83 ± 0.09	3.50 ± 1.20*	3.80 ± 0.51*
Monocytes, ×10 ⁹ /L	0.62 ± 0.10	0.73 ± 0.11	0.60 ± 0.33	1.10 ± 0.66
Lymphocytes, ×10 ⁹ /L	7.50 ± 0.66	6.00 ± 0.71	4.52 ± 0.99*	3.60 ± 0.54*
Platelets, ×10 ⁹ /L	1114 ± 229	768 ± 272	1453 ± 73	1313 ± 260

Values are expressed as means ± SD.
**P* < 0.05 versus db/m + vehicle group.
WBC, white blood cell.

emphasized the predominant role of TGF-β.²⁷ This has been particularly so in kidney disease and especially in diabetic nephropathy in which numerous anti-TGF-β strategies are under development.⁵ However, in addition to the factors responsible for fibrogenesis, others, presumably, might be responsible for dampening the synthetic phase of wound repair. Deficiency of the latter may, accordingly, lead to continued scar formation. Consistent with these actions, recent attention has focused on the ELR[−] chemokines, CXCL9, CXCL10, and CXCL11, as potential mediators, that by binding to their cognate receptor, CXCR3, orchestrate the transition from active fibrogenesis to quiescence.²⁸

In the present study, it was found that CXCL10 reduced collagen production by kidney fibroblasts in response to both high glucose and TGF-β, showing further that CXCL10 inhibited the downstream signaling of the TGF-β receptor pathway. To test the relevance of these findings, rmCXCL10 was then administered to diabetic animals. This elevated the circulating CXCL10 concentrations in db/db and to a greater extent in db/m mice, possibly reflecting a higher rate of DPP4-mediated degradation in diabetes.^{16,29} After receiving CXCL10 over a 4-week period, diabetic mice so treated not only had lower rates of urinary albumin excretion but also displayed less mesangial and tubular basement membrane expansion compared with their vehicle-treated counterparts.

Given that bone marrow-derived cells are the predominant sites of chemokine production and that their secretory products can attenuate kidney fibrosis,^{19,30,31} it was considered whether expression of the ELR[−] chemokines may be reduced in diabetes. Although CXCL10 was detected in the conditioned medium from cultured bone marrow cells from nondiabetic and to a lesser extent in diabetic mice, CXCL9 and CXCL11 were not detectable. Like CXCL10, bone marrow-conditioned medium was antifibrotic *in vitro*, reducing fibroblast collagen synthesis in response to both TGF-β and high glucose. Moreover, findings from this study indicate that the antifibrotic effects of bone marrow-conditioned medium may be abrogated by shRNA-mediated knockdown of CXCR3, the cognate receptor of CXCL10. These findings add reduced anti-fibrotic activity to the list of defects in bone marrow

function in diabetes that include impaired angiogenesis and mobilization.³²

Like most cytokines and growth factors whereby their effects are multifaceted, complex, contextual, and at times contradictory, this is also the case for the ELR[−] chemokines and their receptor CXCR3. Several studies suggest that CXCL10 exerts an organ protective effect. In the lung, for instance, CXCL10 attenuates bleomycin-induced pulmonary fibrosis,^{12,33} and in the kidney its neutralization worsens fibrosis in the unilateral ureteral obstruction model of disease.¹³ In the liver, studies of CXCL10 have, similarly, demonstrated injury reduction in hapten-induced hepatitis,³⁴ acetaminophen-induced toxicity,³⁵ and after carbon tetrachloride administration.³⁶ In contrast, other studies suggest that CXCL10 and CXCR3 activation may not be beneficial but may have a pathogenetic role in

Figure 6 Chemokine expression. A 40-member membrane-based mouse antibody array that included chemokines CXCL9, CXCL10, and CXCL11 was used to assess their relative abundance in the conditioned medium (CM) of bone marrow cells from diabetic db/db and nondiabetic db/m mice. The abundance of CXCL10 was substantially lower in CM derived from diabetic compared with nondiabetic mice. Neither CXCL9 nor CXCL11 were detected in CM derived from either mouse group. Other cytokines that were differentially expressed are indicated as either red dots (higher in db/m) or green dots (higher in db/db). Black dots indicate positive controls, white dots indicate negative controls, and gray dots indicate similar findings in specimens from db/m and db/db mice. B21/22, IL-1ra; C17/18, IL-16; D7/8, M-CSF; D15/16, chemokine (C-C motif) ligand (CCL3); D19/20, macrophage inflammatory protein (MIP)-2; D21/22, CCL5; D23/24, and CXCL12.

