

Original Articles

Blockade of CCL2/CCR2 signalling ameliorates diabetic nephropathy in *db/db* mice

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ABSTRACT

Background. CCL2/C–C chemokine receptor 2 (CCR2) signalling is suggested to play a significant role in various kidney diseases including diabetic nephropathy. We investigated the renoprotective effect of a CCR2 antagonist, RS102895, on the development of diabetic nephropathy in a type 2 diabetic mouse model.

Methods. Six-week-old diabetic *db/db* and non-diabetic *db/m* mice were fed either normal chow or chow mixed with 2 mg/kg/day of RS102895 for 9 weeks. We investigated the effects of CCR2 antagonism on blood glucose, blood pressure, albuminuria and the structure and ultrastructure of the kidney.

Results. Diabetes-induced albuminuria was significantly improved after CCR2 antagonist treatment, and glucose intolerance was improved in the RS102895-treated diabetic mice. RS102895 did not affect blood pressure, body weight or kidney weight. Mesangial expansion, glomerular basement membrane thickening and increased desmin staining in the diabetic kidney were significantly improved after RS102895 treatment.

The up-regulation of vascular endothelial growth factor mRNA expression and the down-regulation of nephrin mRNA expression were markedly improved in the kidneys of RS102895-treated diabetic mice. Increased renal CD68 and arginase II and urinary malondialdehyde in diabetes were effectively attenuated by RS102895 treatment.

Conclusion. Blockade of CCL2/CCR2 signalling by RS102895 ameliorates diabetic nephropathy not only by improving blood glucose levels but also by preventing CCL2/CCR2 signalling from altering renal nephrin and VEGF expressions through blocking macrophage infiltration, inflammation and oxidative stress in type 2 diabetic mice.

INTRODUCTION

Diabetic nephropathy is a leading cause of end-stage renal failure [1] and accounts for significant morbidity and mortality in patients with diabetes. It is likely that the pathophysiology of diabetic nephropathy involves a multifactorial interaction between metabolic and haemodynamic factors

through shared molecular and signalling pathways [2]. Contributing factors include the renin–angiotensin system and related cytokines, hyperglycaemia, reactive oxygen species, intracellular mediators, adiponectin level [3] etc.

CCL2, also known as monocyte chemoattractant protein 1, has emerged as a very important mediator in this process and is over-expressed in the kidneys of diabetic animals [4–7]. We reported that the effects of CCL2 on podocytes may exacerbate the disease process of diabetic albuminuria [8]. Additionally, recent studies employing RS504393 and RO5234444 demonstrate that C–C chemokine receptor 2 (CCR2) inhibitors can block the development of diabetic nephropathy in association with a reduction in kidney macrophage infiltration in type 2 diabetic mice [9, 10]. However, neither study evaluated the ultrastructure of the kidney, nor did they define the mechanism of the anti-albuminuric effect of the CCR2 antagonist or examine nephrin expression.

We investigated the effect of the CCR2 antagonist RS102895 on the functional and structural damages to the kidney in type 2 diabetic *db/db* mice and also examined the effects of RS102895 on other factors affecting albuminuria, including metabolic and haemodynamic factors and the cytokines related to inflammation or oxidative stress.

MATERIALS AND METHODS

Experimental animals

Six-week-old C57BLKS/J *db/db* and *db/m* male mice were purchased from Japan Shizuoka Laboratory Center (Shizuoka, Japan); *db/m* mice were used as controls in all of the experiments. RS102895 was purchased from Sigma-Aldrich (Seoul, Korea). Mice were fed either normal chow or chow mixed with 2 mg/kg/day of the RS102895 for 9 weeks, starting at 8 weeks of age. Daily food intake was monitored at regular intervals to confirm drug administration. In brief, we freshly prepared normal chow and mixed different amounts of RS102895 based on body weight at 2-week intervals after daily food intake. During the experiment, food intake, water intake, urine volume, body weight and blood pressure were measured every month. Tail-cuff blood pressure was recorded as the arithmetic mean of blood pressures recordings determined on at least 3 days, after at least 7 days of training on a non-invasive blood pressure analyser for mice, BP-2000 Blood Pressure Analysis System™ (Visitech Systems, Inc., Apex, NC). All experiments were conducted in accordance with the National Institutes of Health guidelines and with the approval of Yonsei University Institutional Animal Care and Use Committee (Wonju, Korea).

We measured plasma glucose levels to determine the glucose intolerance state of each group at 8 weeks and 16 weeks into the study. After an 8-h fasting period, the plasma glucose levels were measured after dextrose (2 g/kg) injection intraperitoneally. Plasma glucose levels were measured using a glucose oxidase-based method, whereas serum insulin (Shibayagi, Co., Shibukawa, Japan) and adiponectin (Adipogen International, Seoul, Korea) levels were measured using ELISA (enzyme-linked immunosorbent assay) kits. Homeostasis

model assessment (HOMA-IR) was calculated as fasting plasma glucose (mmol/L) × fasting serum insulin (mU/L) ÷ 22.5 and HOMA-β was calculated as 20 × fasting serum insulin (mU/L) ÷ fasting plasma glucose (mg/dL)–3.5. To measure urinary albumin excretion, individual mice were placed once every month in a metabolic cage and urine was collected for 18 h. Urinary albumin concentrations were determined by ELISA (Exocell, Philadelphia, PA) after the correction by urinary creatinine concentrations. Urinary 8-hydroxydeoxyguanosine (8-OHdG) was measured by a competitive enzyme immunoassay kit (Cell Biolabs, Inc., San Diego, CA) that has an 8-OHdG detection sensitivity range of 100 pg/mL to 20 ng/mL. Urinary malondialdehyde (MDA) levels were determined by a rapid and sensitive fluorometric HPLC method (NeoDin Medical Institute, Seoul, Korea). Urine samples were treated with 0.1125 N perchloric acid and 40 mM 4,6-dihydroxy-2-mercaptopyrimidine, and the mixture was at 97°C for 1 h. The reaction was stopped by placing on ice for 20 min. Afterwards, methanol and 20% trichloroacetic acid buffer were added. The samples were then mixed and centrifuged at 13 000 ×g for 6 min, and the supernatant was transferred to an insert vial. Fluorescence was read at an excitation wavelength of 525 nm and emission wavelength of 560 nm. The run time was 2 min, and the flow rate was 1 mL/min. The concentrations of CCL2 (R&D Systems, Minneapolis, MN), interleukin-6 (R&D Systems), TNF-α (BD Biosciences-Pharmingen, San Diego, CA) and superoxide dismutase (Cayman Chemical Company, Ann Arbor, MI) in the renal cortex lysate were measured using a commercial quantitative sandwich ELISA.

