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Inflammation Research

Renoprotective effects of combination of angiotensin converting enzyme inhibitor with mycophenolate mofetil in diabetic rats

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Abstract. *Objective and design:* Previously it was shown that blocking of the renin-angiotensin system (RAS) by angiotensin converting enzyme (ACE) inhibitors, or suppression of inflammatory responses by immunosuppressive drugs such as mycophenolate mofetil (MMF) could attenuate renal injury in diabetic rats. Whether RAS blockade combined with an immunosuppressive drug provides superior renoprotection against diabetic nephropathy has not been clearly delineated.
Materials: Diabetes was induced by injection of streptozotocin after uninephrectomy.
Treatment: Rats were randomly separated into five groups: control, diabetes, diabetes treated with enalapril (an ACE inhibitor, 10 mg/kg/d by gastric gavage), diabetes treated with MMF (10 mg/kg/d by gastric gavage), or diabetes treated with a combination of both agents and were followed for 8 weeks.
Methods: 24 h urinary albumin excretion rate (AER) was determined, renal injury was evaluated, immunohistochemistry for ED-1 macrophage marker, intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) were performed, and expression of transforming growth factor (TGF)-β1 protein was determined by Western blotting analysis.
Results: Diabetes was associated with a considerable increase in AER. Both enalapril and MMF retarded the increase in albuminuria, which was nearly completely abrogated by combination therapy. Increased glomerular volume and tubulointerstitial injury index in diabetic rats was attenuated by treatment with either enalapril or MMF and further reduced by the combination of the two. Elevated malondialdehyde levels in renal tissue were reduced by enalapril or MMF and, more effectively, by combined enalapril with MMF. Renal overexpression of ICAM-1 was not retarded by enalapril and attenuated by MMF or combined enalapril with MMF. Combination therapy was associated with a superior suppression of diabetes-induced macrophage recruitment and overexpression of MCP-1 and TGFβ1 compared to either monotherapy in renal tissue.
Conclusion: The combination of enalapril and MMF confers superiority over monotherapy in renoprotection, a mechanism which may be at least partly correlated with synergistic suppression of increased macrophage recruitment and overexpression of MCP-1 and TGF-β1 in renal tissue in diabetic rats.

Key words: Diabetes – Enalapril – Mycophenolate mofetil – Macrophage – Intercellular adhesion molecule-1 – Monocyte chemoattractant protein-1 – Transforming growth factor -β1

Introduction

Diabetic nephropathy is one of most common microvascular complications of diabetes mellitus and the leading cause of end-stage renal disease in developing countries [1]. Current treatment includes glycemic control, blood pressure control, with emphasis on agents targeting the renin-angiotensin system (RAS), a low-protein diet, and the use of hypolipidemic agents [2–5]. Although these therapeutic options may slow progression, the burden of disease remains large. Therefore, it is important to identify new strategies and additional therapeutic targets for treating diabetic nephropathy.

Diabetic nephropathy is generally considered a nonimmune disease, however, examination of human biopsies and animal models has shown the presence of accumulation of macrophages and overexpression of leukocyte adhesion molecules and chemokines in diabetic kidneys [6–10]. Macrophages that migrate into renal tissue could cause structural damage through the release of proinflammatory and profibrotic cytokines as well as reactive oxygen species (ROS) [11, 12]. A recent study demonstrated that the mechanism of renoprotection of blocking RAS with angiotensin converting enzyme (ACE) inhibitor may be at least partly correlated

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with anti-inflammatory effects via lowering overexpression of monocyte chemotactic protein-1 (MCP-1) in diabetic rats [13]. Mycophenolate mofetil (MMF), a drug used to prevent allograft rejection, suppresses macrophage infiltration and proliferation and suppresses the expression of intercellular adhesion molecule-1 (ICAM-1) in the experimental setting of renal ablation [14]. MMF has beneficial effects on the progression of glomerular injury in diabetic rats [15]. The renoprotective effects of MMF could not be attributed to a hemodynamic or metabolic effect, and partially resulted from its anti-proliferative and immunosuppressive properties. Mizoribine acts through similar mechanisms to MMF to cause immunosuppression, it was reported recently that mizoribine also provides treatment against renal injury in OLETF rats, a non-insulin-dependent diabetic model [16].

However, renal protection with either ACE inhibitor or MMF as monotherapy is suboptimal. A recent study has demonstrated that combined MMF and losartan (an angiotensin II receptor blocker, ARB) therapy resulted in greater reduction in macrophage infiltration, glomerulosclerosis, and interstitial expansion in the 5/6 ablation model [17]. Considering the individual renoprotective efficacy of MMF and ACE inhibitor and the fact that these agents act at distinct steps of the cascade leading to end-stage renal disease in diabetic state, we investigated whether simultaneous treatment with MMF and ACE inhibitor affords better renal protection than monotherapy with either drug alone. To test this hypothesis, combined MMF and enalapril (an ACE inhibitor) therapy was instituted in uninephrectomised diabetic rats.

