Original Article

Protective effect of COMP-angiopoietin-1 on cyclosporine-induced renal injury in mice

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Abstract

Background. Peritubular capillary injury induces chronic hypoxia in the renal tubulointerstitium, and renal peritubular capillary dysfunction is an early event that contributes to tubulointerstitial fibrosis. Cyclosporine A (CsA) is a potent immunosuppressant and improves survival of renal allografts. However, the limitation of CsA use is chronic nephrotoxicity. A soluble, stable and potent angiopoietin-1 (Ang1) variant, cartilage oligomeric matrix protein (COMP)-Ang1 has been developed. We investigated whether COMP-Ang1 ameliorates CsA-induced renal injury.

Methods. CsA-treated mice were injected with recombinant adenovirus expressing either COMP-Ang1 or LacZ. Histology, inflammatory, haemodynamic and fibrotic parameters, and signalling pathway were evaluated.

Results. Histologic examination showed that COMP-Ang1 significantly decreased CsA-induced tubular damage and tubulointerstitial fibrosis. CsA-induced increases in macrophage infiltration and expression of MCP-1 and ICAM-1 after CsA treatment were significantly reduced by COMP-Ang1. Treatment with COMP-Ang1 also decreased the CsA-induced increases in TGF-β1 and Smad 2/3 levels while increasing Smad 7 levels. Laser–Doppler sonographic findings and endothelial factor VIII staining revealed that COMP-Ang1 preserved the integrity of peritubular vasculature and intrarenal haemodynamics from the CsA-induced renal injury. COMP-Ang1 inhibited tubular cell apoptosis while increasing tubular cell proliferation in CsA-induced renal injury.

Conclusions. These results indicate that COMP-Ang1 exhibited a protective effect on damaged peritubular capillaries, haemodynamic alteration and inflammation in CsA-induced renal injury. Thus, COMP-Ang1 may be useful as a therapeutic and prophylactic agent for specific protection against endothelial dysfunction and inflammation.

Keywords: COMP-angiopoietin-1; cyclosporine A; endothelial cells; renal fibrosis

Introduction

Cyclosporine A (CsA), which is a potent and effective immunosuppressant, significantly improves 1-year survival of renal allografts. However, the limitation of long-term CsA use is chronic nephrotoxicity, which remains an unresolved problem. Chronic CsA nephrotoxicity is characterized by tubular atrophy, inflammatory cell infiltration, striped tubulointerstitial fibrosis, afferent arteriolopathy and progressive renal impairment [1].

Injury to the renal microvasculature and tubulointerstitial results in extracellular matrix expansion and tubular atrophy, which are the main structural determinants of progressive renal diseases including CsA-induced renal injury. It has been suggested that renal ischaemia and vasoconstriction mediate CsA-induced renal injury through activation of the intrarenal renin–angiotensin system, increased release of endothelin-1 and dysregulation of nitric oxide [2–5]. The endothelial injury and dysfunction induced by CsA may also play an important role in the pathogenesis of CsA nephrotoxicity.

Angiopoietin-1 (Ang1) is a ligand acting on endothelial cells via the Tie2 tyrosine kinase receptor. Ang1 displays essential roles in regulating vascular growth, development, maturation, permeability and inflammation. We have demonstrated that Ang1 increases endothelial cell survival and shows an anti-inflammatory effect [6,7]. We have developed a soluble, stable and potent Ang1 variant, termed COMP-Ang1. The amino terminal of COMP-Ang1 consists of the short coiled-coil domain of a cartilage oligomeric matrix protein (COMP). COMP-Ang1 is more potent than...
native Ang1 in phosphorylating the Tie2 receptor and signalling via Akt in primary cultured endothelial cells [8]. COMP-Ang1 antagonizes radiation-induced apoptosis in microcapillary endothelial cells of the intestinal villi and prolongs cell survival [9]. Recently, we have demonstrated that COMP-Ang1 treatment preserves renal microvasculature in the unilateral ureteral obstruction model and displays an anti-inflammatory effect in diabetic db/db mice, resulting in the decrease of the progression of renal fibrosis [10,11].

In the present study, we investigated whether COMP-Ang1 ameliorates CsA-induced renal injury of mice. Our results indicate that treatment with COMP-Ang1 protects endothelial damage, haemodynamic alteration and inflammation in CsA-induced renal injury.