Renal pathology

The renal tissues were fixed for 48 h with 10% paraformaldehyde at 4°C, dehydrated, embedded in paraffin, cut into 3-μm-thick slices and stained with periodic acid-Schiff (PAS). Twenty glomeruli per animal were analysed by using an image processing and analysis system, Image J (National Institutes of Health, Bethesda, MD), under a ×400 magnified field. Glomerular mesangial expansion was scored semi-quantitatively, whereby the percentage of mesangial matrix occupying each glomerulus was rated on a 5 point scale from 0 to 4 as follows: grade 0, normal glomeruli; grade 1, mesangial expansion area up to 25%; grade 2, 25 to 50%; grade 3, 50 to 75%; and grade 4, >75%.

For ultrastructural evaluation, kidney tissue was fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, imbued with uranyl acetate and embedded in epoxy resin (epon). The specimen was thin-sectioned and examined under a transmission electron microscope (JEM-1200EX II, JEOL, Tokyo, Japan). Electron micrographs of five to ten glomeruli per kidney were randomly taken at 1000× and 10 000× for each mouse. Mean glomerular basement membrane (GBM) thickness was obtained from the measurements at three different sites of cross-sectioning, with the aid of Image J according to published methods [11, 12]. Tangentially-sectioned GBM was excluded from the analysis.

For immunohistochemical staining, paraffin-embedded renal tissues sliced into 3-μm-thick sections were microwaved

for 5 min twice in a 0.1 M citrate buffer after dewaxing. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol. Non-specific binding was blocked with 10% normal goat serum in PBS. Kidney sections were stained with an anti-desmin (1:100, Abcam, Cambridge, UK) antibody or a monoclonal antibody to F4/80 (1:200, Serotec, Oxford, UK) in a humidified chamber at 4°C. Polymer horseradish peroxidase-linked anti-goat (Vector Laboratories, Burlingame, CA) or anti-rabbit IgG (Golden Bridge International, Mukilteo, WA) was used as the secondary antibody. Sections were covered with 3,3-diaminobenzidine substrate solution (Sigma-Aldrich) and then dehydrated in ethanol, cleared in xylene and mounted without counterstaining. All of these sections were examined in a masked manner using light microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan) equipped with a digital camera (Olympus DP72; Olympus Optical). Desmin expression was scored semi-quantitatively by estimating the percentage of desmin-positive epithelial cells located in the outer cell layer of the glomerular tuft. Desmin staining was graded as follows: 0, 0%; 1+, 1–25%; 2+, 26–50%; 3+, 51–75% and 4+, 76–100% [13]. Twenty glomeruli from each mouse were scored, and the final desmin score was calculated by the arithmetic mean.

Real-time quantitative RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. cDNA was synthesized from 1 µg of total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and polymerase chain reaction (PCR) reaction was performed in a 20 µL reaction mixture containing 10 ng of cDNA template, SYBR® Green PCR Master Mix-Plus (Toyobo, Osaka, Japan), and primers. The sequence of each primer was as follows: mouse nephrin, forward: 5' GAG GAG GAT CGA ATC AGG AA 3' and reverse: 5' GGT CCA CTT CTG CTG TGC TA 3'; mouse vascular endothelial growth factor (VEGF), forward: 5' GTA CAT CTT CAA GCC GTC CTG TGT 3' and reverse: 5' TCC GCA TGA TCT GCA TGG TG 3'; mouse β-actin, forward: 5' GGA CTC CTA TGT GGG TGA CG 3' and reverse: 5' CTT CTC CAT GTC GTC CCA GT 3'. For all real-time PCR analysis, β-actin mRNA was used to normalize RNA inputs. Fold-change expression with respect to control was calculated for all samples. Each sample was tested in triplicate.

Western blotting

The kidney was homogenized in RIPA lysis buffer and spun at 14 000 g to pellet the nuclei and large cellular fragments. The supernatant protein concentrations were measured by the BCA assay (Bio-Rad, Hercules) and equalized with the addition of Laemmli buffer, before sodium dodecyl sulphate (SDS)-based electrophoresis through a 6–10% SDS polyacrylamide gel. After electrical wet transfer of the proteins to a nitrocellulose membrane, CD68 or arginase II was probed with the appropriate primary antibody: mouse anti-CD68 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-arginase II (1:1000, Santa Cruz Biotechnology) or goat anti-β-actin (1:5000, Abcam). After incubation with the appropriate horseradish peroxidase-conjugated secondary

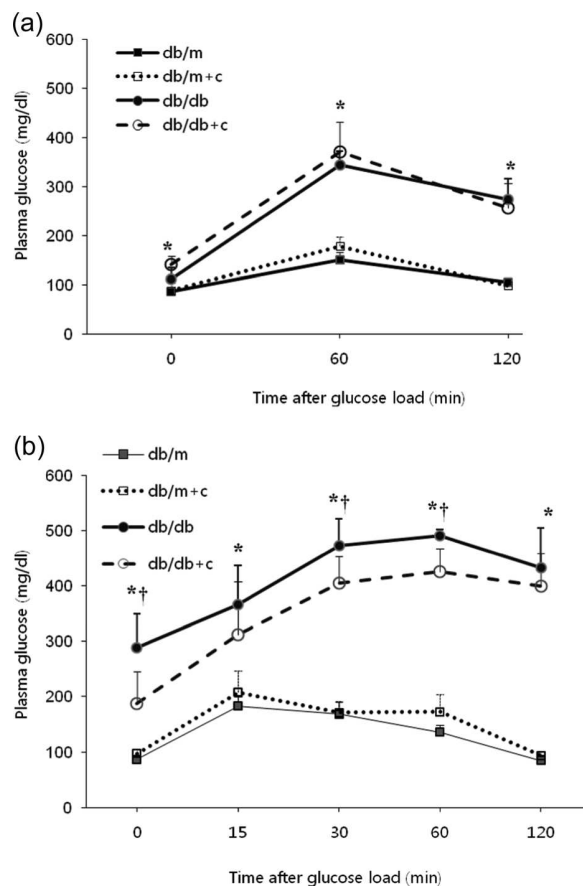


FIGURE 1: Effects of RS102895 on glucose intolerance in experimental animals. Intraperitoneal glucose tolerance test was performed at (a) 8 weeks and (b) 16 weeks of age. *P < 0.05 versus untreated non-diabetic mice; †P < 0.05 versus treated non-diabetic mice with RS102895.

antibody, anti-rat or anti-mouse IgG (both GE Healthcare, Piscataway, NJ), the chemiluminescent reaction was developed with SuperSignal West Pico (Pierce, Rockford, IL).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences between the groups were examined for statistical significance using ANOVA with the Tukey correction (SPSS 11.5; SPSS, Chicago, IL) for multiple comparisons when appropriate. A P-value of <0.05 was considered to be a statistically significant difference.