Materials and methods

Animals

Adult male Munich-Wistar rats, with initial weights of 180 to 200g (Grade II, Certificate No 01) were obtained from Experimental Animal Center of Anhui Medical University. The research protocol was in accordance with the principles approved by the animal ethics committee of Anhui Medical University. Animals were housed at a temperature of 22±1 °C and humidity of 65 %–70 %, and were submitted to a 12 h light/dark cycle, and allowed free access to standard laboratory chow (Experimental Animal Center of Anhui Medical University, Hefei, China) and tap water.

Experimental protocol

All rats were initially subjected to removal of the right kidney under anesthesia by intraperitoneal injection of sodium pentobarbital (Haerbin Pharmaceutical Factory, Haerbin, China) at a dose of 50 mg/kg to hasten the development of diabetic nephropathy as described previously [18, 19], and were rendered diabetic two weeks later by intraperitoneal injection of streptozotocin (Sigma Chemical Co., St. Louis, Mo, USA) at a dose of 65 mg/kg, diluted in citrate buffer 0.1 mol/L (pH 4.0). Two days later, the diabetic state was confirmed by measurement of tail blood glucose (BG) levels using a reflectance meter (one touch II, lifescan LTD, China). Diabetic rats received subcutaneous injections of long-acting insulin (Protophane, Novo Nordisk Pharmaceuticals, Bagsvaerd, Denmark), in doses adjusted individually (ranging from 1 to 4 units/day) to maintain BG levels between 200 and 400 mg/dl, and to avoid ketonuria. BG levels were measured twice a week. Five experimental groups were studied: non-diabetic uninephrectomised rats (group C, $n = 10$), uninephrectomised rats made diabetic (group DM, $n = 10$), diabetes treated with enalapril (10 mg/kg/d by gastric gavage, group DM+E, $n = 10$), diabetes treated with MMF (10 mg/kg/d by gastric gavage, group DM+MMF, $n = 10$), or diabetes treated with a combination of both agents (group DM+E+MMF, $n = 10$). The dose of ACE inhibitor was chosen to be antialbuminuric effect, and effects with the maximal dose of enalapril were not examined, so that effects of the maximal dose of enalapril alone was not compared with combination of enalapril with MMF in the present experiments. In preliminary experiments, the effects of different concentrations of MMF (5 to 20 mg/kg/d) on albuminuria in diabetic rats were compared. MMF at dose 10 mg/kg/d exhibited better effect than 5 mg/kg/d but similar effects at 20 mg/kg/d, 10 mg/kg/d was used in this study. Enalapril (Merck&Co. Inc., Rahway, NJ, USA) was dissolved in the drinking water, MMF (Roche, Brussels, Belgium) was dissolved in dimethylsulfoxide (5 %) and then in olive oil.

Metabolic parameter and tissue collection

After eight weeks, body weight was measured at the conclusion of the experiment. Rats were then anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and placed on a temperature-regulated table, the right jugular artery was catheterized, this arterial catheter was used for blood sampling. BG levels were determined with autoanalyzer (Beckman Instruments, Palo Alto, CA, USA). The kidneys were perfused in vivo via the abdominal aorta with 100 ml of normal saline at 4 °C, while the left renal vein was punctured to permit the perfusate to drain, the kidneys were removed immediately and sectioned midfrontally into two pieces. One fragment was fixed in 10 % formalin and processed in paraffin for subsequent histologic assessment and immunohistochemistry studies, while the other was stored at –80 °C for evaluation of malondialdehyde (MDA) level and Western blotting analysis.

Urinary albumin excretion rate

Prior to sacrifice, animals were placed in metabolic cages for collection of urine over 24 h for measurement of albumin concentration. Urinary albumin concentrations were measured by enzyme-linked immunoabsorbent assay using an anti-rat albumin antibody (Nephrat, Exocell, Philadelphia, Pa., USA) and 24 h urinary albumin excretion rate (AER) was calculated by multiplying the urinary protein excretion by 24 h urine volume.

Oxidative stress

Oxidative stress was studies by determination of renal content of MDA. MDA were measured by using commercially available kits according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Results in renal MDA levels were expressed as nmol MDA per milligram protein (nmol/mg prot). The protein content was determined according to Bradford's method [20], with bovine serum albumin used as a standard. To allow a comparison of the combination of the drugs to the control group, results were shown as relative to control (percent of control).

Renal pathology

Formalin-fixed kidney sections (2 μm) were stained with periodic acid-schiff (PAS) reagent to identify kidney structure and hematoxylin to distinguish cell nuclei. Digital images of glomeruli and interstitial areas were obtained from microscopy (magnification, ×400). The glomerular cross-sectional area (A_G) was measured in 50 glomerutar profiles per rat by using computerized image analysis system (Beijing Aeronautic and Aerospace University, Beijing, China). The glomerular volume (V_G) was then calculated as: $V_G = \beta/K[A_G\, I^{3/2}$, where $\beta = 1.38$ is the size distribution coefficient and $K = 1.1$ is the shape coefficient for glomeruli

idealized as a sphere [21]. Tubulointerstitial area in the cortex was evaluated and graded as: 0, normal; 1, the area of interstitial inflammation and fibosis, tubular atrophy and dilation with cast formation imvolving <25% of the field; 2, lesion area between 25 % and 50 % of the field; and 3, lesions involving >50 % of the field. The indices for tubulointerstital injury (TII) were calculated by averaging the grades assigned to all tubule fields [22]. All measurements and scoring were performed on blinded slides.