**Subjects and methods**

**Animals**

Male ICR mice were purchased from Charles River Korea (Seoul, Korea) 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 7–8 weeks of age, weighing 25–30 g. All mice received a low-salt diet (0.01% sodium chloride, Teklad Premier, Madison, WI, USA) throughout the experiment period.

**Study design**

For adenoviral treatment, 1 × 10⁹ pfu per mouse of Ade-COMP-Ang1 or Ade-LacZ diluted in 50 µL of sterile 0.9% NaCl was injected intravenously through the tail vein. Recombinant adenovirus expressing COMP-Ang1 or LacZ was constructed using the pAdEasy vector system (Qbiogene, Carlsbad, CA, USA). Serum levels of COMP-Ang1 started to increase at 1 day after Ade-COMP-Ang1 injection and remained at the elevated levels for 2 weeks in control mice (Figure 1). Ade-COMP-Ang1 or Ade-LacZ was injected two times at 2-week intervals (Figure 2). CsA (Chong Kun Dang Pharm., Seoul, Korea) was diluted in mineral oil (Sigma Chemical Co., St Louis, MO, USA) to a final concentration of 15 mg/mL. To obtain optimal dose of CsA (20 mg/kg) for inducing renal injury, we performed preliminary experiments using three different doses of CsA (10, 15 and 20 mg/kg). As described in previous study [10,11], adenoviral treatment (Ade-LacZ) or Ade-COMP-Ang1 itself as vehicle did not affect results. Based on these results, mice were randomized to three groups and treated for 1 month: (1) the vehicle group (N = 32) in which mice received subcutaneous injection of mineral oil daily; (2) the CsA + LacZ group (N = 16) in which mice received both CsA (subcutaneous injection of 20 mg/kg per day) and Ade-LacZ; (3) the CsA + COMP-Ang1 group (N = 16) in which mice received both CsA and Ade-COMP-Ang1. On Day 30 of experiment, mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), and blood was collected by cardiac puncture. Urea nitrogen and creatinine levels in blood were measured using an enzymatic method (Neodine Laboratories, Seoul, Korea). The whole blood CsA level was measured by a monoclonal radioimmunoassay (Neodine Laboratories). Blood pressure of conscious mice at a steady state was measured using a programmable tail–cuff sphygmomanometer (BP98-A; Softron, Tokyo, Japan).

**Histologic examination**

The mouse kidney was sectioned in blocks and fixed in 10% formalin, and then dehydrated in graded concentrations of alcohols and embedded in paraffin. The kidney block was cut into 5-µm sections and stained with periodic acid–Schiff (PAS). Two observers unaware of the origin of each kidney section performed examination. Tubular injury was examined in PAS-stained sections and scored using the percentage of cortical tubules showing tubular atrophy, tubular dilatation, epithelial desquamation, epical blebbing and brush border loss in 10 randomly chosen, non-overlapping fields per kidney section to obtain ~80% of the kidney section. At least one glomerulus per field was included to examine the cortical lesion. The degree of the tubular injury was designated as score 0, 0 to 5% damaged tubules of the cortical tubules; score 1, involvement of >5 to 25% of the cortical tubules; score 2, involvement of >25 to 50% of the cortical tubules; score 3, involvement of >50 to 75% of the cortical tubules; score 4, extensive damage involving >75% of the cortical tubules.
Interstitial fibrotic area was assessed in kidney cortical sections on slides by staining with Masson’s trichrome. To examine the fibrotic area, a total of 10 randomly chosen, non-overlapping fields (×200 magnification) per kidney section were assessed semiquantitatively depending on the percentage of positive staining of the cortical area using a computer-assisted colour image analyser (analysis; Soft Imaging System GmbH, Münster, Germany).

Immunohistochemical analysis

Isolated kidney tissue was fixed by immersion in 4% paraformaldehyde and embedded in paraffin blocks. The tissue sections were cut and placed on glass slides, deparaffinized with xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min, the slides were rinsed with PBS and the samples were treated with pepsin at 42°C for 5 min.

