Renoprotective effect of COMP-angiopoietin-1 in db/db mice with type 2 diabetes

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Abstract

Background. Inflammatory processes have been recently seen as underlying the pathogenesis of diabetic nephropathy. Angiopoietin-1 (Ang1) plays essential roles in regulating vascular growth, development, maturation, permeability and inflammation. We have developed a soluble, stable and potent Ang1 variant, cartilage oligomeric matrix protein (COMP)-Ang1.

Methods. In this study, db/db mice were treated with recombinant adenovirus expressing either COMP-Ang1 or LacZ. Histology, inflammatory, metabolic, and fibrotic parameters and signalling pathway were evaluated.

Results. COMP-Ang1 reduced albuminuria and decreased mesangial expansion, thickening of the glomerular basement membrane and podocyte foot process broadening and effacement. COMP-Ang1 suppressed both renal expression of intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 and monocyte/macrophage infiltration in diabetic db/db mice. COMP-Ang1 also reduced renal tissue levels of transforming growth factor-β1 (TGF-β1), α-smooth muscle actin, fibronectin, as well as Smad 2/3 expression, but increased Smad 7 expression. In human umbilical vein endothelial cells (HUVECs) grown in high glucose concentrations of glucose, recombinant COMP-Ang1 protein decreased nuclear factor-κB (NF-κB) expression. COMP-Ang1-mediated inhibition of increased NF-κB-DNA binding in nuclear extracts from HUVEC grown in high glucose was significantly blocked by soluble Tie2 receptor-Fc. In addition, COMP-Ang1 significantly decreased fasting blood glucose level, epididymal fat weight to body weight ratio, and epididymal adipocyte size in diabetic db/db mice. After intraperitoneal glucose challenge, COMP-Ang1 significantly lowered plasma glucose levels. However, there was no difference in serum insulin levels.

Conclusion. We conclude that COMP-Ang1 delayed the fibrotic changes in the kidney of diabetic db/db mice through its anti-inflammatory or metabolic effects.

Keywords: COMP-angiopoietin-1; diabetic db/db mouse; inflammation; kidney

Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease worldwide, and many treatments, such as those involving glycaemic and blood pressure control reagents, may slow but not halt disease progression. Therefore, diabetic nephropathy is a serious problem for patients and healthcare systems [1].

Several mechanisms have been suggested to underlie development of diabetic nephropathy. Many metabolic factors, such as high glucose, advanced glycation end products (AGEs), reactive oxygen species (ROS) and angiotensin II, stimulate mesangial cells, resulting in overproduction of extracellular matrix, injury to podocytes and increased proteinuria. Haemodynamic factors in glomeruli also play an important role in glomerular injury seen in diabetic nephropathy.

Recently, involvement of inflammatory processes has emerged as a factor underlying the pathogenesis of diabetic nephropathy. Nuclear factor-κB (NF-κB) activation, intercellular adhesion molecule-1 (ICAM-1) expression and macrophage infiltration may play significant roles in this condition [2]. Diabetic nephropathy is also associated with increased renal expression of tumour necrosis factor-α (TNF-α) and urinary albumin excretion.
Angiopoietin-1 (Ang1) is a ligand acting on endothelial cells via the Tie2 tyrosine kinase receptor. Ang1 has essential roles in regulating vascular growth, development, maturation, permeability and inflammation. Ang1 can reduce endothelial permeability and antagonize vascular endothelial growth factor (VEGF) functions such as increasing vascular permeability and induction of angiogenesis. It has been known that Ang1 is detected in podocytes in normal glomeruli and Tie2 is expressed on glomerular capillary endothelial cells [3]. Yamamoto et al. [4] have suggested that the expression of Ang1 was detected in undiseased mice, but not significantly altered in streptozotocin-induced diabetic mice. In contrast, the protein level of Ang2, an endogenous antagonist of Ang1, was increased in diabetic mice as compared with undiseased mice. The level of Tie2 was mildly decreased in diabetic mice as compared with non-diabetic control mice [4].

We previously demonstrated that Ang1 reduces leucocyte adhesion to endothelial cells by reducing expression of adhesion molecules [5]. Takazawa et al. [6] reported that increased Ang1 expression in the kidney was associated with reduced monocyte/macrophage infiltration in experimental models of anti-Thy-1 mesangioproliferative glomerulonephritis. Although Ang1 is known to promote endothelial stabilization and have anti-inflammatory effects, it is unclear whether Ang1 is therapeutically useful in reducing diabetic renal injury.

Recently, we developed a soluble, stable and potent Ang1 variant, cartilage oligomeric matrix protein (COMP)-Ang1. The amino-terminal of COMP-Ang1 consists of the short coiled-coil domain of COMP. COMP-Ang1 is more potent than native Ang1 in phosphorylating the Tie2 receptor and signalling via Akt in primary cultured endothelial cells [7]. COMP-Ang1 antagonizes radiation-induced apoptosis in microvascular endothelial cells of the intestinal villi and prolongs cell survival [8]. Thus, COMP-Ang1 may be useful as a therapeutic agent for specific protection against endothelial dysfunction and inflammation.