Immunohistochemistry

Immunostaining of ED-1 (macrophage marker, equal to CD68 in the human), MCP-1 and ICAM-1 in renal tissue sections was conducted using the streptavidin-biotin-peroxidase complex (SABC) method. The primary antibodies that were used included a monoclonal mouse anti-rat ED-1 antibody (a monoclonal IgG_1 antibody to cytoplasmic antigen present in macrophages, Serotec, Oxford, UK; diluted 1: 50), a polyclonal rabbit anti- MCP-1 antibody (Santa Cruz Biotechnology, CA, USA; diluted 1: 400) and a polyclonal rabbit anti-ICAM-1 antibody (Santa Cruz Biotechnology, CA, USA; diluted 1: 200). Three μm paraffin sections of kidneys were microwave-treated at 800 w for 12 min in 0.1 mol/L sodium citrate (pH 6.0) and incubated for 20 min with 0.6 % H_2O_2 followed by avidin and biotin block and 20 % normal sheep serum to prevent non-specific detection. Sections were then incubated overnight at 4 °C with primary antibody in 1 % bovine serum albumin. After washing in phosphate-buffered saline (PBS), sections were incubated with biotinylated goat antibodies (1: 200) for 1 h followed by SABC solution and developed with 3.3-diaminobenzidine to produce a brown color. Normal IgG was used as a negative control in the staining. Sections were then counterstained with Mayer's hematoxylin and examined by two independent observers blinded to the disease status of the animal. Quantitative analysis of ED-1-positive cells in glomeruli was performed under ×400 magnification and expressed as cells/glomerular cross section (gcs), for each section, 50 sequential glomerular profiles were examined. ED-1-positive cells in tubulointerstitium were counted in 25 consecutive high power (×400) interstitial fields by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscopy and expressed as cells/mm^2 [23]. Immunostaining of MCP-1 and ICAM-1 in glomeruli was evaluated using the following semiquantitative scale: 0 = diffuse, very weak or absent staining; 1 = staining involving less than 25 %; 2 = staining involving 25 to 50 %; 3 = staining involving 50 % to 75 % and 4 = staining involving 75 % to 100 % [24]. Immunostaining of MCP-1 and ICAM-1 in tubulointerstitium was quantified using computerized image analysis system (Beijing Aeronautic and Aerospace University, Beijing, China) by evaluating the positively stained area of the sections under the same light intensity for microscopy [25]. All scoring was performed on blinded slides. To allow a comparison of the combination of the drugs to the control group, all results were shown as relative to control (percent of control).

Western blotting analysis

Kidney samples were homogenized in lysis buffer [PBS, 1 % nonidet P-40 (NP-40), 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 100 ug/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), Sodium orthovanadate] at 4 °C throughout all procedceres, and sonicated for 70 s, then add 300 μg PMSF per gram of tissue and incubate on ice for 30 min, followed by centrifugation at 15,000 rpm for 20 min at 4 °C. The protein content was determined according to Bradford's method [20], with bovine serum albumin used as a standard. Protein samples (30 μg) were boiled with 2 × sample buffer containing 5 % β-mercaptoethanol for 5 min, separated by size on 15 % polyacrylamide gel under SDS denaturing conditions, and transferred to a nitrocellucose membrane at 90 V for 2 h. The nitrocellulose membranes were stained with ponceau S to assess the efficiency of transfer. Non-specific binding was blocked by incubation in block buffer (5 % non-fat dry milk, 0.05 % Tween-20, 1 × tris□Cl-buffered saline) overnight at 4 °C, The membranes were hybridized with a 1: 1000 dilution of polyclonal rabbit anti-rat TGF-β1 (Santa Cruz Biotechnology, CA, USA), then incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG (1: 500). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Life Science, Little Chalfont, UK). Housekeeping protein β-actin was used as a loading control. Positive immunoreactive bands were quantified densitometrically (Leica Q500IW image analysis system) and expressed as ratio of TGF-β1 to β-actin in optical density units. To allow a comparison of the combination of the drugs to the control group, results were shown as relative to control (percent of control).

Statistical Analysis

Data were expressed as the means±SEM unless otherwise specified. One-way analysis of variance (ANOVA) with pairwise comparisons according to the Tukey method was used in this study. Because of a positively skewed distribution, urinary albumin excretion rate (AER) was logarithmically transformed before statistical analysis and expressed as the geometric mean ×/÷ tolerance factor. Differences were considered significant if the P value was less than 0.05.