After treatment with a blocking buffer, the samples on the slides were incubated overnight at 4°C with a primary antibody against fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution: 1:100), monocyte chemoattractant protein-1 (MCP-1) (Fitzgerald, Concord, MA, USA; dilution: 1:100), intercellular adhesion molecule-1 (ICAM-1) (Santa Cruz Biotechnology; dilution: 1:50), F4/80 (a 160 kD glycoprotein expressed in murine macrophages; Serotec Inc., Oxford, UK; dilution: 1:50), endothelial factor VIII (Chemicon international, Temecula, CA, USA; dilution: 1:100) or proliferating cell nuclear antigen (PCNA) (Fitzgerald; dilution: 1:100). The primary antibody was visualized using the Vectastain ABC-Elite peroxidase detection system (Vector Laboratories, Burlingame, CA, USA), followed by reaction with diaminobenzide as chromogen and counterstaining with haematoxylin (Sigma Chemical Co.). Sample evaluation was performed by an observer unaware of the sample origin. The number of F4/80- or PCNA-positive cells in each section was determined by counting positively stained cells in 10 × 400 fields per slide. To evaluate the immunostaining for fibronectin, ICAM-1, MCP-1 or endothelial factor VIII, a total of 10 randomly chosen, no overlapping fields (×200 magnification) per mouse were assessed using a computer-assisted colour image analyser (analysis; Soft Imaging System GmbH, Münster, Germany).

TUNEL assay

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated uridine triphosphate (dUTP) nick-end labelling (TUNEL) and the number of apoptotic cells, as defined by chromatin condensation or nuclear fragmentation (apoptotic bodies), were counted. Apoptosis was detected in the specimen using the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International) according to the manufacturer’s protocol. TUNEL-positive cells were counted in the cortical tubular cells at 10 × 400 fields per slide.

RT-PCR

The primers were designed by computer assistance according to the gene bank. TGF-β1: forward, 5′-GAGAGCCCTGGATACCAAATATTG-3′; reverse, 5′-GTGTGGTCCAGGCTCCAAAATAG-3′; the size of the amplified fragment is 173 bp. Internal control GAPDH: forward, TGCCACAGTCGAAGCTGAGA; reverse, CTCTGAGTGGCAGTGATGG; the size of the amplified fragment is 387 bp. Total RNA from each sample was isolated using the TRIzol solution (Gibco Inc., USA) and quantified with the ratio of absorption values of RNA samples at 260 nm and 280 nm. For each sample, 2 µg of total RNA was reverse transcribed into first strand of cDNA in a 20-µL reaction system at 37°C for 50 min. Then the polymerase chain reaction was performed from the synthesized cDNA in a 25-µL solution containing 4 µL of cDNA, 1 µL of 25 mmol primers (up-stream and down-stream) of TGF-β1, 0.5 µL of 25 mM primers (up-stream and down-stream) of GAPDH, 10 mM dNTP 1 µL, 25 mM MgCl₂ 4 µL, 10× buffer 5 µL and 1 µL of Taq DNA polymerase (Promega, Madison, WI, USA). Amplification was performed in a thermal cycler (MJ Research Co, USA) under the following conditions: 26 cycles of denaturation at 94°C for 10 s, annealing at 58°C for 30 s, extension at 72°C for 60 s, followed by a final extension for 5 min. PCR product (25 µL) was electrophoresed on a 1.5% agarose gel, and stained with ethidium bromide.

ELISA of COMP-Ang1 and TGF-β1

Blood samples 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 [12].

TGF-β1 levels in kidney tissue were determined by using ELISA kits (R&D Systems, Minneapolis, MN, USA). To activate latent TGF-β, protein was incubated with 1 N HCl at room temperature for 10 min. The acidified samples were neutralized by adding 1.2 N NaOH/0.5 M HEPES, and then assayed immediately. A standard curve was also constructed from standards provided by the manufacturer.