In the present study, we investigated the renoprotective effects of COMP-Ang1 on the renal injury seen in type 2 diabetic db/db mice. Our results indicate that long-term and sustained COMP-Ang1 treatment produced by adenoviral delivery decreases renal injury seen in db/db mice.

Materials and methods

Generation of COMP-Ang1 recombinant protein and Ade-COMP-Ang1

Recombinant Chinese hamster ovary cells expressing COMP-Ang1 (CA1-2; production rate, ∼30 mg/l) were established as previously described [7,8]. Recombinant adenovirus expressing COMP-Ang1 or LacZ was constructed using the pAdEasy vector system (Qbiogene).

Animals

Male db/db mice and age-matched non-diabetic db/m littermates were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred in our pathogen-free animal facility. Animal care and experimental procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. At the start of the experiments, the mice were 8–10 weeks of age.

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated using methods previously described [5]. Isolated HUVECs were incubated at 37°C with sterile M199 containing 20% fetal bovine serum. The endothelial cell identity of each culture was confirmed by immunofluorescent detection of factor VIII. HUVECs were maintained in M199 medium supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum at 37°C in 5% CO2. The primary cultured cells used here were between passages three and five. Before assaying NF-kB expression, HUVECs were incubated for 24h with M199 (containing 5 mM glucose) plus 5% newborn calf serum (GIBCO BRL) and 5% human serum (medium test), with 16.5 mM or with 25 mM glucose. Glucose was virtually endotoxin free (LPS < 0.001 ng/ml; Sigma Chemical Co., St Louis, MO, USA). COMP-Ang1 recombinant protein (20 ng/ml), soluble Tie2 receptor-Fc (sTie2-Fc; 2 μg/ml; Regeneron Pharmaceuticals, Tarrytown, NY, USA) or control buffer was added to the medium with glucose treatment (5, 16.5 or 25 mM). Control samples received buffer in place of Ang1rp.

Study design

The db/db mice were randomized into two groups of 15 mice. For adenoviral treatment, ×10⁷ pfu of Ade-COMP-Ang1 or Ade-LacZ (vehicle) diluted in 50 μl of sterile 0.9% NaCl was injected intravenously through the tail vein. Serum levels of COMP-Ang1 started to increase 1 day after Ade-COMP-Ang1 injection and remained at the elevated levels for 2 weeks in non-diabetic db/m mice (Figure 1). Ade-COMP-Ang1 or Ade-LacZ was repeatedly injected four times at 2-week intervals. Non-diabetic db/m mice (n=15) were injected with saline as vehicle. Body weight, blood glucose, systolic blood pressure and food intake were measured initially and every 2 weeks. Blood glucose levels were measured in tail vein blood by Precision Xtra Plus (Abbott Laboratories, MediSense Products, Bedford, MA, USA). An intraperitoneal glucose tolerance test was performed after 8 weeks of Ade-COMP-Ang1 treatment. After 18 h fasting, tail blood samples (5 μl) were collected at 0 and 30 min, 1, 2, 3, 4 and 5 h after the intraperitoneal glucose challenge (1.5 g/kg), and plasma glucose was measured using the Autokit Glucose (Wako Pure Chemical, Osaka, Japan). Blood pressure of conscious mice at a steady state was measured using a programmable tail-cuff sphygmomanometer (BP98-A; Softron, Tokyo, Japan). After 8 weeks, mice were placed in metabolic cages to collect 24-h urine samples for albumin. Urinary albumin was measured with indirect competitive enzyme-linked immunosorbent assay (ELISA) system (Exocell, Philadelphia, PA, USA).
Cruz Biotechnology, Santa Cruz, CA, USA; dilution

The effacement of podocyte foot process was semiquantitatively determined by the podocyte number/5

points of glomerular basement membrane (GBM) were

analysed by a computer-assisted colour image analyser (LUZEX F; Nikon, Tokyo, Japan). The middle

glomerular area was determined along the outline of the
capillary loop using a computer-assisted colour image analyser (LUZEX F; Nikon, Tokyo, Japan). The middle

piece of the right kidney was fixed in 0.1 mol/l cacodylate buffer with 1% glutaraldehyde and 2% paraformaldehyde

and tubulointerstitial area showing positive staining: score 0,

unaware of sample origin. The number of F4/80-positive

cells in each section was determined by counting positively

stained cells in 10

/measured. The middle piece of the left kidney was

fixed in 4% paraformaldehyde to measure glomerular or

mesangial area (mesangial matrix) by light microscopy. Thirty glomeruli cut at their vascular poles were used for

the morphometric analysis. The extent of the mesangial area was measured by assessing the periodic acid-Schiff (PAS)-

positive and nuclei-free area in the mesangium. The

glomerular area was determined along the outline of the

capillary loop using a computer-assisted colour image analyser (LUZEX F; Nikon, Tokyo, Japan). The middle

piece of the right kidney was fixed in 0.1 mol/l cacodylate buffer with 1% glutaraldehyde and 2% paraformaldehyde

for measurement of basement membrane thickness (BMT)

by electron microscopy. Ten photographs were taken in each glomerulus at a magnification of 3000×. Five points of
glomerular basement membrane (GBM) were chosen in each photograph and the thickness was measured.