Results

Clinical and metabolic parameters

Rats in DM group had reduced body weight gain and increased blood glucose level. No effects on body weight and blood glucose were observed with monotherapy treatment or enalapril and MMF combination. Kidney enlargement was

Table 1. Clinical and metabolic parameters in rats after treatment with enalapril, MMF or combined enalapril and MMF[a]

Group	Dose (mg/kg)	BG (mg/dl)	BW (g)	KW (g)	KW/BW (mg/g)	AER[b] (mg/24h)
C		107.33 ± 7.48	358.75 ± 22.02	1.81 ± 0.07	4.20 ± 0.42	0.48 ×/÷ 1.3
DM		401.96 ± 22.43**	261.11 ± 22.81*	2.29 ± 0.12*	9.38 ± 0.84**	1.26 ×/÷ 1.1**
DM+E	10	356.87 ± 24.59	300.87 ± 15.39	1.98 ± 0.10$^{\#}$	6.09 ± 0.36$^{\#}$	0.86 ×/÷ 1.1$^{\#}$
DM+MMF	10	396.98 ± 28.12	287.52 ± 29.72	1.93 ± 0.09$^{\#}$	6.98 ± 0.59$^{\#}$	0.74 ×/÷ 1.2$^{\#}$
DM+E+MMF	both agents	355.83 ± 25.67	280.94 ± 24.89	1.81 ± 0.08$^{\#\dagger}$	5.31 ± 0.43$^{\#\#\dagger}$	0.52 ×/÷ 1.2$^{\#\#\dagger}$

[a] Values are presented as means±SEM from 10 rats in each group.
[b] Because of a positively skewed distribution, AER was logarithmically transformed before statistical analysis and expressed as the geometric mean ×/÷ tolerance factor. Abbreviations are: BG, blood glucose; BW, body weight; KW, kidney weight; KW/BW, kidney weight/body weight; AER, albumin excretion rate. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* C, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* DM, $^{\dagger}p < 0.05$ *vs.* DM+E and DM+MMF.

observed in DM group, which was significantly reduced by treatment with enalapril or MMF and further lowered by the combination of the two. In DM group, albuminuria was significantly increased when compared to C group, treatment with enalapril or MMF attenuated the increase in albuminuria in the diabetic rats, but this level was still higher than that observed in control rats. The combination of enalapril and MMF was associated with a further reduction in albuminuria than was seen with either drug administrated alone, the similar AER level to that observed in control animals (Table 1). Treatment with MMF alone or in combination with enalapril did not affect SGOT, SGPT, and survival of diabetic rats (data not shown).

Renal histology

Rats in DM group had an increase in the glomerular volume when compared with the values in C group. Enalapril or MMF treatment ameliorated the increase of the glomerular volume, and the combination of the two was associated with further reduction in glomerular volume (Fig. 1, 2A). Rats in DM group had an increase in TII when compared to C group, enalapril or MMF treatment was associated with a significant reduction in TII as compared with DM group, the combination of the two was associated with a further reduction in TII as compared with DM group (Fig. 1, 2B).

Oxidative stress

The renal content of MDA was higher in DM group than that in C group, enalapril or MMF treatment was associated with a significant reduction in the MDA content of the kidney. The combination of enalapril and MMF was associated with a further reduction in content of MDA than was seen with either drug administrated alone (Fig. 3).

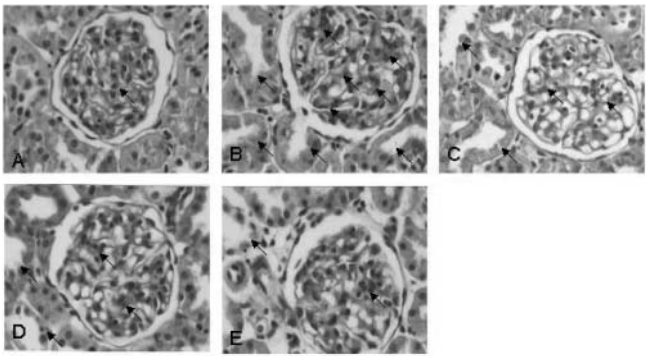

Fig. 1. A representative microphotograph of kidney tissue stained with Periodic acid-Schiff (arrowheads) from C (A), DM (B), DM+E (C), DM+MMF (D) and DM+E+MMF (E) rats. C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Original magnification × 400

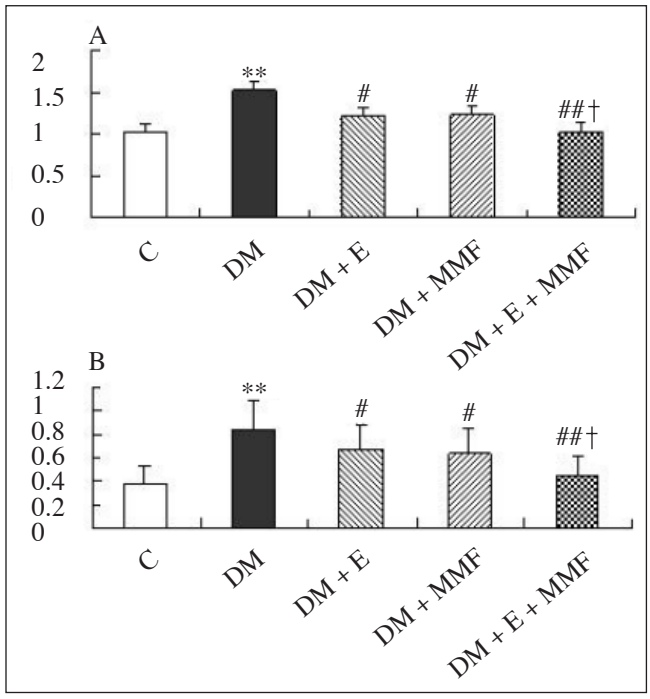

Fig. 2. Glomerular volume (A) and tubulointerstitial injury indices (B) in C, DM, DM+E, DM+MMF and DM+E+MMF rats. C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Values are presented as means ±SEM from 10 rats in each group. $^{**}p < 0.01$ *vs*. C; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. DM; $^{\dagger}p < 0.05$ *vs*. DM+E and DM+MMF.

