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Nephrin and podocin loss is prevented by mycophenolate mofetil in early experimental diabetic nephropathy

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ABSTRACT

Several works in the setting of early experimental diabetic nephropathy using anti-inflammatory drugs, such as mycophenolate mofetil (MMF), have shown that prevention of the development or amelioration of renal injury including proteinuria. The exact mechanisms by which anti-inflammatory drugs lower the albuminuria have no still to clarify well. In this study, diabetes was induced by injection of streptozotocin after uninephrectomy. Rats were randomly divided into three groups: control group, diabetic group and diabetic group treated with MMF. Elevated 24 h urinary albumin excretion rate was markedly attenuated by MMF treatment. In diabetic rats receiving no treatment, there were increase in ED-1+ cells in the glomeruli, which were effectively suppressed by MMF treatment. The expression of nephrin and podocin protein was reduced in the glomeruli from diabetic rats, and MMF treatment significantly increased the expression of nephrin and podocin. The expression of IL-1, TNF-α and 3-NT protein in the glomeruli were significantly increased in diabetic rats, which were all significantly inhibited by MMF treatment. Our results show that MMF could decrease urinary albumin excretion, which mechanism may be at least partly correlated with upregulated expression of nephrin and podocin in the glomeruli of diabetic rat.

1. Introduction

Diabetic nephropathy has become one of the main cause of end-stage renal disease [1,2]. Experimental and clinical evidence suggests that inflammation plays a role in the pathogenesis of diabetic nephropathy, in addition to, or in concert with the associated hemodynamic and metabolic changes [3–6]. Proteinuria is not only a cardinal manifestation of glomerular injury in diabetes, but also an important pathogenetic factor in the progression of renal dysfunction [7]. Recently, we and others in the setting of early experimental diabetic nephropathy using different drugs, such as mycophenolate mofetil (MMF), methotrexate, and erythromycin, have shown that prevention of the development or amelioration of renal injury including proteinuria in diabetes is associated with anti-inflammatory actions [8–11]. However, the exact mechanisms by which anti-inflammatory drugs lower the albuminuria have no still to clarify well.

The glomerular vasculature consists of three structures that act in concert to prevent the development of albuminuria and proteinuria. These structures are the fenestrated endothelium, the glomerular basement and the epithelial slit diaphragm. Several studies have addressed the mechanisms involved in the loss of glomerular permselectivity in patients with diabetes. Many of them focused on the role of glomerular basement membrane components with particular regard to glomerular anionic sites [12,13]. However, the central role of the podocyte slit diaphragm in maintaining the size-selective barrier has been outlined by recent studies on different slit diaphragm-associated proteins, such as nephrin, CD2-associated protein, podocin, and α-actinin-4 [14]. Recently, several experimental reports showed the presence of altered nephrin and podocin expression in different models of diabetic nephropathy, such as rats with streptozotocin (STZ)-induced diabetes and non-obese diabetic mice [15–17].

More recently, the study *in vitro* indicated that macrophages as well as macrophage-derived cytokines markedly suppressed activity of the nephrin gene promoter and thereby repress expression of its protein, revealed that inflammation play a crucial role in the injury of podocytes. Therefore, the aim of this study was to test the hypothesis that anti-inflammatory drugs such as MMF attenuates proteinuria through prevention loss of nephrin and podocin in early experimental diabetic nephropathy.

2. Materials and methods

2.1. Reagents

MMF was provided by Roche Pharmaceuticals Inc. (Brussels, Belgium). STZ was purchased from Sigma Chemical Co (St. Louis, Mo, USA). Microalbumin assay kit was purchased from Exocell

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Inc (Philadelphia, Pa., USA). Rabbit anti-interleukin (IL)-1, anti-tumor necrosis factor (TNF)-α, nephrin and podocin polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-CD68-recognizing rat macrophages (ED-1) was obtained from Serotec (Oxford, UK). Anti-3-nitrotyrosine (3-NT) was purchased from Upstate Technology (Lake Placid, NY, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG as well as fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were Boster Biotechnology (Wuhan, China). Chemiluminescence kit was from Amersham Life Science (Little Chalfont, UK).

2.2. Animals

Adult male Munich–Wistar rats, with initial weights of 180–200 g (Grade II, Certificate No. 01) were obtained from the 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 and tap water.