The effacement of podocyte foot process was semiquantitatively determined by the podocyte number/5 µm length

of GBM on electron microscopy. Epididymal fats were

removed and weighted. The ratio of epididymal fat to body

weight was measured. The middle piece of the left kidney was

fixed in 4% paraformaldehyde for 20 min, and then incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by chemiluminescent detection according to the manufacturer’s protocol (Amersham Pharmacia Biotech, London, UK). Membranes were reprobed with anti-actin antibody to verify equal loading of protein. Signals were analysed by densitometric scanning (LAS-1000, Fuji Film, Tokyo, Japan).

**Immunohistochemical analysis**

Isolated kidney tissue was fixed by immersion in 4% paraformaldehyde and embedded in paraffin blocks. Tissue sections were cut and placed on glass slides, deparaffinized with xylene and rehydrated with graded ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min, slides were rinsed with PBS, and samples were treated with pepsin at 42°C for 5 min. After treatment with blocking buffer, slides were incubated overnight at 4°C with a primary antibody for ICAM-1 (Santa Cruz Biotechnology; dilution 1:50), α-smooth muscle actin (α-SMA) (Alomone Laboratories, Jerusalem, Israel; dilution 1:50), fibronectin (Santa Cruz Biotechnology; dilution 1:100), TGF-β1 (Santa Cruz Biotechnology; dilution 1:100), monocytic chemotaxin protein-1 (MCP-1) (Fitzgerald, Concord, MA, USA; dilution 1:100), F4/80 (a 160 kDa glycoprotein expressed in murine macrophages; Serotec Inc., Oxford, UK; dilution 1:50). The primary antibody was visualized using the Vectastain ABC-Elite peroxidase detection system (Vector Laboratories, Burlingame, CA, USA), followed by reaction with diaminobenzidine as chromogen and counterstaining with haematoxylin (Sigma Chemical Co.). Sample evaluation was performed by an observer unaware of sample origin. The number of F4/80-positive cells in each section was determined by counting positively stained cells in 10 × 400 fields per slide. To evaluate the immunostaining for α-SMA, fibronectin or MCP-1, a total of 20 randomly chosen glomeruli per mouse were graded. Each score, primarily reflected changes in the extent of staining and, depends on the percentage of the glomeruli and tubulointerstitial area showing positive staining: score 0, 0–5% stained; score I, >5–25%; score II, >25–50%; score III, >50–75%; score IV, >75%.

**Western blot analysis**

Samples were mixed with sample buffer, boiled for 10 min, separated by 12% sodium dodecyl sulphate (SDS)-
polyacrylamide gel electrophoresis under denaturing conditions, and electroblotted to nitrocellulose membranes. Membranes were incubated with blocking buffer containing 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST) buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and then incubated with an antibody against transforming growth factor-β1 (TGF-β1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100), an anti-ICAM-1 monoclonal antibody (Santa Cruz

Biotechnology; dilution 1:2000), a Smad 2/3 antibody (Cell Signaling Technology Inc.; dilution 1:1000) or a Smad 7 antibody (Cell Signaling Technology Inc.; dilution 1:1000). Membranes were washed with phosphate-buffered saline (PBS) and incubated with horseradish peroxidase-conjugated secondary antibody. Signals were visualized by chemiluminescent detection according to the manufacturer’s protocol (Amersham Pharmacia Biotech, London, UK). Membranes were reprobed with anti-actin antibody to verify equal loading of protein. Signals were analysed by densitometric scanning (LAS-1000, Fuji Film, Tokyo, Japan).

**Cytosolic and nuclear protein extraction for analysis of NF-κB**

HUVECs were lysed in a hypotonic buffer (10 mmol/l HEPES, pH 7.9, 1.5 mmol/l MgCl2, 10 mmol/l KCl, 0.5 mmol/l dithiothreitol, 0.5 mmol/l phenylmethyl sulphonyl fluoride and 0.6% NP-40) and centrifuged at 1500 g for 15 min at 4°C. The supernatant was used as cytosolic protein. Pellets were lysed in 15 µl of a high-salt buffer (20 mmol/l HEPES, pH 7.9, 420 mmol/l NaCl, 25% glycerol, 1.5 mmol/l MgCl2, 0.2 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l phenylmethyl sulphonyl fluoride and 0.5 mmol/l dithiothreitol) for 20 min on ice. Storage buffer (75 µl; 20 mmol/l HEPES, pH 7.9, 100 mmol/l NaCl, 20% glycerol, 0.2 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l phenylmethyl sulphonyl fluoride and 0.5 mmol/l dithiothreitol)
was added. The resulting nuclear pellets were agitated by vortexing and centrifuged at 16000 g for 20 min. The resulting supernatant was used as soluble nuclear proteins.