RESULTS

CCR2 Antagonist improves blood glucose levels

At 8 weeks of age, diabetic mice were not more hyperglycaemic, by fasting blood glucose, than their non-diabetic controls. The diabetic mice did display a significant increase in blood glucose levels after a glucose challenge (Figure 1a). At 16 weeks of age, the diabetic mice were overtly more hyperglycaemic than the non-diabetic mice. The disparity in blood glucose was even more pronounced when the mice were

administered intraperitoneal glucose, such that the diabetic mice showed a marked increase in the postprandial blood sugar whereas the non-diabetic mice displayed almost no change. RS102895 treatment significantly improved the blood glucose levels of diabetic mice, although they remained more hyperglycaemic than the control mice (Figure 1b). HOMA-IR, the insulin resistance index, was markedly increased in the diabetic mice compared with non-diabetic mice but was significantly improved with RS102895 treatment. HOMA- β , which shows insulin secretory capacity of pancreatic β cells, was not impaired in diabetic mice compared with non-diabetic mice and was unaffected by RS102895 treatment (Table 1).

The type 2 diabetic *db/db* mice were more obese than non-diabetic *db/m* control mice, regardless of whether they were treated with RS102895 or not, throughout the entire experimental period (data not shown). The absolute kidney weight of diabetic mice was significantly higher than that of control mice, perhaps indicative of diabetic renal hypertrophy. Treatment with RS102895 did not affect body or kidney weight (data not shown).

CCR2 Antagonist did not affect blood pressure

To assess haemodynamic changes, systolic blood pressure was measured in all mice every month. Blood pressure was not different between diabetic mice and controls. Treatment with RS102895 did not affect blood pressure (Figure 2).

CCR2 Antagonist ameliorates diabetic albuminuria

To assess the effect of CCR2 blockade on albuminuria, we measured the total urinary albumin excretion rate and the urinary albumin-to-creatinine ratio in the 18-h urine collections. The albumin excretion rate was markedly increased in the diabetic *db/db* mice compared with the non-diabetic *db/m* control mice, whether the albuminuria was expressed per day or corrected for the urinary creatinine. Urinary albumin excretion was significantly ameliorated after RS102895 treatment (Figure 3).

CCR2 Antagonist reduces diabetic mesangial expansion

Mesangial hypertrophy assessed by the percentage of mesangial matrix or mesangial expansion score was markedly increased in the untreated diabetic mice. RS102895 significantly reduced the percentage of mesangial matrix and mesangial expansion score. Representative photomicrographs of the kidneys stained with PAS in the non-diabetic control and diabetic mice with or without RS102895 treatment are shown in Figure 4.

We also evaluated the effects of RS102895 on the renal tubulointerstitial pathology. The tubulointerstitial areas in the diabetic *db/db* mice and the non-diabetic control mice both appeared normal, and there was no acute or chronic tubular damage. There were no inflammatory cells, fibrosis or atrophic changes in the tubulointerstitium in either the untreated or the RS102895-treated diabetic mice (data not shown).

CCR2 Antagonist prevents diabetic GBM thickening

The thickness of whole GBM and lamina densa was markedly increased in the diabetic *db/db* mice versus non-diabetic control mice. The GBM thickness was effectively normalized in the diabetic mice that were treated with RS102895, a finding that can be seen in the representative electron photomicrographs (Figure 5).

CCR2 Antagonist improves diabetic podocyte injury

Podocyte foot process effacement was observed in the diabetic *db/db* mice, but the podocyte dysmorphology was prevented by RS102895 treatment (Figure 5). Desmin expression, indicating damage to the podocytes, was significantly increased in untreated diabetic mice. Treatment with RS102895 significantly improved diabetes-induced podocyte injury as estimated by the desmin score (Figure 6).

CCR2 Antagonist ameliorates nephrin down-regulation in the diabetic kidney

The effect of RS102895 on nephrin, a major component of the slit diaphragm, was evaluated. Nephrin mRNA expression in kidneys analysed by real-time quantitative RT-PCR was significantly decreased in diabetic mice versus non-diabetic control mice. Treatment of the diabetic mice with RS102895 significantly restored the nephrin mRNA expression as compared with the untreated diabetic mice (Figure 7).

CCR2 Antagonist attenuates VEGF up-regulation in diabetic kidneys

The effect of CCR2 blockade on VEGF, a cytokine that plays a major role in the development of diabetic glomerular hyperpermeability, was also evaluated by real-time quantitative RT-PCR. VEGF mRNA expression in the kidney significantly increased in untreated diabetic mice compared with non-diabetic control mice. However, RS102895 treatment markedly attenuated the diabetes-induced increase of VEGF expression in the *db/db* mice (Figure 8).

Table 1. RS102895 improves insulin resistance in *db/db* diabetic mice

Parameters	<i>db/m</i>	<i>db/m + c</i>	<i>db/db</i>	<i>db/db + c</i>
HOMA-IR	0.43 \pm 0.08	0.35 \pm 0.05	7.51 \pm 1.23*	3.66 \pm 0.55**
HOMA- β	6.34 \pm 1.09	6.25 \pm 1.68	3.99 \pm 0.95	4.93 \pm 1.10

Data are expressed as mean \pm SEM.
*P < 0.001 versus non-diabetic control mice (*db/m*), **P < 0.01 versus untreated diabetic mice (*db/db*).

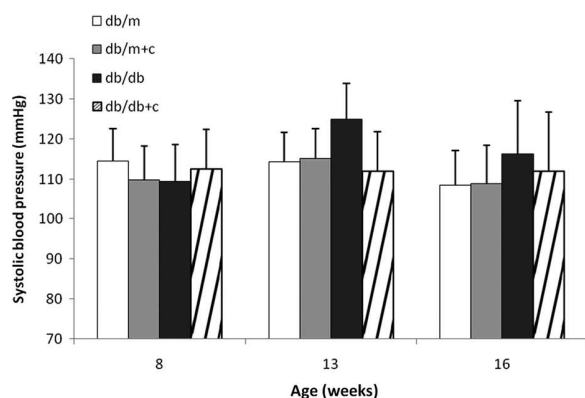


FIGURE 2: Effects of RS102895 on blood pressure in experimental animals. RS102895 treatment did not affect blood pressure at any time periods.

CCR2 Antagonist improves oxidative stress markers in diabetes

A marker of oxidative stress, urinary MDA corrected for the urinary creatinine, was markedly increased in diabetic mice, but this parameter was significantly improved by RS102895 (Figure 9a). Another marker of reactive oxygen species, urinary 8-OHdG excreted in 24 h, was markedly increased in diabetic mice, which was unaffected by RS102895 treatment (Figure 9b). The antioxidant enzyme superoxide dismutase in the kidney tissue was markedly decreased in diabetic mice, but this parameter was significantly improved by RS102895 (Figure 9c).