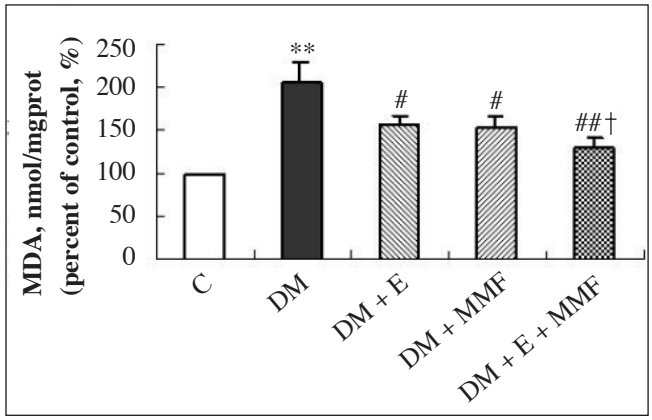

Fig. 3. MDA content in renal tissue in C, DM, DM+E, DM+MMF and DM+E+MMF rats. C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Values are presented as means ± SEM from 10 rats in each group. $^{**}p < 0.01$ *vs*. C; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. DM; $^{\dagger}p < 0.05$ *vs*. DM+E.

Renal macrophage infiltration

ED-1-positive cells (representing macrophage) infiltrated the glomeruli of DM group at eight weeks. ED-1-positive cells infiltrating the periglomerular and tubulointerstitial

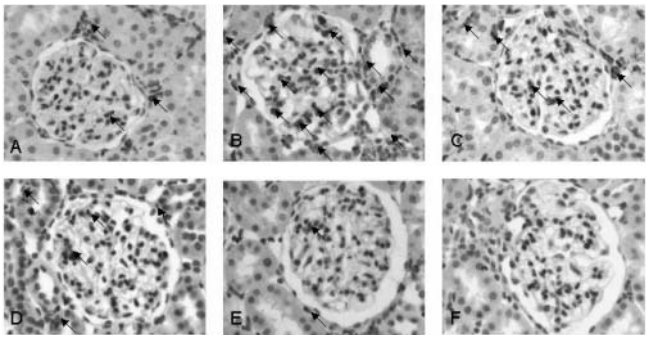

Fig. 4. A representative microphotograph of immunostaining (arrowheads) for ED-1 in renal tissue from C (A), DM (B), DM+E (C), DM+MMF (D) and DM+E+MMF (E) rats. Negative control (F). C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Original magnification × 400.

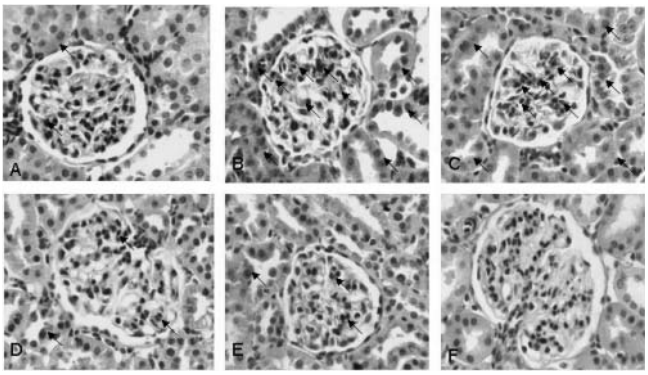

Fig. 6. A representative microphotograph of immunostaining (arrowheads) for ICAM-1 in renal tissue from C (A), DM (B), DM+E (C), DM+MMF (D) and DM+E+MMF (E) rats. Negative control (F). C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Original magnification × 400