For Western analysis, samples (30 μg of protein per lane) were loaded on 10% SDS-polyacrylamide gel. After electrophoresis at 120 V for 90 min, separated proteins were electroblotted to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in TBST buffer for 1 h and incubated overnight at 4°C with anti-NF-κB p65 (Upstate Biotech, Lake Placid, NY, USA; dilution 1:1000). Then, the blots were analysed as aforementioned.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay (EMSA) was performed as described previously [10]. Nuclear extracts (10 μg) were incubated with or without unlabelled competitor at room temperature for 5 min. Subsequently, biotin-labelled NF-κB binding site oligomer 5'AGTTGAGGGGACTTTCCAGGCC was added, and the incubation was continued for 30 min. The reaction mixture was resolved on a 6% non-denaturing polyacrylamide gel with Tris-borate EDTA (TBE) as an electrophoresis buffer. The gel was transferred to nylon hybridization transfer membrane (Hybond-N+; Amersham Pharmacia Biotech, Buckinghamshire, UK) and cross-linked at 120 mJ/cm² using a UV cross-linker (Stratagene, La Jolla, CA, USA), and subjected to streptavidin-horseradish peroxidase conjugate-based detection method as described by the manufacturer (EMSA Gel-Shift kit; Panomics, Redwood City, CA). Supershift experiments were conducted using specific antibodies (anti-NF-κB p50 antibody and anti-NF-κB p65 antibody, Santa Cruz Biotechnology). Reactions were identical to gel shift reaction conditions except 2 μg of anti-NF-κB p65 or p50 antibody was added to the binding reactions after addition of labelled probe. The reaction mixtures were incubated for 2 h at 4°C before electrophoresis. Specificity was verified for NF-κB binding by conducting identical control assays with up to 2 μg of normal rabbit IgG (Santa Cruz Biotechnology).

**ELISA of COMP-Ang1 and TGF-β1**

Blood samples (0.5 ml) were taken from the mice at 1, 3, 5, 7, 14 and 28 days after administering the COMP-Ang1 adenovirus. The blood samples were collected by cardiac puncture, and the serum concentrations of COMP-Ang1 were determined as described previously [11].

TGF-β1 levels in kidney tissue were determined by using ELISA kits (Endogen, Woburn, MA, USA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. To activate latent TGF-β, protein was incubated with 0.5 N acetic acid/10 M urea. After mixing and incubation for 10 min at room temperature, the acidified samples were neutralized by adding 2.7 N NaOH/1 M HEPES, and then the samples were assayed immediately. In all cases, a standard curve was constructed from standards provided by the manufacturer.

**Statistical analysis**

Data are expressed as mean ± SD. Multiple comparisons were examined for significant differences using ANOVA, followed by individual comparisons with the Tukey post-test. Statistical significance was set at P < 0.05.

**Results**

**Effect of Ade-COMP-Ang1 on characteristics of study groups**

Characteristics of three groups of mice at age 16 weeks are presented in Table 1. Body weights of db/db mice were significantly greater than those of db/m controls. Body weights of the Ade-COMP-Ang1 treated group were less than those in the db/db group. Kidney weights were also significantly greater in db/db compared with db/m mice. The Ade-COMP-Ang1 treated group showed significantly lower kidney weights than the db/db mouse group. As expected, food intake of db/m controls was significantly less than that of the db/db and Ade-COMP-Ang1 treated db/db group. Levels of fasting blood glucose were significantly lower in the Ade-COMP-Ang1 treated db/db group than in the db/db (Table 1). After intraperitoneal glucose challenge, db/db mice treated with Ade-COMP-Ang1 maintained significantly low plasma glucose levels (Figure 2A). Serum insulin levels of the db/db and Ade-COMP-Ang1 treated db/db mice were significantly higher than those of the db/m mice, and there was no significant difference between db/db and Ade-COMP-Ang1 treated db/db mice (Table 1). However, neither serum creatinine nor BUN levels were significantly different between groups. Systolic blood pressures of db/db mice were significantly higher than those of the db/m mice, however there was no significant difference between db/db and Ade-COMP-Ang1 treated db/db mice and Ade-COMP-Ang1 treated db/db mouse group (Table 1). The ratio of epididymal fat to body weight was significantly lower in the Ade-COMP-Ang1 treated db/db mice than that of the db/db mouse (Table 1). In addition, Ade-COMP-Ang1