Additionally, systemic and urinary adiponectin levels were measured, because adiponectin levels correlate tightly with insulin resistance and the development of albuminuria [3]. Whereas serum adiponectin was decreased in diabetic mice compared with control mice (0.6-fold versus non-diabetic control), the urinary adiponectin-to-creatinine ratio was not different between diabetic and non-diabetic mice. RS102895 treatment did not affect either serum or urinary adiponectin levels (Figure 9d).

CCR2 Antagonist prevents renal macrophage infiltration in diabetes

The effect of CCR2 blockade on renal macrophage accumulation was evaluated by western blot. The amount of CD68 and arginase II, corrected for β -actin, in the kidney tissues was found to be increased in the diabetic mice compared with non-diabetic controls (Figure 10a). The increase in renal CD68 and arginase II with diabetes was significantly diminished by RS102895 treatment (Figure 10a). Renal CCL2 levels were correlated with urinary albumin excretion ($r = 0.49$, $P < 0.05$) and were significantly increased in diabetic mice (2.0-fold versus non-diabetic control, Figure 10b). RS102895 treatment did not change renal CCL2 levels in the diabetic mice (Figure 10b). Other inflammatory markers in kidney tissue, i.e. IL-6 (Figure 10c) and TNF- α (Figure 10d), were also measured. Whereas IL-6 was markedly increased in diabetic mice compared with control mice, TNF- α was not different between diabetic and non-diabetic mice. Treatment of the

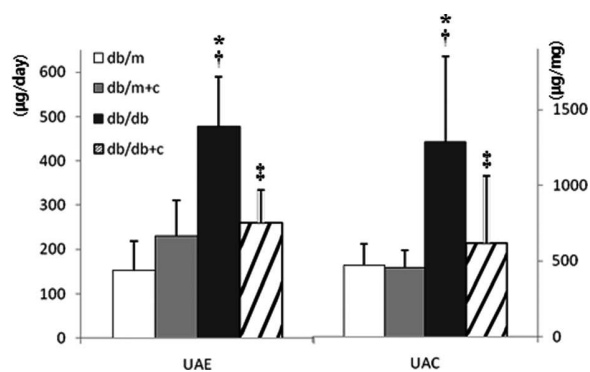


FIGURE 3: Effects of RS102895 on albuminuria in experimental animals. Total urinary albumin excretion (UAE) or urinary albumin creatinine ratio (UAC) at 16 weeks were significantly attenuated in diabetic mice treated with RS102895. * $P < 0.05$ versus untreated non-diabetic mice; † $P < 0.05$ versus treated non-diabetic mice with RS102895; ‡ $P < 0.05$ versus untreated diabetic mice.

diabetic mice with RS102895 significantly restored the IL-6 and TNF- α as compared with the untreated diabetic mice.

The number of F4/80-positive macrophages was negligible in the kidneys of non-diabetic control mice. There was a substantial increase in the number of renal macrophages in the diabetic *db/db* mice. The infiltration of glomerular macrophages was reduced in the RS102895-treated diabetic mice (Figure 11).

DISCUSSION

Chemokines play important roles in the recruitment of inflammatory cells to the kidney. Increased infiltration of monocytes and macrophages has been observed in the glomeruli and the interstitium of the diabetic kidney in human and animal studies [14–17], and chemokines and their receptors have been shown to be involved in diabetic renal injury [18, 19].

Monocyte recruitment to the sites of inflammation is regulated, in large part, by CCL2. CCL2 attracts macrophages by signalling through the CCR2 chemokine receptor and has been reported to have an important role in the pathogenesis of diabetic nephropathy [7, 8]. It is produced by podocytes, mesangial and tubular epithelial cells and mediates renal interstitial inflammation, tubular atrophy and interstitial fibrosis [5, 20, 21].

As diabetic nephropathy is a multifactorial disease, successful treatment requires the targeting of multiple mediators. Combination therapy was more effective at suppressing the renal injury in crescentic glomerulonephritis than either a CCR2 antagonist (RS102895) or an angiotensin II receptor blocker alone [22]. The CCR2 antagonists—RS504393 [9], RO5234444 [10] and RS504393 [23]—have recently been studied as novel therapies directed against the development and progression of diabetic nephropathy. Sayyed *et al.* demonstrated that CCR2 inhibitors (RO5234444) can block the development of diabetic nephropathy in association with a

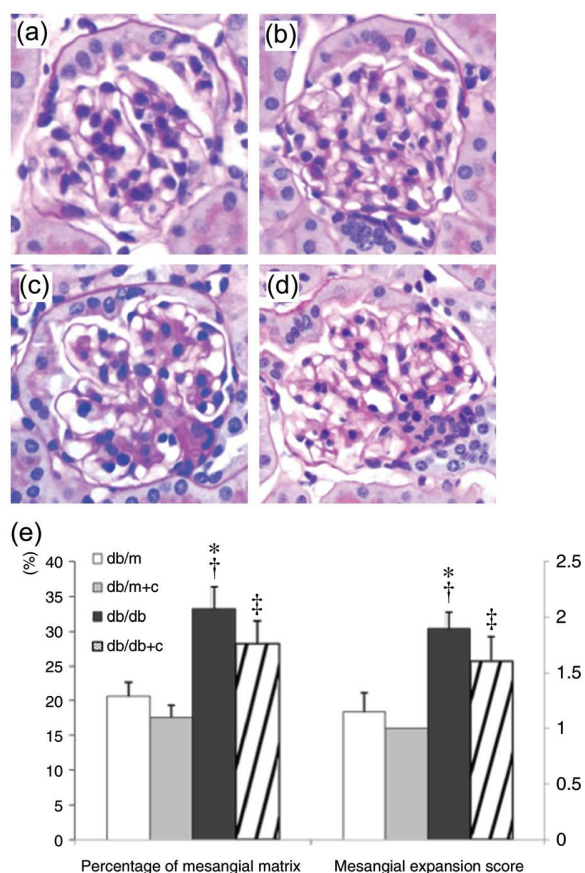


FIGURE 4: Effects of RS102895 on mesangial matrix expansion in experimental animals. Representative images after periodic acid-Schiff staining are shown in untreated non-diabetic mice (a), non-diabetic mice treated with RS102895 (b), untreated diabetic mice (c) and diabetic mice treated with RS102895 (d). Original magnification: $\times 400$. (e) Mesangial matrix expansion shown as percentage of mesangial matrix or mesangial expansion score increased in the untreated diabetic group. The RS102895 did significantly reduce mesangial matrix expansion. * $P < 0.05$ versus untreated non-diabetic mice; $\dagger P < 0.05$ versus treated non-diabetic mice with RS102895; $\ddagger P < 0.05$ versus untreated diabetic mice.

reduction in kidney macrophage infiltration in type 2 diabetic mice [10]. The results are consistent with our study in which RS102895, another CCR2 antagonist, improves albuminuria, reduces mesangial expansion and limits podocyte injury in *db/db* mice. The current study is distinct from previous studies [11, 12, 23] in which RS102895 has not been investigated for its effects on the diabetic kidney disease that develops in *db/db* mice. Additionally, we investigated several albuminuria-related factors including glucose intolerance, oxidative stress, adiponectin, cytokines such as VEGF, nephrin expression and desmin staining as well as GBM ultrastructure.

