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Vitamin D suppresses macrophage infiltration by down-regulation of TREM-1 in diabetic nephropathy rats

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ABSTRACT

This study intends to investigate the effect of active vitamin D (VD) on the expression of triggering receptor expressed on myeloid cells-1 (TREM-1) in the renal tissues of diabetic nephropathy (DN) rats and to explore the impact of TREM-1 on macrophage adhesion and migration. We find that the expressions of TREM-1 and CD68 protein are higher in DN rats compared with rats in the normal control group and that these changes are decreased in the DN + VD group. In vitro, the capacity for macrophage adhesion and migration and the expression of TREM-1 are increased under high-glucose conditions, but VD inhibits this progress. TREM-1 siRNA decreases high-glucose-induced macrophage adhesion and migration, whereas over-expression of TREM-1 inhibits its action. However, VD cannot suppress high glucose-induced TREM-1 expression and macrophage adhesion and migration when TREM-1 is over-expressed. These results demonstrate that VD can suppress macrophage adhesion and migration by reducing the expression of TREM-1.

1. Introduction

Diabetic Nephropathy (DN), which is characterized by inflammatory cell infiltration and pro-inflammatory factor over-expression, is a chronic inflammatory disease (Duran-Salgado and Rubio-Guerra, 2014; Liu et al., 2014; Gnudi, 2015). Macrophages are key inflammatory cells with important roles in the development and progression of DN (Zhang et al., 2017). Currently, several therapeutic approaches for DN, such as the control of blood pressure, glucose and lipids, are unsatisfactory (Lim, 2014). Therefore, finding a new target in DN treatment has become a direction of research.

Active vitamin D (VD) has been reported as a novel immunomodulator for the prevention and treatment of DN through the regulation of immunocytes and cytokines (Thomas and Cooper, 2010). In recent years, numerous studies have identified the effects of VD analogues in the prevention of albuminuria and podocyte injury in animal models (Gravellone et al., 2011; Goncalves et al., 2014). Our prior research also indicated that calcitriol, a bioactive 1, 25-dihydroxyvitamin D3, effectively decreased proteinuria and exerted a renoprotective effect in STZ-induced DN rats (Zhang et al., 2015). Furthermore, Sanchez-Niño has found that VD reduced macrophages infiltration, the expression of inflammation mediators and the extracellular matrix deposition in the same model (rats with STZ) (Sanchez-Nino et al., 2012). However, the mechanism of anti-inflammatory effect of VD is unclear.

Triggering receptor expressed on myeloid cells 1 (TREM-1) is a newly identified member of the immunoglobulin superfamily receptors and is found on macrophages. It appears to be a novel biomarker for the diagnosis and treatment of inflammatory diseases (Bosco et al., 2016). Zhao (Zhao et al., 2013) found that the TREM-1 level was two-fold higher in DN patients compared to normal subjects using antibody microarray technology. Recently, evidence has shown that TREM-1 is involved in the regulation of macrophage function (Lo et al., 2014; Yuan et al., 2014). The goal of this study is to investigate the effect of VD on the expression of TREM-1 in the renal tissue of DN rats and to explore the effect of TREM-1 on macrophage adhesion and migration.

2. Materials and methods

2.1. Animal experiments

All animal care and experimental protocols were in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China. The experimental protocol was

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approved by the Ethical Committee of Southeast University. All rats experiments were performed under anaesthesia.

Six-week-old healthy male Sprague–Dawley (SD) rats weighing 200–220 g were obtained from Shanghai Slac Laboratory Animal (Shanghai, China). After one week of acclimation, the rats were randomly divided into four groups: (1) NC (normal control group, n = 16), (2) VD (normal treated with vitamin D, n = 16), (3) DN (DN rats, n = 20) and (4) DN + VD (DN rats treated with vitamin D, Calcitriol 0.1 μg/kg/d, by gavage, n = 20). DN was induced with a single intraperitoneal injection of STZ (sigma) dissolved in 0.1 M citrate buffer (pH 4.5) at 58 mg/kg, and the control rats received only the 0.1 M citrate buffer solution. Three days later, the diabetic state was confirmed by measuring the tail blood glucose (BG) level. Rats with a blood glucose level that greater than 16.7 mmol/L were considered diabetic rats. Calcitriol (1,25-dihydroxyvitamin D_3 Soft Capsules) was purchased from Shanghai Roche Company (Shanghai, China). Calcitriol was administered orally at a daily dose of 0.1 μg/kg after the induction of DN. The rats were sacrificed at 8 weeks after treatment. During the treatment period, body weight was measured weekly. Blood glucose was monitored with a blood glucose monitoring system (Bayer) using one drop of tail blood. Urine samples were collected at 24 h in metabolic cages. Blood samples were taken for the measurement of biochemical parameters, and the kidneys were collected for histological examination and molecular assays.