| Table 1. Characteristics of experimental animals after 8 weeks treatment |
|--------------------------|--------------------------|--------------------------|--------------------------|
|                          | db/m                     | db/db + LacZ             | db/db + Ang1             |
| Body weight (g)          | 25.6 ± 1.7               | 53.9 ± 2.6*              | 51.3 ± 1.1**             |
| Kidney weight (g)        | 0.28 ± 0.03              | 0.44 ± 0.04*             | 0.35 ± 0.02**            |
| Food intake (g/24 h)     | 4.6 ± 0.9                | 8.5 ± 0.5*               | 8.5 ± 0.4*               |
| Serum insulin (μU/ml)    | 6.9 ± 0.8                | 40.7 ± 4.3*              | 39.2 ± 5.6*              |
| Fasting blood glucose (mg/dl) | 83 ± 27              | 554 ± 66*                | 366 ± 85**               |
| BUN (mg/dl)              | 31.2 ± 7.8               | 31.2 ± 8.0               | 31.4 ± 9.5               |
| SCR (mg/dl)              | 0.36 ± 0.05              | 0.39 ± 0.07              | 0.42 ± 0.08              |
| Systolic BP (mmHg)       | 104 ± 1.6                | 112 ± 4.1*               | 112.2 ± 2.9*             |
| Ratio of epididymal fat | 0.01 ± 0.00              | 0.11 ± 0.01*             | 0.09 ± 0.01**            |

Diabetic db/db mice were treated for 8 weeks with either Ade-LacZ (db/db + LacZ) or Ade-COMP-Ang1 (db/db + Ang1). Values are expressed as mean ± SD. *P < 0.05 vs db/m mice; **P < 0.01 vs Ade-LacZ-treated db/db mice. db/m, control db/m mice. BUN, blood urea nitrogen; SCR, serum creatinine; BP, blood pressure. n = 5–15 in each group.
treated \( db/db \) mice had a greater number of larger adipocytes than \( db/+/db \) mice (Figure 2B and C).

**Ade-COMP-Ang1 treatment decreases urinary albumin excretion in \( db/db \) mice**

Albuminuria is an index that precedes and predicts the development of diabetic nephropathy. As shown in Figure 3, urinary albumin excretion in diabetic \( db/db \) mice was approximately 6.5-fold greater than that seen in control \( db/m \) mice. Treatment with Ade-COMP-Ang1 significantly decreased urinary albumin excretion in diabetic \( db/db \) mice (Figure 3).

**Ade-COMP-Ang1 treatment reduces mesangial expansion and thickening of GBM in \( db/db \) mice**

Striking characteristics of diabetic nephropathy are mesangial expansion, which results from accumulation of extracellular matrix proteins, and thickening of the GBM. We examined whether Ade-COMP-Ang1 treatment reduces either of these effects in diabetic \( db/db \) mice. The glomeruli of diabetic \( db/db \) mice showed an increase in accumulation of PAS-positive matrix in the mesangium compared with glomeruli of \( db/m \) mice. Diabetic \( db/db \) mice treated with Ade-COMP-Ang1 showed relatively normal appearing glomeruli with mild mesangial matrix expansion compared with diabetic \( db/db \) mice (Figure 4A–F). In control \( db/m \) mice, the mesangial matrix fraction of the total glomerular tuft area was 6.6 ± 1.5%. However, that fraction significantly increased to 12.6 ± 2.2% in diabetic \( db/db \) mice at age 16 weeks. With diabetic \( db/db \) mice, Ade-COMP-Ang1 treatment significantly decreased the mesangial matrix fraction to 7.4 ± 3.3% (Figure 4G). Electron microscopic findings revealed that GBM thickness significantly increased from 178 ± 26 nm in the control \( db/m \) mice to 358 ± 19 nm in diabetic \( db/db \) mice. In addition, diabetic \( db/db \) mice showed extensive fusion of podocyte foot processes. Ade-COMP-Ang1 treatment significantly reduced GBM thickening to 236 ± 24 nm in diabetic \( db/db \) mice and blocked extensive fusion of podocyte foot processes (Figure 5).

**Ade-COMP-Ang1 treatment inhibits up-regulation of renal ICAM-1 and MCP-1 expression in \( db/db \) mice**

Immunostaining analysis showed that ICAM-1 was very weakly expressed in glomerular and peritubular cells.
COMP-angiopoietin-1 in diabetic nephropathy

capillaries and the tubular brush border of the control
$\text{db/m}$ mice, but was markedly increased in the
glomeruli and interstitium of diabetic $\text{db/db}$ mice. In
contrast, ICAM-1 expression was significantly
decreased in diabetic $\text{db/db}$ mice treated with
Ade-COMP-Ang1 (Figure 6A–C). Consistent with
the immunostaining results, western blot analysis also
showed that Ade-COMP-Ang1 decreased ICAM-1
protein levels in renal tissues obtained from diabetic
$\text{db/db}$ mice (Figure 6G).