The CCR2 antagonist, RS102895, had a protective effect in *db/db* mice with diabetic nephropathy. RS102895 attenuated urinary albumin excretion, a finding consistent with our previous *in vitro* studies that reported CCL2/CCR2 signalling as capable of inducing podocyte injury and increasing podocyte permeability to albumin [8]. As albuminuria is an important

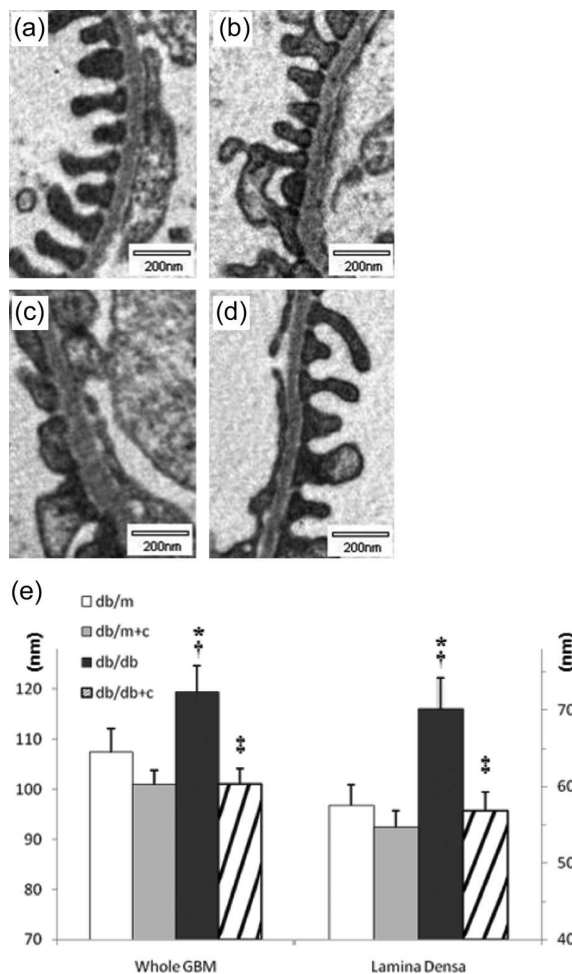


FIGURE 5: Glomerular basement membrane (GBM) thickness was markedly reduced in the diabetic mice that were treated with RS102895. Representative images demonstrating GBM are shown in untreated non-diabetic mice (a), non-diabetic mice treated with RS102895 (b), untreated diabetic mice (c), and diabetic mice treated with RS102895 (d). Original magnification: $\times 10\,000$. (e) Thickness of whole GBM and lamina densa increased in diabetic mice. RS102895 attenuated GBM thickness. * $P < 0.05$ versus untreated non-diabetic mice; $\dagger P < 0.05$ versus treated non-diabetic mice with RS102895; $\ddagger P < 0.05$ versus untreated diabetic mice.

clinical parameter for the assessment of renal disease progression, CCR2 antagonism can be reasonably expected to inhibit the progression of renal disease in diabetes.

To assess the renoprotective effect of RS102895, we conducted a histopathologic and biochemical evaluation that included immunohistochemical stains, electron microscopy, real-time quantitative RT-PCR, ELISA and western blot. Animal and human studies have demonstrated that diabetes leads to ultrastructural alterations in the glomerular filtration barrier and glomerulosclerosis [24]. These structural changes correlate with increasing albuminuria, an early feature of diabetic kidney disease. We could observe the key pathological changes of diabetic nephropathy in untreated diabetic mice, such as the augmentation of extracellular matrix, the expansion of the mesangium, the effacement of podocyte foot

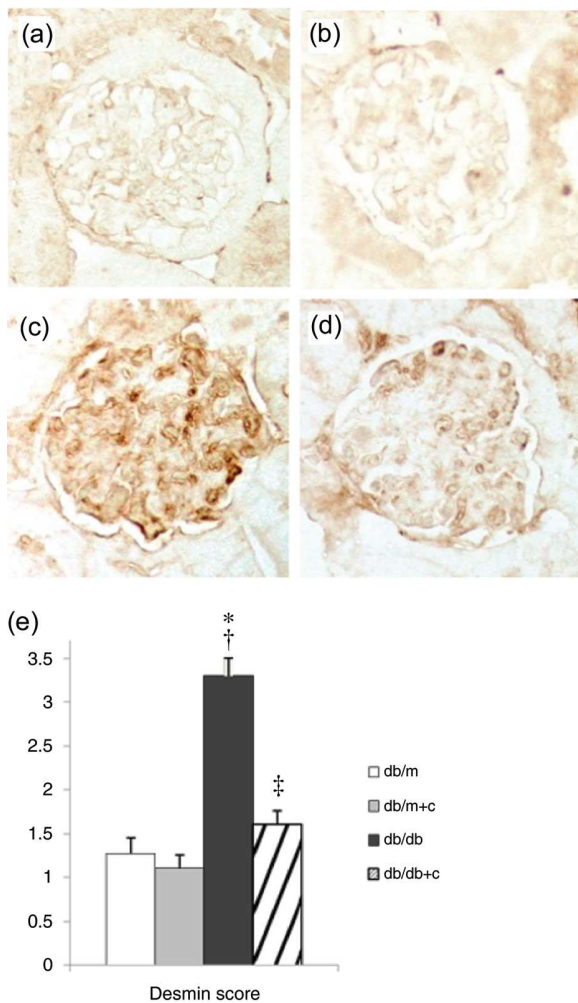


FIGURE 6: Desmin expression, indicating damage to the podocytes, increased in untreated diabetic mice. Figures represent the glomeruli of non-diabetic mice, either untreated (a) or treated with RS102895 (b) and diabetic mice, either untreated (c) or treated with RS102895 (d). Original magnification: $\times 400$. (e) Treatment with RS102895 reduced desmin scores with statistical significance. * $P < 0.05$ versus untreated non-diabetic mice; † $P < 0.05$ versus treated non-diabetic mice with RS102895; ‡ $P < 0.05$ versus untreated diabetic mice.

processes and the thickening of the GBM. Treatment with RS102895 markedly attenuated the morphological changes as well as the increased GBM thickness in diabetic mice. The results raise the question of how a CCR2 antagonist ameliorates the structural and functional injury in diabetic nephropathy. Several pathogenetic mechanisms were assessed:

First, RS102895 did not affect the systemic blood pressure. Among the multifactorial interactions in diabetic nephropathy, an improvement of haemodynamic factors was not responsible for the renoprotective effect of RS102895.