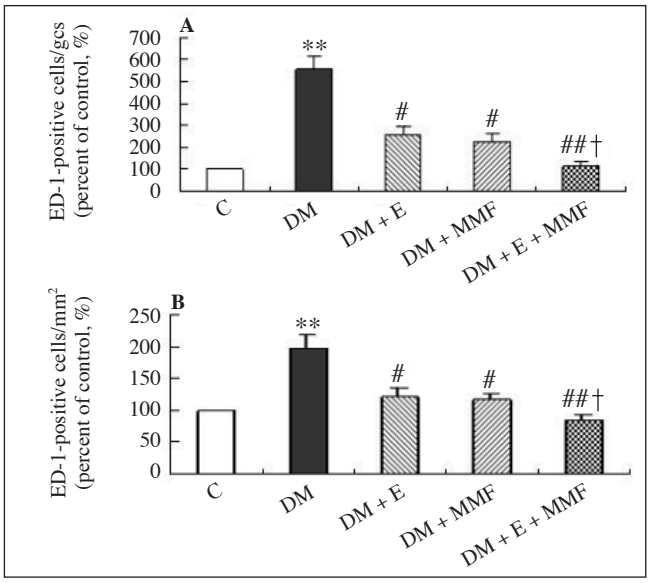

Fig. 5. ED-1-positive cells in glomeruli (A) and tubulointerstitium (B) in renal tissue in C, DM, DM+E, DM+ MMF and DM+E+MMF rats. C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Values are presented as means ±SEM from 10 rats in each group. $^{**}p < 0.01$ *vs.* C; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* DM; $^{\dagger}p < 0.05$ *vs.* DM+E and DM+MMF.

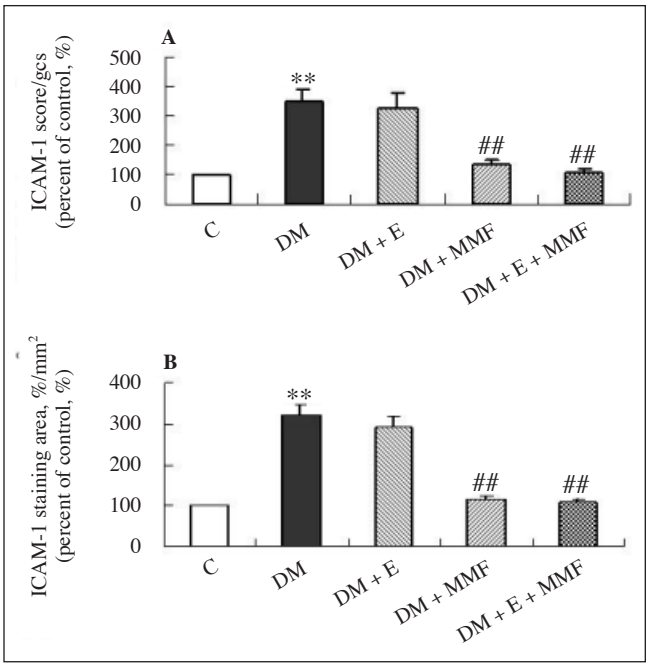

Fig. 7. The score of ICAM-1 (A) in glomeruli and immunostaining area of ICAM-1 (B) in tubulointerstitium in renal tissue in C, DM, DM+E, DM+MMF and DM+E+MMF rats. C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Values are presented as means ± SEM from 10 rats in each group. $^{**}p < 0.01$ *vs.* C; $^{\#\#}p < 0.01$ *vs.* DM.

areas was also observed in DM group. ED-1-positive cell infiltration into the glomeruli and tubulointerstitial areas was suppressed by enalapril or MMF at eight weeks of treatment and further reduced by combination of enalapril and MMF (Fig. 4, 5 A and B).

Renal ICAM-1 and MCP-1 Expression

ICAM-1 protein immunostaining was observed in glomeruli and, to a lesser degree, tubulointerstitium in C group. Immunostaining for ICAM-1 was increased in DM group in glomeruli and tubulointerstitium and reduced by treatment with MMF. However, treatment with enalapril has no effect on overexpression of ICAM-1, the combination of enalpril and MMF did not result in further reduction in renal ICAM-1 expression (Fig. 6, 7A and B). In the kidneys of C group,

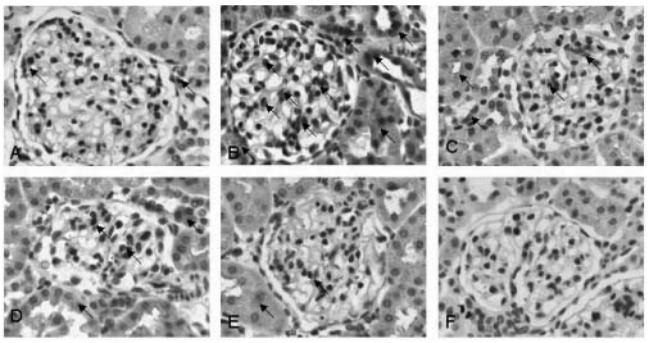

Fig. 8. A representative microphotograph of immunostaining (arrowheads) for MCP-1 in renal tissue from C (A), DM (B), DM+E (C), DM+MMF (D) and DM+E+MMF (E) rats. Negative control (F). C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Original magnification × 400.