Immunohistochemical staining of MCP-1 protein in
the kidney revealed no staining of MCP-1 in kidneys
obtained from control $\text{db/m}$ mice, but stronger staining
in proximal tubules in kidneys from diabetic $\text{db/db}$
mice. Ade-COMP-Ang1 treatment significantly
reduced MCP-1 staining in diabetic $\text{db/db}$ mice
(Figure 6D–F). Ade-COMP-Ang1 treatment decreases renal
macrophage infiltration in $\text{db/db}$ mice

Increased numbers of infiltrated renal macrophage,
as assessed by F4/80-positive cells, were identified in
the glomeruli and interstitium of diabetic $\text{db/db}$ mice.
In control $\text{db/m}$ mice, macrophage infiltration was
observed in the tubulointerstitium with few macro-
phages detected in the glomeruli. Treatment with

Fig. 3. Effect of COMP-Ang1 on urinary albumin excretion
in diabetic mice. Mice were placed in metabolic cages to collect
24-h urine samples for albumin measurement. Control $\text{db/m}$
mice, $\text{db/m}$; diabetic $\text{db/db}$ mice treated with Ade-LacZ,
$\text{db/db} + \text{LacZ}$; diabetic $\text{db/db}$ mice treated with Ade-COMP-Ang1,
$\text{db/db} + \text{Ang1}$. Values are expressed as mean ± SD. *$P < 0.05$ vs $\text{db/m}$
mice; **$P < 0.05$ vs Ade-LacZ-treated $\text{db/db}$ mice. $n = 10$ in each
group.

Fig. 4. Effect of COMP-Ang1 on mesangial matrix expansion in diabetic mice. Representative photomicrographs (magnification: A–C, 400×;
D–F, 1000×) of periodic acid-Schiff (PAS)-stained kidney sections from: (A, D) normal $\text{db/m}$ mice ($\text{db/m}$); (B, E) diabetic $\text{db/db}$ mice treated
with Ade-LacZ ($\text{db/db} + \text{LacZ}$); (C, F) diabetic $\text{db/db}$ mice treated with Ade-COMP-Ang1 ($\text{db/db} + \text{Ang1}$). Note diffusely expanded
evaculular mesangial matrix in the $\text{db/db}$ mice treated with Ade-LacZ and marked reduction of this expansion in $\text{db/db}$ mice treated with
Ade-COMP-Ang1. (G) Quantitation of extravascular mesangial matrix expansion is expressed as PAS-positive mesangial material per total
glomerular tuft cross-sectional area (mesangial matrix area/total glomerular tuft area 100×). An average value was obtained from analyses of
30 glomeruli per mouse. Values are expressed as mean ± SD. *$P < 0.05$ vs $\text{db/m}$ mice; **$P < 0.05$ vs Ade-LacZ-treated $\text{db/db}$ mice. $n = 9$ in
each group.
Ade-COMP-Ang1 significantly decreased macrophage infiltration within both the glomeruli and the tubulointerstitium (Figure 7).

**Ade-COMP-Ang1 treatment reduces up-regulation of renal TGF-β1 expression in db/db mice**

As TGF-β1 is an important regulator in the process leading to glomerular sclerosis as well as progression of tubulointerstitial fibrosis, we investigated whether Ade-COMP-Ang1 treatment regulates renal TGF-β1 expression in diabetic db/db mice. As shown in Figure 8, glomerular TGF-β1 expression was markedly increased in db/db mice. Increased TGF-β1 expression was attenuated in db/db mice treated with Ade-COMP-Ang1. To corroborate this finding, we measured TGF-β1 production in renal tissue by ELISA. Increased levels of total TGF-β1 protein seen in renal tissue of diabetic db/db mice were significantly decreased in db/db mice treated with Ade-COMP-Ang1 (Figure 8D).

**Ade-COMP-Ang1 treatment alters expression of Smad proteins in db/db mice**

Inhibition of TGF-β/Smad pathway is associated with amelioration of renal insufficiency and glomerulosclerosis in the db/db mice [12]. Western blot analyses of Smad 2/3 and Smad 7 protein levels were performed in db/db mice treated with Ade-COMP-Ang1. Compared with control db/m mice, renal Smad 2/3 protein levels were increased in diabetic db/db mice. Ade-COMP-Ang1 treatment reduced significantly increased Smad 2/3 protein levels in diabetic db/db mice. Smad 7 protein levels were decreased in db/db mice compared with control db/m mice. Ade-COMP-Ang1 treatment increased significantly Smad 7 protein levels compared with diabetic db/db mice (Figure 9). These results indicate that Ade-COMP-Ang1 treatment alters levels of Smad proteins known to mediate kidney phenotypes seen in db/db mice.

**Ade-COMP-Ang1 treatment decreases α-SMA and fibronectin expression in db/db mice**

To evaluate the therapeutic effect of Ang1 in diabetic nephropathy, expression levels of α-SMA and fibronectin were examined. Immunostaining analysis showed increased glomerular and interstitial expression of α-SMA and fibronectin in diabetic db/db mice compared with control db/m mice, whereas expression of α-SMA and fibronectin was markedly decreased in Ade-COMP-Ang1 treated diabetic db/db mice compared with untreated db/db mice (Figure 10).
We performed a western blot analysis for NF-κB p65 protein in cytosolic and nuclear fractions and EMSA for NF-κB DNA binding activity of HUVECs grown in high glucose. Western blot analysis revealed that NF-κB protein levels in nuclear extracts from HUVECs in high glucose were increased compared with the levels seen in control glucose concentration. Such increased NF-κB p65 levels in nuclear extracts were decreased by COMP-Ang1 treatment. In contrast, levels of NF-κB p65 protein in cytosolic extracts from HUVECs were decreased under high glucose compared with the levels seen in control glucose concentration. These decreased NF-κB p65 levels were increased by COMP-Ang1 treatment (Figure 11A). EMSA analysis showed increased NF-κB-DNA binding in nuclear extracts from HUVECs in high glucose compared with cells grown in control glucose concentrations. Such increased NF-κB-DNA binding was decreased by COMP-Ang1 administration. However, COMP-Ang1-mediated inhibition of NF-κB-DNA binding was significantly blocked by sTie2-Fc (Figure 11B). These results suggest that Ade-COMP-Ang1 acts through a process that is the molecular link between the Tie2 and the NF-κB system.