Secondly, RS102895 diminished the oxidative stress. Urinary MDA and renal SOD were markedly changed in diabetic mice and RS102895 restored these indicators of

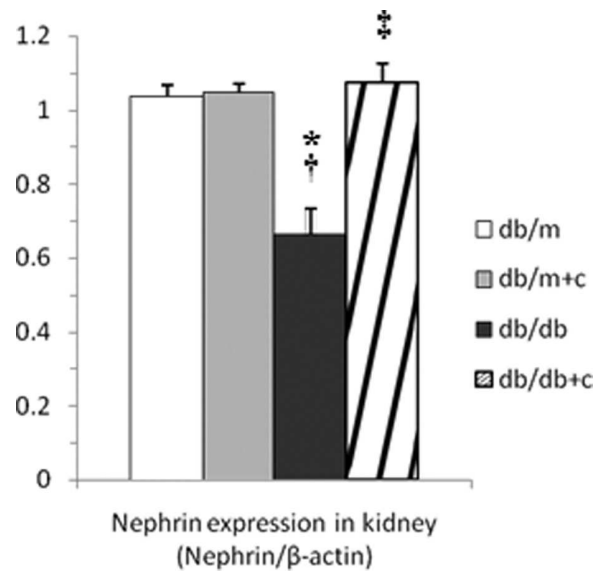


FIGURE 7: Nephryn mRNA expression in the kidney decreased in untreated diabetic mice. However, RS102895 treatment recovered nephryn expression in diabetic mice. * $P < 0.05$ versus untreated non-diabetic mice; † $P < 0.05$ versus treated non-diabetic mice with RS102895; ‡ $P < 0.05$ versus untreated diabetic mice.

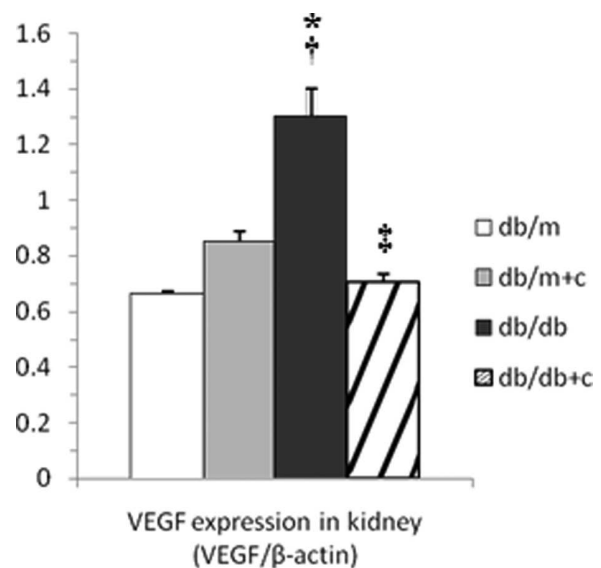


FIGURE 8: Vascular endothelial growth factor (VEGF) mRNA expression in the kidney increased in untreated diabetic mice. However, RS102895 treatment attenuated increase of VEGF expression in diabetic mice. * $P < 0.05$ versus untreated non-diabetic mice; † $P < 0.05$ versus treated non-diabetic mice with RS102895; ‡ $P < 0.05$ versus untreated diabetic mice.

oxidative stress, although we could not observe the significant differences of urinary 8-OHdG between diabetic and non-diabetic mice. We conclude that part of the renoprotective effect of RS102895 derives from the reduction of oxidative stress.

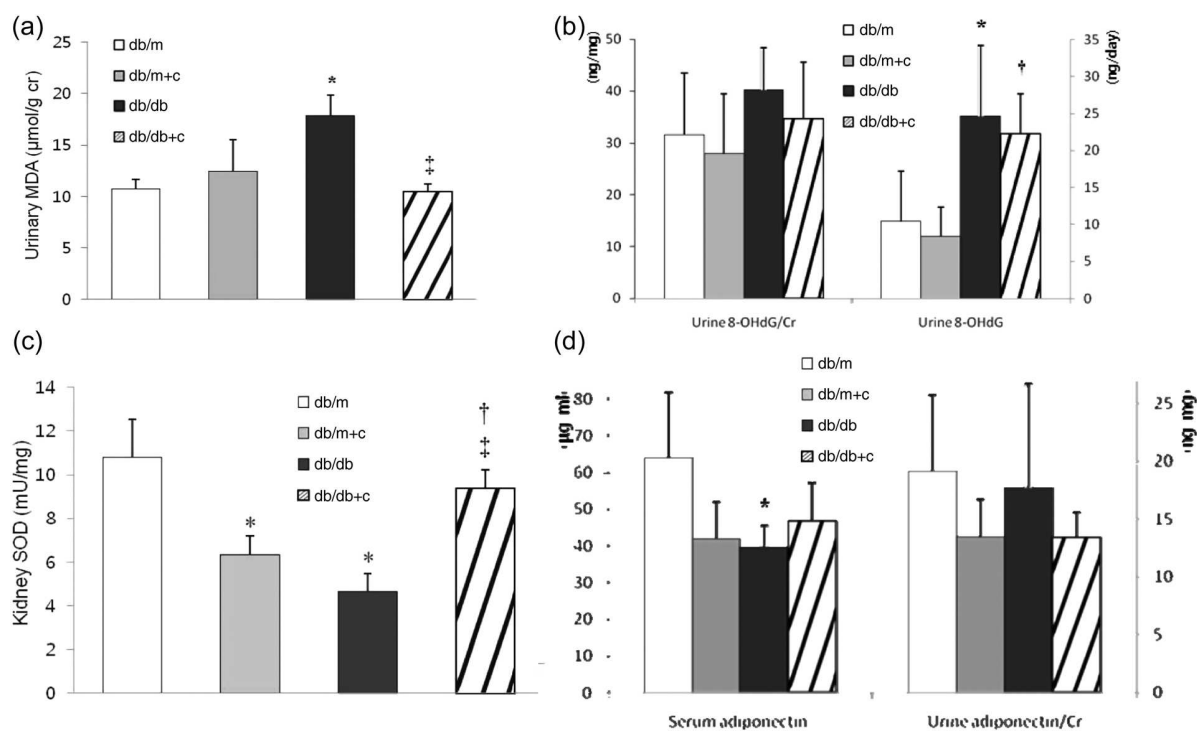


FIGURE 9: Effects of RS102895 on oxidative stress markers and adiponectin in experimental animals. (a) Urinary malondialdehyde (MDA), corrected for the urinary creatinine, was markedly increased in diabetic mice and RS102895 significantly improved urinary MDA. (b) Urinary 8-OHdG excreted in 24 h was also markedly increased in diabetic mice, was unaffected by RS102895. (c) The antioxidant enzyme superoxide dismutase in the kidney tissue was markedly decreased in diabetic mice, but was significantly improved by RS102895. (d) Effects of RS102895 on serum adiponectin and urinary adiponectin to creatinine ratio in experimental animals. * $P < 0.05$ versus untreated non-diabetic mice; † $P < 0.05$ versus treated non-diabetic mice with RS102895; ‡ $P < 0.05$ versus untreated diabetic mice.