2.2. Cellular experiments

The mouse macrophage cell line RAW264.7 was purchased from Shanghai Bogoo Biotechnology Company (Shanghai, China) and was routinely cultured in RPMI 1640 media (containing 11.1 mM glucose) supplemented with 10% foetal bovine serum (Sciencell, Santiago, USA) and incubated at 37 °C in 5% CO_2. First, RAW264.7 cells were stimulated with 30 mM of glucose for 12 h, 24 h, 48 h and 72 h, then cells were treated with increased concentrations of glucose (11.1 mM, 20 mM, 25 mM, 30 mM and 35 mM) for 48 h. The expression of TREM-1 was measured to ascertain the optimum dose and time point. Second, the effect of VD on macrophage TREM-1 expression was examined. RAW264.7 cells were incubated with 30 mM glucose for 48 h with or without 10^{-8} mol/L 1,25-dihydroxyvitamin D3 (VD) (Sigma, St. Louis, USA). At the same time, the effect of VD on macrophage adhesion and migration was examined. Then, TREM-1 siRNA and plasmid were pre-treated with macrophages, and macrophage adhesion and migration were examined again. The cells were randomly divided into different groups showed in Table 1.

2.3. Serum and urine chemistry analyses

Blood urea nitrogen (BUN) and creatinine (Scr) were analysed by an automatic biochemistry analyser (Hitachi, Tokyo, Japan). Urinary proteinuria was measured using an ELISA Kit (Jiancheng, Nanjing, China) according to the manufacturer's method.

2.4. Renal histology analyses

Kidney sections were stained with periodic acid-Schiff (PAS) trichrome staining and were then examined by light microscopy (magnification, × 400) in a blinded manner. An semi-quantitative analysis of mesangial hyperplasia was evaluated in twenty randomly selected areas using the Image-Pro Plus image analysis system. The percentage of mesangial hyperplasia is the ratio of the pink mesangial area and the total glomerular area in each glomerulus.

2.5. Immunohistochemistry

Immunohistochemistry was performed on paraffin sections using a microwave-based antigen retrieval technique. Sections were incubated with primary mouse anti-CD68 (Santa Cruz Biotechnology, California, USA) followed by incubation with an appropriate secondary antibody. The immunostaining was visualized using diaminoben zidine tetrahydrochloride, and the slides were counterstained with haematoxylin.

2.6. Western blot

Proteins from renal tissues or mouse macrophages were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with primary antibodies against TREM-1 (Bioss, Beijing, China) and β-actin at 4 °C overnight. After three washes with PBST/5 min, the nitrocellulose membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1–2 h. Finally, the membranes were visualized with an enhanced chemiluminescence advanced system (GE Healthcare, Buckinghamshire, UK) and captured on X-ray film. Immunoreactive bands were quantified with densitometry using Image J software (NIH, Bethesda, USA).

2.7. Immunofluorescence staining

For immunofluorescence, RAW264.7 cells were seeded on a capsule and allowed to adhere overnight. After incubation with different intervention reagents for 48 h, the cells were washed three times with PBS and then fixed with 4% paraformaldehyde. After the PBS washes, the cells were blocked for 10 min. The cells were washed and incubated with anti-TREM-1 (Bioss, Beijing, China) overnight at 4 °C. Then, the cells were washed and incubated with anti-rabbit secondary antibody (Jackson, Colorado, USA) for 2 h at room temperature. After staining the nuclei with DAPI, the cells were visualized using an IX70 fluorescence microscope (OLYMPUS, Tokyo, Japan).

2.8. Migration assays

Transwell migration assays were performed (Costar polycarbonate filters, 5 μm pore size). Macrophages (0.5×10^5 cells/

Table 1
Groups.

Pretreatment	Group							
— —	1) NC	2)HG	3)HG+Ethanol	4)HG+VD + Ethanol	5)VD+Ethanol	6)Ethanol	7)Mannitol	8)— —
siRNA	1) NC	2)HG	3)HG+Ethanol	4)HG+VD + Ethanol	5)VD+Ethanol	6)Ethanol	7)Mannitol	8)NTC
overexpression	1) NC	2)HG	3)HG+Ethanol	4)HG+VD + Ethanol	5)VD+Ethanol	6)Ethanol	7)Mannitol	8)NTC

1)NC: 11.1 mM glucose; 2)HG: 30 mM glucose; 3)HG + Ethanol: 30 mM glucose+10^{-3} mol/L Ethanol; 4)HG + VD + Ethanol: 30 mM glucose+10^{-8} mol/L $1,25(OH)_2D_3$+10^{-3} mol/L Ethanol; 5)VD + Ethanol: 10^{-8} mol/L $1,25(OH)_2D_3$+10^{-3} mol/L Ethanol; 6)Ethanol: 10^{-3} mol/L Ethanol; 7)Mannitol: 11.1 mM glucose+19 mM mannitol; 8) NTC: nontarget control.

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