Discussion

In the present study, we demonstrate that COMP-Ang1 has a renoprotective effect mediated by its anti-inflammatory activity, and improves metabolic status in diabetic db/db mice. COMP-Ang1 treatment relieves albuminuria and decreases mesangial expansion, GBM thickening and podocyte foot process broadening and effacement. Renal expression of ICAM-1 and MCP-1 and monocyte/macrophage infiltration are reduced by COMP-Ang1 treatment in db/db mice as are levels of TGF-β1, α-SMA and fibronectin. COMP-Ang1 treatment also reduces expression of Smad 2/3, but, in contrast, increases expression of Smad 7. In HUVEC grown under high glucose concentrations, COMP-Ang1 decreases NF-κB expression and soluble Tie2 receptor blocks that decrease. In addition, COMP-Ang1 significantly decreased fasting blood glucose level, epididymal fat weight to body weight ratio, adipocyte cell surface area, and epididymal adipocyte size in diabetic db/db mice. After intraperitoneal glucose challenge, COMP-Ang1 significantly lowered plasma glucose levels without changes in serum insulin levels between COMP-Ang1 treated and untreated db/db mice.
Hallmarks of developing diabetic nephropathy are an accumulation of extracellular matrix components in the mesangium and interstitium, GBM thickening, and an increase in urinary albumin excretion, leading to glomerulosclerosis and interstitial fibrosis. It has been suggested that the pathogenesis of diabetic nephropathy is associated with overexpression of several growth factors, AGE products and activation of protein kinase C. However, recent studies suggest that inflammatory mechanisms are involved in the development and progression of diabetic nephropathy. Okada et al. [14] report that ICAM-1-deficient mice are resistant to renal injury after induction of diabetes, and ICAM-1 is critically involved in the pathogenesis of diabetic nephropathy. Chow et al. [15] also report that increased expression of ICAM-1, promotes macrophage infiltration, in type 2 diabetic db/db mice. It has been shown that methotrexate has protective effects on renal injury in experimental diabetic rats via its anti-inflammatory actions through inhibition of NF-κB activation, and consequent reduction of ICAM-1 and macrophage infiltration [16]. In addition, several clinical studies have indicated a correlation between diabetic nephropathy and up-regulation of pro-inflammatory cytokines. These findings strongly demonstrate an association of pro-inflammatory cytokines, adhesion molecules and inflammatory cells in the development of diabetic renal injury. High glucose concentrations and hyperglycaemia induce leucocyte adhesion to the endothelium through up-regulation of cell-surface expression of adhesive proteins in a NF-κB-dependent fashion.

We previously demonstrated that Ang1 reduces VEGF-stimulated leucocyte adhesion to endothelial cells by reducing ICAM-1, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin expression [5]. Joussen et al. [17] have demonstrated that Ang1 inhibits leucocyte adhesion, endothelial injury, ICAM-1 expression and blood-retinal barrier breakdown in mice with diabetic retinopathy. Witzenbichler et al. [18] have reported that Ang1 has a protective effect in lipopolysaccharide-induced endotoxic shock by an improvement in haemodynamic function, reducing lung injury, decreasing expression of inflammatory adhesion molecules, and preserving eNOS activity in the lung tissue. A direct molecular link between Tie2 and NF-κB may be important in the anti-inflammatory and stabilizing activity of Tie2 in
endothelial cells and microvessels [13]. In this study, we demonstrate that COMP-Ang1 treatment decreases ICAM-1 and MCP expression in glomerular and peritubular capillaries of diabetic \( \text{db/db} \) mice. COMP-Ang1 also reduces increased infiltration of F4/80-positive monocyte/macrophages in these mice. Our findings indicate that COMP-Ang1 suppresses NF-\( \kappa \)B activation in HUVECs grown in high glucose. However, COMP-Ang1-induced suppression for NF-\( \kappa \)B activation is reversed by sTie2-Fc. Thus, these results suggest that Ang1 has a protective effect in diabetic nephropathy through the anti-inflammatory process that is the molecular link between the Tie2 and the NF-\( \kappa \)B system.