Thirdly, RS102895 attenuated renal macrophage infiltration. The anti-inflammatory effect of other CCR2 antagonist compounds was recently studied and reported in diabetic nephropathy. Treatment with RS102895 significantly inhibited the glomerular macrophage accumulation, according to a prior study [22]. Also, Awad *et al.* demonstrated that RS504393, another CCR2 antagonist, significantly ameliorated albuminuria, renal dysfunction, histological changes and glomerular macrophage recruitment [23]. They suggested that the renoprotective effect of CCR2 antagonism correlates with a significant reduction of kidney macrophage infiltration. The fact that the increased CD68 in diabetic kidneys was effectively attenuated by a CCR2 antagonist suggests that a similar mechanism might underlie the salient effects of RS102895 in the present study. Recently, dramatic alterations in arginine metabolism due to increased activity of arginase were shown in diabetic vascular dysfunction, and the renal protective effect of arginase II blockade was highly correlated with a significant reduction of kidney macrophage infiltration in a diabetic renal injury model [25]. In the current study, the diabetes-induced arginase II and the successful attenuation by RS102895 suggests that an additional renoprotective mechanism of CCR2 antagonism might derive from the inhibition of renal macrophage infiltration.

Fourthly, CCL2/CCR2 signalling directly affected nephrin expression and VEGF regulation in the diabetic kidney. Nephrin, a major component of the slit diaphragm and a determinant of the glomerular filtration barrier integrity, was decreased in untreated diabetic mice, as expected. Interestingly, CCR2 antagonism restored nephrin expression in diabetes in our study. This finding might help explain the anti-albuminuric mechanism of CCR2 antagonism in diabetes. Diabetic microalbuminuria also parallels with endothelial dysfunction, and a prominent endothelial growth factor, VEGF, is up-regulated in diabetic nephropathy, even beyond the constitutive expression levels by the podocytes. The preservation of vascular integrity seems to depend upon a fine balance in the regulation of VEGF expression/activity. Too little or too much of this cytokine can be detrimental, resulting in glomerular capillary pathology and increased vascular permeability [26]. In agreement with previous studies, VEGF mRNA expression increased in the untreated diabetic kidney. Interestingly, RS102895 treatment attenuated the induction of VEGF expression in the diabetic mice. Together, the results suggest that RS102895 has anti-albuminuric effects via nephrin restoration and VEGF regulation in the kidney.

Finally, RS102895 improved postprandial glucose. High glucose stimulates the local activation of the renin-angiotensin system. Angiotensin II in turn stimulates podocyte-