Western blot analysis

Samples were mixed with a sample buffer, boiled for 10 min, separated by 12% SDS–polyacrylamide gel electrophoresis under denaturing conditions and electroblotted to nitrocellulose membranes. Membranes were incubated with a blocking buffer containing 5% nonfat dry milk in the TBST buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20), and then incubated with an antibody against Smad 2/3 antibody (Cell Signaling Technology Inc., Beverly, MA, USA; dilution: 1:1000), or Smad 7 antibody (Cell Signaling Technology Inc; dilution 1:1000). Membranes were washed with phosphate-buffered saline (PBS) and incubated with a 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 an anti-actin antibody to verify equal loading of protein. Signals were analysed by densitometric scanning (LAS-3000, Fuji Film, Tokyo, Japan).
**COMP-Ang-1 in cyclosporine nephrotoxicity**

Fig. 3. Effect of COMP-Ang1 on tubulointerstitial morphology in CsA-induced renal injury. (A) Periodic acid-Schiff staining. COMP-angiopoietin-1 (COMP-Ang1) relatively preserved the normal architecture of the kidney and significantly reduced cyclosporine A (CsA)-induced tubulointerstitial injury. Arrows indicate epical blebbing, hyaline casts and tubular atrophy. (B) Quantitative analysis of tubulointerstitial injury. Note the significantly less tubulointerstitial injury in the CsA + COMP-Ang1 group compared to that in the CsA + LacZ group. VH, vehicle. *P < 0.05 versus the vehicle group; **P < 0.05 versus the CsA + LacZ group. Magnification: 400×.

**Measurement of maximal renal artery velocity and cortical blood flow**

The blood flow in the superficial renal cortex was assessed using a laser–Doppler flow probe (1.2-mm diameter, type N; Transonic Systems, Ithaca, NY, USA). Maximal renal artery velocity was measured with a 10- to 15-MHz linear transducer (ACUSON Sequoia C512; Siemens, Malvern, PA, USA).

**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 COMP-Ang1 on physiologic changes in the experimental groups**

Serum creatinine, BUN, CsA whole blood trough level and blood pressure were measured at Day 30 of the experiment. Treatment of CsA-treated mice with Ade-COMP-Ang1 did not affect the CsA whole blood trough level. Although CsA-induced hypertension was not induced in this model, blood pressure in the CsA-treated group was increased compared to that in the vehicle group. There was no significant difference in serum creatinine and BUN between vehicle, CsA + LacZ and CsA + COMP-Ang1 groups (Table 1).

**Effect of COMP-Ang1 on tubulointerstitial morphology in CsA-induced renal injury**

The kidneys of mice treated with CsA showed marked histological changes such as severe epical blebbing, hyaline casts, tubular atrophy, glomerular basement membrane thickening and tubulointerstitial fibrosis. In contrast, treatment with Ade-COMP-Ang1 relatively preserved the normal architecture of the kidney and markedly reduced CsA-induced tubulointerstitial injury (Figure 3A and B).

**Effect of COMP-Ang1 on tubulointerstitial fibrosis in CsA-induced renal injury**

Examination of the effect of COMP-Ang1 on renal fibrosis revealed that collagen deposition in the kidneys of CsA-treated mice is increased by 24% compared to that in the kidneys of vehicle-treated mice. Treatment of CsA-treated mice with Ade-COMP-Ang1 resulted in a significant reduction (~40%) of the collagen deposition compared to that in the kidneys of CsA-treated mice (Figure 4A and C). In addition, treatment with Ade-COMP-Ang1 significantly reduced fibronectin expression compared to that in the kidneys from CsA-treated mice (Figure 4B and D). These...
Table 1. Effect of COMP-Ang1 on physiologic changes in the experimental groups

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<th>Blood pressure (mmHg)</th>
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<th>BUN (mg/dL)</th>
<th>Serum Cr (mg/dL)</th>
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<td>VH</td>
<td>92 ± 5</td>
<td>0 ± 0</td>
<td>16.6 ± 2.6</td>
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<td>CsA + LacZ</td>
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<td>1698 ± 492</td>
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<td>CsA + COMP-Ang1</td>
<td>95 ± 6</td>
<td>1853 ± 299</td>
<td>17.2 ± 3.7</td>
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Values are expressed as mean ± SD. N = 5–8 in each group. *P < 0.05 versus the vehicle group.

VH, vehicle; CsA + LacZ, group received both CsA and Ade-LacZ; CsA + COMP-Ang1, group received both CsA and Ade-COMP-Ang1; CsA, cyclosporine A; BUN, blood urea nitrogen; Cr, creatinine.