2.3. Experimental protocol

All rats were initially subjected to removal of the right kidney under anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg) to hasten the development of diabetic nephropathy as described previously, and were rendered diabetic two weeks later by intraperitoneal injection of STZ at a dose of 65 mg/kg, diluted in citrate buffer 0.1 M (pH 4.0). Two days later, the diabetic state was confirmed by measurement of the tail blood glucose level using a reflectance meter (one touch II, lifescan LTD, China). Diabetic rats received daily injections of long-acting insulin, in doses adjusted individually (ranging from 1 to 4 U), to maintain blood glucose levels between 200 and 400 mg/dl and to avoid ketonuria. Blood glucose levels were measured twice a week. Three experimental groups were studied: non-diabetic uninephrectomised rats (C group, $n = 10$), uninephrectomised rats made diabetic (DM group, $n = 10$) and diabetic rats treated with MMF (DM + MMF group, $n = 10$). MMF (10 mg/kg) was orally administrated once a day for 8 weeks. MMF was dissolved in dimethylsulfoxide (5%) and then in olive oil.

2.4. Metabolic parameters and tissue collection

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 and used for blood sampling. Blood glucose levels were determined with a glucose analyzer. The kidney was perfused *in vivo* via the abdominal aorta with 100 ml of normal saline at 4 °C. The left renal vein was punctured to permit the perfusate to drain and the kidney was removed immediately and snap-frozen in isopentane (−70 °C) for subsequent histologic studies. The remaining kidney was stored at −70 °C for Western blotting analysis.

2.5. Urinary albumin excretion

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

2.6. Immunohistochemistry

Immunofluorescence analysis of nephrin and podocin in the renal tissue was performed on 4 μm cryostat sections. Sections were fixed in acetone/ethanol (4:1) solution for 10 min and washed in phosphate-buffered saline (PBS). The samples were incubated with 10% normal goat serum in PBS at room temperature for 1 h, and after that with either anti-nephrin antibody, or antibody to podocin in 1% normal goat serum in PBS for 2 h at room temperature. The slides were washed with PBS and the primary antibody was incubated with FITC-conjugated goat anti-rabbit IgG diluted in 1% normal goat serum in PBS for 1 h at room temperature. Nephrin and podocin immunoreactivities were analyzed by measuring fluorescence intensity by digital image analysis of images obtained by using a low-light video camera (Beijing Aeronautic and Aerospace University, Beijing, China).

Immunoperoxidase staining for ED-1+ macrophages was conducted on 3 μm sections of formalin-fixed renal tissue using antigen retrieval (microwave oven heating in 0.1 M sodium citrate pH 6.0 for 10 min) followed by a three-layer streptavidin–biotin peroxidase complex staining method. Quantitative analysis of ED-1+ macrophages in the glomeruli was performed under 400× magnification and expressed as cells/glomerular cross section (gcs). For each section, 50 sequential glomerular profiles were examined. All scoring was performed on blinded slides.

2.7. Western blotting analysis

Glomeruli were isolated by differential sieving and then lysed in SDS–PAGE sample buffer, boiled, centrifuged, and supernatant recovered. The protein content was estimated by the dye binding assay of Bradford [18], with bovine serum albumin used as a standard. A total of 30 μg of protein of each sample was separated by SDS–PAGE, electroblotted onto nitrocellulose membranes, incubated with blocking buffer (5% non-fat dry milk, 0.05% Tween 20, 1× Tris–Cl-buffered saline) for 1 h, and then incubated with primary antibody overnight at 4 °C. The membranes were then incubated with a HRP-labeled goat anti-rabbit IgG. The bound secondary antibody was detected by enhanced chemiluminescence. Housekeeping protein β-actin was used as a loading control. Positive immunoreactive bands were quantified densitometrically (Leica Q500IW image analysis system) and normalized for β-actin.

2.8. Statistical analysis

Data were expressed as means ± S.E.M unless otherwise stated. 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 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.

3. Results

Rats in the DM group had reduced body weight gain and increased blood glucose levels. No effects on body weight and blood glucose were observed with MMF treatment, although treatment was associated with a reduced body weight gain. The ratio of kidney weight to body weight was significantly higher in the DM group than that in the C group rats. Kidney enlargement was significantly attenuated by treatment with MMF. In the DM group, AER was significantly increased when compared to the C group. Treatment with MMF attenuated the increase in AER from the diabetic rats (Table 1).