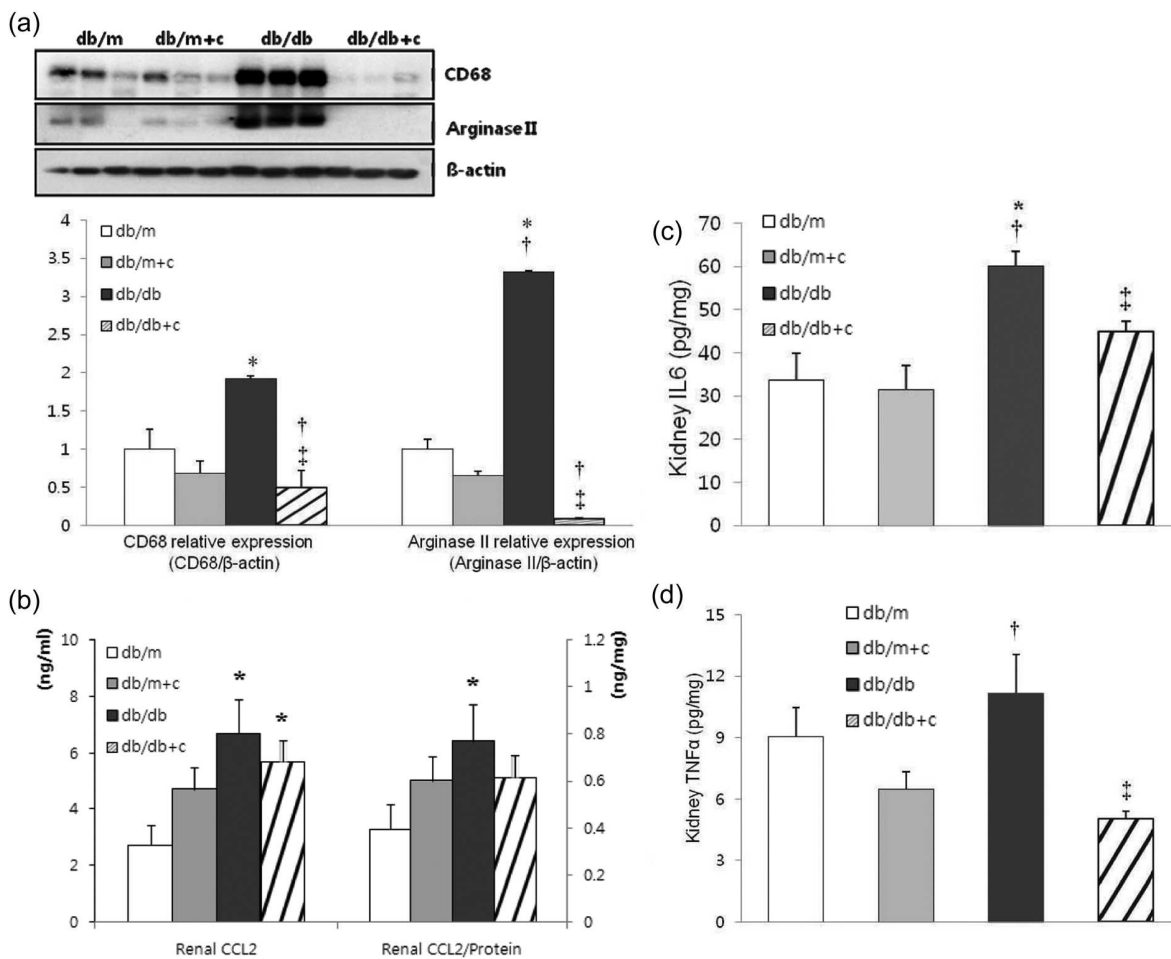


FIGURE 10: The effect of CCR2 blockade on renal macrophage accumulation and inflammatory cytokines. (a) The amount of CD68 and arginase II evaluated by western blot, corrected for β -actin, in the kidney tissues was found to be increased in the diabetic mice compared with non-diabetic controls. The increase in renal CD68 and arginase II with diabetes was significantly diminished by RS102895 treatment. (b) Renal CCL2 levels were significantly increased in diabetic mice. RS102895 treatment did not change renal CCL2 levels in the diabetic mice. Other inflammatory markers in kidney tissue, IL-6 (c) and TNF- α (d) were also measured by ELISA. * $P < 0.05$ versus untreated non-diabetic mice; $\dagger P < 0.05$ versus treated non-diabetic mice with RS102895; $\ddagger P < 0.05$ versus untreated diabetic mice.

derived VEGF, suppresses nephrin expression and induces TGF- β 1 leading to podocyte apoptosis and fostering the development of glomerulosclerosis [27]. In this study, the CCR2 antagonist improved glucose intolerance in diabetic mice. Although the glucose-lowering mechanism has not been elucidated, insulin resistance may be linked to the CCR2 receptor through systemic inflammation since RS102895 effectively improved HOMA-IR in the present study. The CCL2/CCR2 pathway has been suggested to be involved in adipose tissue inflammation and insulin resistance [28].

Additionally, we investigated adiponectin levels in the urine. Adiponectin, a 30-kDa circulating plasma protein primarily secreted by adipocytes, is a known insulin sensitizer. Blood adiponectin levels are reduced with increasing visceral obesity and tightly correlated with insulin resistance and the development of type 2 diabetes. Further, albuminuria was associated with low adiponectin levels in hypertensive and obese patients.

We also found decreased blood adiponectin levels in the obese diabetic *db/db* mice. However, Koshimura *et al.* demonstrated that circulating adiponectin levels are increased in patients with overt diabetic nephropathy [29]. This discrepancy might be due to the reduced clearance of adiponectin because the mean serum creatinine in the overt diabetic nephropathy group was 2.0 versus 0.7 mg/dL in the control group of Koshimura's study [29]. Recently, Sharma *et al.* showed that adiponectin is a key regulator of albuminuria, likely acting through the adenosine monophosphate-activated protein kinase pathway to modulate oxidative stress in podocytes [3]. Adiponectin knockout mice were reported to have increased albuminuria with podocyte foot process effacement. Considering the anti-albuminuric effect of CCR2 antagonists in diabetic nephropathy, we ascertained whether RS102895-treated *db/db* mice showed restoration of the adiponectin level. Though serum adiponectin was reduced in diabetes, blockade of CCR2 did not restore systemic or urinary adiponectin levels. The reason for the discrepancy is uncertain but could arise because

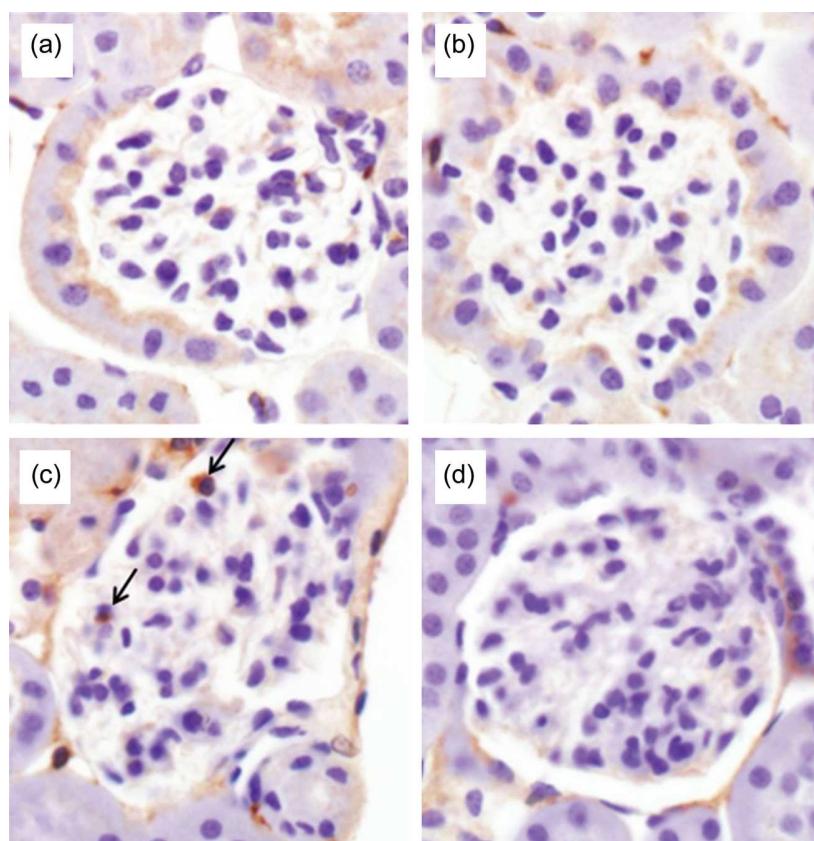


FIGURE 11: The number of F4/80 positive macrophages evaluated by immunohistochemistry was negligible in the kidneys of non-diabetic normal control mice (a) and RS102895-treated non-diabetic mice (b). Compared with the non-diabetic normal control mice, there was a substantial increase in renal macrophages (arrows) in the diabetic mice (c). The infiltration of glomerular macrophages was reduced in the RS102895-treated diabetic mice (d). Original magnification: $\times 400$.

CCR2 works independently of adiponectin to exacerbate diabetic albuminuria. In addition, our study is one of the few to measure urinary adiponectin in conjunction with plasma adiponectin, rather than the plasma level alone.

Moreover, elevated urinary albumin excretion occurred not only with the decline in renal nephrin expression but also with the increase in desmin score. These results reinforce the multifactorial nature of diabetic kidney disease and indicate that both podocyte injury and inflammation contribute to the pathogenesis of diabetic nephropathy.

Altogether, this study in type 2 diabetic *db/db* mice suggests that the CCR2 antagonist, RS102895, improves the pathological (e.g. mesangial matrix expansion, GBM thickening and podocyte foot process effacement) and the functional (e.g. increased albuminuria) impairments of diabetic nephropathy. Glucose intolerance was also improved by treatment, but this accounts for the only part of the beneficial effects that accrue to RS102895. CCR2 inhibition may be a potential therapeutic modality in the treatment of human diabetic nephropathy.

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CONFLICT OF INTEREST STATEMENT

None declared.

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