Fig. 4. Effect of COMP-Ang1 on tubulointerstitial fibrosis in CsA-induced renal injury. (A) Masson's trichrome staining. Increased collagen deposition induced by CsA significantly decreased in the COMP-Ang1-treated kidney. Arrows indicate the fibrotic area. (B) Immunohistochemical findings of fibronectin protein. In parallel with the fibrotic area, COMP-angiopoietin-1 (COMP-Ang1) significantly reduced fibronectin expression in the cyclosporine A (CsA)-treated kidney. (C) Quantitative analysis of the fibrotic area. Note the significantly lower fibrotic area in the CsA + COMP-Ang1 group than in the CsA + LacZ group. (D) Quantitative analysis of fibronectin expression. Note that increased immunoreactivity of fibronectin protein in the CsA + LacZ group was decreased in the CsA + COMP-Ang1 group. VH, vehicle. *P < 0.05 versus the vehicle group; **P < 0.05 versus the CsA + LacZ group. Magnification: 400×.

results show that COMP-Ang1 ameliorated the CsA-induced renal fibrosis.

Effect of COMP-Ang1 on inflammation in CsA-induced renal injury

Immunohistochemical staining of MCP-1 protein showed no staining of MCP-1 in the kidneys from vehicle-treated mice, whereas strong staining in damaged tubules in the kidneys from CsA-treated mice was observed. Treatment of CsA-treated mice with Ade-COMP-Ang1 significantly reduced MCP-1 staining in the kidneys (Figure 5A and C). Immunostaining analysis of ICAM-1 revealed that ICAM-1 was expressed at very low levels in peritubular capillaries and the tubular brush border in the kidneys from the vehicle-treated mice. In contrast, the level of ICAM-1 expression was markedly increased in the interstitium of the kidneys from CsA-treated mice. The increased ICAM-1 expression in
Effect of COMP-Ang1 on MCP-1 and ICAM-1 in CsA-induced renal injury.

(A) Immunohistochemical findings of monocyte chemoattractant protein (MCP)-1 protein. COMP-angiopoietin-1 (COMP-Ang1) significantly reduced MCP-1 expression in the cyclosporine A (CsA)-treated kidney. (B) Immunohistochemical findings of intercellular adhesion molecule (ICAM)-1 protein. COMP-Ang1 significantly decreased ICAM-1 expression in the CsA-treated kidney. (C) Quantitative analysis of MCP-1 expression. The increased immunoreactivity of MCP-1 protein in the CsA + LacZ group was decreased in the CsA + COMP-Ang1 group. (D) Quantitative analysis of ICAM-1 expression. The increased immunoreactivity of ICAM-1 protein in the CsA + LacZ group was decreased in the CsA + COMP-Ang1 group. VH, vehicle. *P < 0.05 versus the vehicle group; **P < 0.05 versus the CsA + LacZ group. Magnification: 400×.

The interstitium of the kidneys from CsA-treated mice was significantly reduced by treatment with Ade-COMP-Ang1 (Figure 5B and D). Number of infiltrated renal macrophages, as assessed by F4/80-positive cells, was significantly increased in the interstitium of the kidneys from CsA-treated mice compared to that in the kidneys from vehicle-treated mice where a few of macrophages in the tubulointerstitium were found (Figure 6). Treatment of CsA-treated mice with Ade-COMP-Ang1 significantly decreased the number of infiltrated macrophages in the kidneys (5 ± 2 versus 13 ± 2 cells per high power field, P < 0.05).