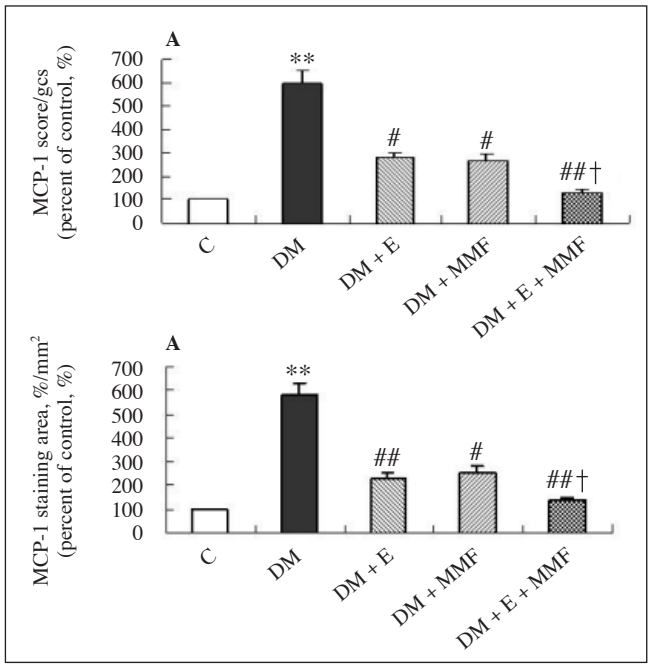

Fig. 9. The score of MCP-1 (A) in glomeruli and immunostaining area of MCP-1 (B) in tubulointerstitium in renal tissue in C, DM, DM+E, DM+MMF and DM+E+MMF rats. C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Values are presented as means ± SEM from 10 rats in each group. $^{**}p < 0.01$ *vs.* C; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* DM; $^{\dagger}p < 0.05$ *vs.* DM+E and DM+MMF.

there was minimal staining for MCP-1, diabetes was associated with a prominent increase in renal MCP-1 immunostaining, which was seen in glomeruli and tubulointerstitium, enalapril and MMF treatment reduced overexpression of MCP-1 in glomeruli as well as in tubulointerstitium, which was nearly completely abrogated by combination therapy (Fig. 8, 9A and B).

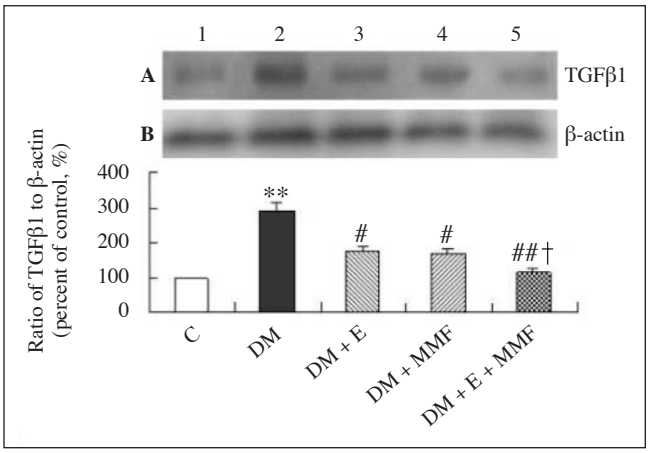

Fig. 10. Western blot analysis of TGF-β1 protein (A) and densitometric analysis (B) in renal tissue in C, DM, DM+E, DM+MMF and DM+E+MMF rats. Lane 1 shows control; lane 2 shows DM; lane 3 shows DM+E; lane 4 shows DM+MMF; lane 5 shows DM+E+MMF. C, non-diabetic uninephrectomised rats; DM, uninephrectomised rats made diabetic; DM+E, diabetes treated with enalapril; DM+MMF, diabetes treated with MMF; DM+E+MMF, diabetes treated with a combination of both agents. Values are presented as means ± SEM from 10 rats in each group. $^{**}p < 0.01$ *vs.* C; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$*vs.* DM; $^{\dagger}p < 0.05$ *vs.* DM+E and DM+MMF.

Renal TGF-β1 expression

Western blotting analysis noted that an increase in the amount of immunoreactive peptide was seen in kidney for DM group rats compared to that from C group rats. Densitometric analysis of the Western blotting showed a 2.92 fold increase in the amount of TGF-β1 from DM group rats with respect to C group rats, treatment with enalapril or MMF as well as combination of the two could reduced TGF-β1 protein expression by approximately 39.73 %, 42.47 % and 60.27 %, respectively (Fig. 10 A and B).

Discussion

Our present study shows that the combination of an agent that interrupts the RAS with one that possesses immunosuppressive effect provide superior renoprotection in a model of experimental diabetes than either agent alone. This has been shown for a range of functional and structural parameters including albuminuria, glomerular and tubulointerstitial injury. In the present study, increased macrophage infiltration in glomeruli and tubulointerstitium as shown by ED-1-positive cells is found to correlate with glomeruli hypertrophy and tubulointerstitial injury. Our study demonstrates that enalapril and MMF treatment alone can only partially inhibit recruitment of macrophage in diabetic rats, combination of the two nearly completely reduce recruitment of macrophage.