Figure 7 Bone marrow secretions. **A** and **B**: Kidney fibroblast (NRK-49F) collagen production was assessed by quantifying their incorporation of ^3H -proline at baseline and in response to stimulating them with high glucose (HG; 25 mmol/L; **A**) or 10 ng/mL transforming growth factor (TGF)- β (**B**). The effects of bone marrow conditioned medium (CM) on ^3H -proline incorporation were then assessed after siRNA-mediated silencing of CXCR3, the cognate receptor of CXCL10. Both HG (**A**) and TGF- β (**B**) stimulated ^3H -proline incorporation were reduced by CM. Silencing attenuated the ability of CM to reduce ^3H -proline incorporation in response to HG and TGF- β , whereas scramble shRNA had no effect. Data are expressed as means $\pm$ SEM. * $P < 0.05$ versus control; † $P < 0.05$ versus HG; ‡ $P < 0.05$ versus HG + CM; § $P < 0.05$ versus TGF- β ; ¶ $P < 0.05$ versus TGF- β + CM.

inflammatory and autoimmune disease.³⁷ Here, no proinflammatory changes were seen in circulating leukocyte numbers in association with CXCL10 administration, and in the kidney an increase was observed in the relative abundance of CD206⁺ that identifies pro-reparative M2 macrophages.³⁸ Although M2 macrophages secrete the profibrotic growth factor, TGF- β , they also secrete a range of other factors that diminish the activity of M1 macrophages, thereby attenuating myofibroblast formation and further fibrogenesis.³⁹

This study has several limitations that relate principally to animal models. The characteristic features of human diabetic nephropathy such as nodular glomerulosclerosis, arteriolar hyalinosis, robust interstitial fibrosis, and declining glomerular filtration rate, as described by the Animal Models of Diabetes Consortium are not seen in rodent models of the disease.⁴⁰ The db/db mouse does, however, develop some typical pathologic changes with mesangial expansion, podocyte loss, tubular

basement membrane thickening, and albuminuria that render it a useful model for studying the early changes of diabetic kidney disease.⁴¹ Cleavage of the two NH₂-terminal amino acids of CXCL10 by the near-ubiquitous ectoenzyme DPP4 renders it biologically inactive.^{29,42} Given that DPP4 activity is increased in diabetes,¹⁶ it seems likely that increased degradation as well as reduced secretion may contribute to the lower plasma concentration of CXCL10 that were observed in diabetic mice. However, because the assay that was used measures total CXCL10 that includes both active and inactive forms, the level of assayable CXCL10 after rmCXCL10 administration to db/db mice might be substantially higher than its biologically active form. Unfortunately, the cross-reactivity between human and mouse of a recently developed assay that distinguishes cleaved from uncleaved forms of human CXCL10 has not been established.⁴³ As such, it is uncertain how closely plasma concentrations of circulating biologically

Figure 8 Kidney CXCL10 and CXCR3 expression. **A–C**: Expression CXCL10 and CXCR3 in kidney was assessed by immunoblot (**A**) and quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; **B** and **C**). No difference among groups was noted for either CXCL10 (**B**) or CXCR3 (**C**). **D** and **E**: Immunohistochemistry showed expression of CXCR3 predominantly in the parietal epithelium of glomeruli in both nondiabetic db/m and diabetic db/db mice. Data are expressed as means $\pm$ SEM. Scale bar = 100 μm . Original magnification, $\times 160$.

active CXCL10 in db/m and rmCXCL10-treated db/db mice approximated each other. Because of its abundance in the bone marrow secretions, CXCL10 was studied. However, it is certainly possible that the other ELR⁺ chemokines, CXCL9 and CXCL11, may have produced similarly beneficial effects when administered to diabetic animals. Although examining the effects of CXCL10 in a diabetic CXCR3-deficient mouse would potentially substantiate the role of this chemokine in diabetic nephropathy, these mice have been bred on a nephropathy-resistant C57BL/6 background, necessitating several generations of cross breeding with db/m mice to produce sufficient numbers because db/db mice are infertile. Although no evidence of an inflammatory or autoimmune diathesis was observed with CXCL10, effects on specific lymphocyte subpopulation that are known to be modulated by it were not studied.⁴⁴ Finally, although rmCXCL10 significantly reduced both albuminuria and glomerular expansion, it failed to completely inhibit them. Although a reduction in CXCL10 may contribute to the pathology of diabetic kidney disease, it is unlikely to be the sole pathogenetic factor.

Conclusions

The present study demonstrates that a relative deficiency of CXCL10 may contribute to the pathogenesis of experimental diabetic nephropathy and that its augmentation presents a novel strategy for its potential amelioration.

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Supplemental Data

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