Effect of COMP-Ang1 on TGF-β1, Smad 2/3 and Smad 7 in CSA-induced renal injury

As TGF-β1 is a key cytokine implicated in the pathogenesis of renal fibrosis, we examined whether treatment with Ade-COMP-Ang1 regulates renal TGF-β1 expression in CsA-treated mice. The level of renal TGF-β1 mRNA was markedly increased in CsA-treated mice compared to that in vehicle-treated mice. However, treatment with Ade-COMP-Ang1 significantly reduced the level of renal TGF-β1 mRNA increased by CsA treatment (Figure 7A). To corroborate this finding, we measured TGF-β1 production in kidney tissue by an immunoassay. Increased levels of total TGF-β1 protein seen in the kidney tissue of CsA-treated mice were significantly decreased in CsA-treated mice treated with Ade-COMP-Ang1 (Figure 7B). Levels of renal Smad 2/3 proteins were increased in CsA-treated mice compared to those in vehicle-treated mice. Treatment with Ade-COMP-Ang1 reduced significantly the increased levels of Smad 2/3 proteins in CsA-treated mice (Figure 7B). The level of renal Smad 7 protein was decreased in CsA-treated mice compared to that in vehicle-treated mice. Treatment with Ade-COMP-Ang1 increased significantly the decreased level of renal Smad 7 protein in CsA-treated mice (Figure 8).
Effect of COMP-Ang1 on macrophage infiltration in CsA-induced renal injury. (A) Immunohistochemical findings of F4/80-positive cells. COMP-angiopoietin-1 (COMP-Ang1) significantly reduced the increased number of macrophage in the cyclosporine A (CsA)-treated kidney. (B) Number of F4/80-positive cells in 10 × 400 fields. VH, vehicle. *P < 0.05 versus the vehicle group; **P < 0.05 versus the CsA + LacZ group. Magnification: 400×.

Effect of COMP-Ang1 on cortical blood flow and maximal renal artery velocity in CsA-induced renal injury

As the reduction in renal perfusion may lead to the development of tubulointerstitial fibrosis, we investigated the intrarenal haemodynamic effect of Ade-COMP-Ang1 on CsA-induced renal injury. Treatment with CsA resulted in a significant decrease (∼66%) in renal cortical blood flow compared to that in vehicle-treated mice. Treatment with Ade-COMP-Ang1 increased the reduced renal cortical blood flow in CsA-treated mice by 30% (Figure 9A). Maximal renal artery velocity of CsA-treated mice was significantly decreased compared to that in vehicle-treated mice (0.134 ± 0.02 versus 0.203 ± 0.03 m/s, P < 0.05). However, treatment with Ade-COMP-Ang1 markedly increased the reduced maximal renal artery velocity in CsA-treated mice (0.288 ± 0.04 versus 0.134 ± 0.02 m/s, P < 0.05) (Figure 9B).

Effect of COMP-Ang1 on peritubular endothelial cells in CsA-induced renal injury

We examined endothelial factor VIII staining to determine the correlation between renal peritubular vasculature and haemodynamic alteration in CsA-induced renal injury. Endothelial factor VIII expression was markedly decreased in the kidneys from CsA-induced renal injury. In contrast, treatment with Ade-COMP-Ang1 increased substantially the reduced endothelial factor VIII expression in CsA-treated mice (Figure 10).
Effect of COMP-Ang1 on Smad proteins in CsA-induced renal injury

Western blots and corresponding densitometric analyses of Smad 2/3 and Smad-7 protein. Consistent with the change of the transforming growth factor-β1 level, COMP-angiopoietin-1 (COMP-Ang1) administration decreased the increased Smad 2/3 levels in cyclosporine A (CsA)-treated kidneys, while increasing Smad 7 levels. VH, vehicle. *P < 0.05 versus the vehicle group; **P < 0.05 versus the CsA + LacZ group.

Effect of COMP-Ang1 on tubular cell apoptosis and proliferation in CsA-induced renal injury

To determine whether Ade-COMP-Ang1 protects the tubule from the CsA-induced apoptosis, TUNEL assay was performed. In consistent with the tubulointerstitial injury, tubular apoptosis was increased in the kidneys from CsA-treated mice while treatment with Ade-COMP-Ang1 significantly reduced tubular apoptosis (7 ± 3 versus 12 ± 2 cells per high power field, P < 0.05) (Figure 11A and C). To assess the regeneration of renal tubular cells by treatment with Ade-COMP-Ang1, we examined PCNA staining. PCNA-positive tubular cells were minimal in vehicle-treated mice (1 ± 1 cells per high power field). Treatment with Ade-COMP-Ang1 resulted in an increase in the number of PCNA-positive tubular cells (9 ± 2 cells per high power field) that were further decreased in the kidneys from CsA-treated mice (4 ± 2 cells per high power field) (Figure 11B and D). However, the number of PCNA-positive interstitial cells was decreased by treatment with Ade-COMP-Ang1 (4 ± 2 cells per high power field) compared to CsA treatment (6 ± 3 cells per high power field).