The precise mechanism by which combined MMF with enalapril therapy more effectively prevent macrophage infiltration are not known. In the present study, we show that immunostaining for ICAM-1 and MCP-1 is increased in dia-

betic rats in glomeruli and tubulointerstitium and reduced by treatment with MMF, and enalapril treatment is associated with a significant decrease in MCP-1 expression, and has no effect on overexpression of ICAM-1. Combination therapy is associated with a superior suppression in diabetes-induced overexpression of MCP-1 compared to either monotherapy in renal tissue, the combination of enalapril and MMF can not result in further reduction in renal ICAM-1 expression. The decreased expression of ICAM-1 presented by MMF-treated rats might be explained by the inhibitory action of MMF on adhesion molecule synthesis. MMF is the morpholinoethyl ester of mycophenolic acid (MPA), MPA depletes guanosine triphosphate pools, inhibiting fucose and mannose transfer to membrane glycoproteins, including adhesion molecules [26, 27]. The mechanisms leading to the up-regulation of MCP-1 in various types of renal injury, including diabetic nephropathy, have yet to be fully understood. However, human and rodent mesangial cells can synthesize MCP-1 in response to several factors that are thought be involved in glomerular and tubulointerstitial injury, such as interleukin-1, TNF-α, and low-density lipoprotein. In the case of human mesangial cells, high concentration of glucose as well as advanced glycation end–products (AGEs) have been reported to promote MCP-1 production. An additional influence that may induce synthesis of MCP-1 is the generation of ROS. A growing body of study have shown that oxidant stress is increased in clinical and experimental diabetic nephropathy [28, 29], and our present study, in addition to confirming their findings, have demonstrated that elevated MDA level in renal tissue is reduced by enalapril or MMF and, more effectively, by combined enalapril with MMF in diabetic rats. This effect of combined enalapril with MMF is likely the result of suppression of the inflammatory infiltrate, but by itself, a reduction in ROS would lower the expression of MCP-1.

TGF-ß1 has been consistently implicated as playing a pivotal role in the pathogenesis of diabetic nephropathy [30]. Macrophage may produce TGF-β1, recruiting additional inflammatory cells that may contribute to the propagation of inflammation [31–33]. RAS blockade with either an ACE inhibitor or ARB significantly reduces the expression of TGF-β1 in progressive renal disease, including diabetic nephropathy [34, 35]. In the present study, as glomerular and tubulointerstitial expansion increase and proteinuria progress, the expression of TGF-ß1 is markedly up-regulated, overexpression of TGF-β1 observed in diabetic kidneys is attenuated by enalapril or MMF to a similar lever and further reduced by the combination of the two. These data suggests that both enalapril and MMF inhibit glomerular and tubulointerstitial expansion by controlling renal overexpression of TGF-ß1 in diabetic rats. Also, our studies show that the combination of enalapril and MMF confer superiority over monotherapies on the expression of TGF-ß1. Diabetic state promoted TGF-ß1 production not only by accumulating macrophage but also by renal tubular epithelial cells and mesangial cells themselves. Increased ROS generation, protein kinase C activation, angiotensin II in association with increased glucose metabolism and hemodynamic disorder are considered to be the main upstream signaling molecules of diabetic-induced renal injury [29, 36–39]. In addition, macrophage also produce various types of cytokines and growth factor including TGF-ß1, and they induce tissue injury [40]. Recent studies have reported that the depletion of leukocytes by irradiation decreased the gene expression of TGF-ß1 and type IV collagen in the glomeruli of diabetic rats at 4 weeks after induction of diabetes [6]. MCP-1 has been reported to mediate collagen deposition due to TGF-ß1 in experimental glomerlonephritis, the up-regulatation of MCP-1 and TGF-ß1 could be a common pathway involved in the development of diabetic nephropathy [41]. Because combination therapy is correlated with synergetic suppression on increased macrophage recruitment and overexpression of MCP-1 in renal tissue in diabetic rats, the production of TGF-ß1 may have more effectively been inhibited, resulting in prevention of diabetic nephropathy progression.

The magnitude of tubulointerstitial injury is an important prognostic marker of renal outcome in many forms of renal disease [42–44]. In human diabetic nephropathy, the extent of interstitial fibrosis is strongly associated with mesangial expansion, falling glomerular filtration rate, and increasing proteinuria [45]. In experimental diabetic nephropathy, investigation has focused almost exclusively on the glomeruli and particularly on the mesangial cell, although tubulointerstitial disease also develops in the streptozotocin model [44]. Accumulation of extracellular matrix (ECM), first recognized as thickening of capillary basement membranes, was a characteristic pathologic feature of diabetes and was present in tubulointerstitium as well as glomeruli [46]. In the present study, enalapril or MMF treatment is associated with a significant reduction in tubulointerstitial injury index as compared with group DM, the combination of the two is associated with a further reduction in tubulointerstitial injury index as compared with group DM. Our study show that there is a clear-cut evidence of a superiority of combination therapy on renal structural parameters, especially on the tubulointerstitial injury index.

Potential toxic effects of MMF alone or in combination with enalapril should also be investigated in detail, the mean body weight of MMF alone or in combination with enalapril is slightly higher, although it is not statistically different, than that of diabetic rats. Data from the present study provide that MMF alone or in combination with enalapril do not affect survival, SGOT, and SGPT in diabetic rats.

In conclusion, the combination of enalapril and MMF confers superiority over monotherapy on renoprotection, which mechanism may be at least partly correlated with synergetic suppression on increased oxidative stress and macrophage recruitment as well as overexpression of TGF-β1 in renal tissue. Combination of ACE inhibitor and MMF could have a novel therapeutic value for slowing or preventing the progression of diabetic nephropathy.

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