Infiltrated monocyte/macrophages or damaged intrinsic renal cells release profibrotic cytokine, which induces fibrosis or sclerosis. TGF-\( \beta \)1 plays a central role in the pathogenesis of glomerulosclerosis and tubulointerstitial fibrosis seen in the nephropathy of type 2 diabetes. Increased levels of TGF-\( \beta \) mRNA and protein have been seen in the glomeruli and tubulointerstitium of diabetic animal models with nephropathy. Inhibition of TGF-\( \beta \)1 is a powerful strategy in treating diabetic nephropathy by blocking expression of fibrogenic factors and inhibiting glomerulosclerosis [12]. Our data show that COMP-Ang1 treatment reduces increased renal TGF-\( \beta \)1 levels seen in diabetic \( \text{db/db} \) mice. Furthermore, the increased glomerular and interstitial expression of \( \alpha \)-SMA and fibronectin in diabetic \( \text{db/db} \) mice are markedly decreased by COMP-Ang1 treatment.

Smad proteins function in signalling pathways downstream of TGF-\( \beta \)/Smad pathway decreases renal insufficiency and glomerulosclerosis in diabetic mice [12]. Smad 7 is known to antagonize Smad 2/3 in renal fibrosclerosis. Our results show that COMP-Ang1 treatment reduces increased Smad 2/3 protein levels seen in the kidneys of diabetic \( \text{db/db} \) mice, while increasing Smad 7 expression.

In Ade-COMP-Ang1 treated \( \text{db/db} \) mice, fasting blood glucose level, ratio of epididymal fat weight to body weight, adipocyte cell surface area and adipocyte size of epididymal fat pad were significantly decreased compared with those in Ade-LacZ treated mice. After intraperitoneal glucose challenge, \( \text{db/db} \) mice treated with Ade-COMP-Ang1 showed significantly low plasma glucose levels. However, there was no difference in serum insulin levels between Ade-COMP-Ang1 and Ade-LacZ treated mice. These results suggested that COMP-Ang1 has an insulin sensitizing effect in peripheral tissues without direct effect of
**Fig. 9.** Effect of COMP-Ang1 on changes of Smad 2/3 and Smad 7 in the diabetic mice. Western blots and corresponding densitometric analyses of Smad 2/3 and Smad 7 protein levels in kidneys of control db/m mice (db/m), diabetic db/db mice treated with Ade-LacZ (db/db + LacZ), or diabetic db/db mice treated with Ade-COMP-Ang1 (db/db + Ang1). Data represent mean ± SD from four independent experiments. *P < 0.05 vs db/m mice; **P < 0.05 vs Ade-LacZ-treated db/db mice. n = 5–7 in each group.

**Fig. 10.** Effect of COMP-Ang1 on expression of α-SMA and fibronectin in diabetic mice. Representative immunostaining for (A–C) α-smooth muscle actin (α-SMA) and (D–F) fibronectin in the renal tissue. (A) Control db/m mice (db/m). (B, E) Diabetic db/db mice treated with Ade-LacZ (db/db + LacZ). (C, F) Diabetic db/db mice treated with Ade-COMP-Ang1 (db/db + Ang1). (G, H) The extent of α-SMA and fibronectin immunostaining is assessed semiquantitatively as described in the ‘Materials and methods’ section. Data represent mean ± SD from four independent experiments. *P < 0.05 vs db/m mice; **P < 0.05 vs Ade-LacZ-treated db/db mice. Magnification 400×. n = 5–7 in each group.
COMP-Ang1 on insulin secretion machinery in pancreatic β-cells. Decreased adipocyte cell surface area and relative increase of small-sized adipocytes after COMP-Ang1 treatment may partially explain the slight reduction of body weight. Hyperglycaemia is one of the key players in the development of diabetic nephropathy [19]. High glucose, AGEs and ROS act in concert to induce growth factors and cytokines in diabetic nephropathy [20]. Therefore, the control of blood glucose level is an essential part of every therapeutic regimen to prevent and treat diabetic nephropathy. In this study, although we have focused on the anti-inflammatory effect of COMP-Ang1 on diabetic nephropathy, the metabolic effects of COMP-Ang1 may also contribute to renoprotection in diabetes. Therefore, the metabolic effect of COMP-Ang1 in diabetic mice and its exact mechanism will need to be investigated in the future.

Since Ang1 can reduce endothelial permeability and antagonize VEGF effects in vascular permeability and angiogenesis-inducing activity, further studies are needed to reveal the mechanism by which Ang1 regulates the haemodynamic effect and functions of VEGF in the pathogenesis of diabetic nephropathy.

In conclusion, we propose that COMP-Ang1 has an anti-inflammatory effect by inhibiting the Tie2/NF-κB pathway, which subsequently decreases activation of TGF-β/Smad 2/3 pathway and renal fibrosis. Finally, COMP-Ang1 reduces albuminuria and decreases mesangial thickening of the GBM, and podocyte foot process broadening and effacement. COMP-Ang1 also improves metabolic status in diabetic db/db mice. Taken together, these observations identify COMP-Ang1 as a potentially important therapeutic agent to prevent and treat diabetic nephropathy.

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Conflict of interest statement. None declared.

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