Discussion

In this study, we have demonstrated that COMP-Ang1 preserves renal microcirculation and reduces inflammation in the CsA-treated kidney, resulting in amelioration of CsA-induced renal injury. Tubular injury, interstitial fibrosis and fibronectin expression induced by CsA were significantly reduced by treatment with COMP-Ang1. COMP-Ang1 treatment markedly suppressed the expression of MCP-1 and ICAM-1, and monocyte/macrophage infiltration. COMP-Ang1 also decreased the levels of TGF-β1 and Smad 2/3, and increased Smad 7 levels in the kidneys from CsA-treated mice. CsA-induced reduction of maximal renal artery velocity and cortical blood flow were increased by treatment with COMP-Ang1. Moreover, treatment of CsA-treated mice with COMP-Ang1 markedly preserved peritubular capillary architecture that was disrupted by treatment with CsA. In addition, treatment with COMP-Ang1 significantly inhibited tubular cell apoptosis while increasing the number of proliferating tubular cells.

Peritubular capillary injury induces chronic hypoxia in the renal tubulointerstitium. Renal peritubular capillary dysfunction is an early event that contributes to tubulointerstitial damage. This in turn exacerbates renal fibrosis, which impairs oxygenation to tubular and interstitial cells. Therefore, it is very important to preserve peritubular capillary integrity and blood flow to prevent progressive renal disease [13]. We previously showed that COMP-Ang1 protects renal capillary endothelial cells in the unilateral ureteral obstruction fibrosis model [10]. In the present study, endothelial factor VIII positive cells were markedly decreased in CsA-treated kidneys compared to those in vehicle-treated kidneys. Treatment of CsA-treated mice with COMP-Ang1 increased expression of endothelial factor VIII in cortical peritubular capillaries of CsA-treated kidneys. Consistent with these findings, determination of maximal renal artery velocity and cortical blood flow revealed that COMP-Ang1 increased the reduced cortical microvascular blood flow caused by treatment with CsA. These findings suggest that COMP-Ang1 has a protective effect on the integrity of peritubular vasculature and intrarenal haemodynamics from the impairment caused by treatment with CsA.

Peritubular capillary injury induces chronic hypoxia in the renal tubulointerstitium. Renal peritubular capillary dysfunction is an early event that contributes to tubulointerstitial damage. This in turn exacerbates renal fibrosis, which impairs oxygenation to tubular and interstitial cells. Therefore, it is very important to preserve peritubular capillary integrity and blood flow to prevent progressive renal disease [13]. We previously showed that COMP-Ang1 protects renal capillary endothelial cells in the unilateral ureteral obstruction fibrosis model [10]. In the present study, endothelial factor VIII positive cells were markedly decreased in CsA-treated kidneys compared to those in vehicle-treated kidneys. Treatment of CsA-treated mice with COMP-Ang1 increased expression of endothelial factor VIII in cortical peritubular capillaries of CsA-treated kidneys. Consistent with these findings, determination of maximal renal artery velocity and cortical blood flow revealed that COMP-Ang1 increased the reduced cortical microvascular blood flow caused by treatment with CsA. These findings suggest that COMP-Ang1 has a preservative effect on the integrity of peritubular vasculature and intrarenal haemodynamics from the impairment caused by treatment with CsA.

Tubulointerstitial inflammation is the main feature of chronic CsA-induced renal injury that leads to fibrosis [14]. Infiltration of inflammatory cells into interstitium is a predominant pathologic finding in almost all forms of
progressive renal diseases. Chemokines and adhesion molecules activate, recruit and transmigrate inflammatory cells into the site of renal injury [15–17]. Infiltrating inflammatory cells are important sources of profibrotic molecules such as TGF-β1 [18]. We previously demonstrated that COMP-Ang1 exhibits an anti-inflammatory effect through inhibiting the Tie2/NF-κB pathway, which subsequently decreases activation of TGF-β1/Smad 2/3 signalling and renal fibrosis [10,11]. We also reported that Ang1 decreases VEGF-stimulated leukocyte adhesion to endothelial cells by reducing the expression of ICAM-1, VCAM-1 and E-selectin [6]. In this study, our results showed that COMP-Ang1 reduced the CsA-induced increase in the levels of MCP-1, ICAM-1 and F4/80-positive monocyte/macrophage influx. COMP-Ang1 administration also decreased the increased levels of TGF-β1 and Smad 2/3 in CsA-treated kidneys, while increasing Smad 7 levels. These findings indicate that COMP-Ang1 shows anti-inflammatory and antifibrotic effects in CsA-induced renal injury.

Tubular cell apoptosis is associated with CsA-induced tubular cell death, resulting in renal tubular atrophy and fibrosis [19]. It has also been suggested that CsA activates apoptosis-related genes associated with CsA-induced apoptotic cell death [20]. Consistent with the previous findings, our results showed that treatment with CsA increased TUNEL-positive tubular cells that are significantly reduced by treatment with COMP-Ang1. COMP-Ang1 also

Fig. 9. Effect of COMP-Ang1 on renal cortical blood flow and maximal renal artery velocity in CsA-induced renal injury. (A) Renal cortical blood flow. (B) Maximal renal artery velocity. Upper panels are the Doppler spectrum. Treatment with cyclosporine A (CsA) reduced maximal renal artery velocity and cortical blood flow compared to that of vehicle (VH) treatment. COMP-angiopoietin-1 (COMP-Ang1) increased the reduced cortical blood flow and maximal renal artery velocity in the CsA-treated group at Day 30 of experiment. N = 4 in each group. *P < 0.05 versus the vehicle group; **P < 0.05 versus the CsA + LacZ group.
Fig. 10. Effect of COMP-Ang1 on peritubular endothelial cells in CsA-induced renal injury. (A) Immunohistochemical findings of endothelial factor VIII protein. COMP-angiopoietin-1 (COMP-Ang1) treatment markedly increased the expression of endothelial factor VIII in the cyclosporine A (CsA)-treated group. (B) Quantitative analysis of endothelial factor VIII expression. Note the increased immunoreactivity of endothelial factor VIII protein in the CsA + COMP-Ang1 group was decreased in the CsA + LacZ group. VH, vehicle. ∗$P < 0.05$ versus the vehicle group; ∗∗$P < 0.05$ versus the CsA + LacZ group. Magnification: 400×.

Fig. 11. Effect of COMP-Ang1 on tubular cell apoptosis and proliferation in CsA-induced renal injury. (A) Immunohistochemical staining of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) assay. TUNEL-positive cells were increased by cyclosporine A (CsA) while COMP-angiopoietin-1 (COMP-Ang1) significantly reduced TUNEL-positive cells in the CsA + LacZ group. (B) Immunohistochemical staining of proliferating cell nuclear antigen (PCNA). PCNA-positive renal tubular cells increased in the CsA + LacZ group, but further increase was shown in the CsA + COMP-Ang1 group. (C) Quantitative analysis of TUNEL-positive cells. Note that increased number of TUNEL-positive cells in the CsA + LacZ group was decreased in the CsA + COMP-Ang1 group. (D) Quantitative analysis of PCNA-positive cells. Note that the number of PCNA-positive cells was further increased in the CsA + COMP-Ang1 group. VH, vehicle. ∗$P < 0.05$ versus the vehicle group; ∗∗$P < 0.05$ versus the CsA + LacZ group. Magnification: 400×.
increased the number of proliferating tubular cells. These results suggest that COMP-Ang1 inhibits tubular cell apoptosis while increasing tubular cell proliferation in CsA-induced renal injury.

In the present study, the mice model used allows only conclusions about CsA nephrotoxicity, which is a major limitation in this study. In kidney transplantation, the CsA effects are mediated via immunologic as well as non-immunologic mechanisms. The potential of COMP-Ang1 in prevention of renal function in kidney transplantation could be clarified using an allograft model in the future.

In summary, our results indicate that COMP-Ang1 exhibits a protective effect on damaged peritubular capillaries, haemodynamic alteration and inflammation in CsA-induced renal injury. In parallel with these protective effects, COMP-Ang1 decreases CsA-induced tubular apoptosis and tubulointerstitial fibrosis. On the basis of these findings, we suggest that COMP-Ang1 can be a strong candidate as a therapeutic and prophylactic agent for specific protection against endothelial dysfunction and inflammation.

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