Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Expression and effect of TLR4 in rats with diabetic nephropathy

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ARTICLE INFO

Article history:
Received 10 May 2013
Received in revised form 15 June 2013
Accepted 15 July2013
Available online 20 August 2013

Keywords:
Diabetic nephropathy
TLR4
Glomerulus

ABSTRACT

Objective: To observe the expression of TLR4 in kidney tissue of rats with diabetic nephropathy and discuss the role of TLR4 in the occurrence and development of the diabetic nephropathy. **Methods:** A total of 60 clean male SD rats were selected and randomly divided into the modeling group and control group after 1 week of breeding, including 30 rats in each group. Biochemical indices as well as the protein expression of TLR4 were observed and compared between two groups at 2 w, 4 w, 6 w, 8 w and 12 w after the modeling, and the correlation between TLR4 and each biochemical indexes was analyzed. **Results:** Rats in the modeling group had higher levels of blood glucose, 24-hour urine protein and blood urea nitrogen after the modeling, and showed the increase in the serum creatinine, kidney/body weight ratio, CRP and serum TNF-α at 4w after the modeling, with the significant difference compared to results of the control group (P<0.05). The cross-section area and mean volume of glomerulus in the modeling group at 4 w, 6 w, 8 w and 12 w were significantly higher than those in the control group, with the statistically significant difference (P<0.05). The expression of TLR4 at each time point in the control group was relatively low. Rats in the modeling group had the high expression of TLR4 in kidney's glomerular basement membrane, proximal convoluted tubule and renal interstitial area since 2 w, with the significant difference compared to the control group (P<0.05). The expression in rats of the modeling group was higher than the one of the control group since the 2nd week. As the time flied, its expression increased, with the statistically significant difference between two groups (P<0.05). There was certain correlation between the protein expression of TLR4 and the increased serum titer of 24-hour urine protein excretion, serum creatinine, CRP and TNF-α. **Conclusions:** TLR4 may activate the immuno-inflammatory reactions to play a role in the occurrence and development of the diabetic nephropathy.

1. Introduction

Diabetes mellitus (DM) is the metabolic endocrine disease characterized by the disturbance of fat and glucose metabolism and rise of glucose level in the plasma, which will result in the injuries of multiple organs in the end stage. Diabetic nephropathy (DN) is one of common microvascular complications for the DM and it has become the leading cause for the end stage renal disease in recent years[1,2]. Presently, it is considered that the incidence of DN is influenced by many aspects, including the hemodynamic disorders, genetic factor and disorder of biochemical metabolism[3]. In recent years, theories of inflammation and immunity have attracted much attention. Researches indicate that the occurrence and development of DM are closely related to the platelet activation and endothelial cell loss. Toll-like receptors (TLRs) are the key transmembrane proteins to transfer the information of antigen recognition from outside to inside of the cell, as an important factor in the immune reaction[4]. TLR is the main part of mediated immune and inflammatory reaction, while TLR4 plays an important role both in the recognition of lipopolysaccharide

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and signal transduction of mediated inflammatory reaction[5]. Researchers pay more attention to its role in the kidney diseases. This paper studies the mechanism of TLR4 in the DN, in order to find the new method to treat the DN.

2. Materials and methods

2.1. Experimental animals

A total of 60 clean male SD rats (purchased from the provincial experimental animal center, number of quality certification: JZDW2011-0197) with the weight of 160-200 g were selected. All rats had sufficient water every day under 20-24 ℃ and 40%-70% humidity. The model of DN rat was established after 1 week of breeding.

2.2. Main reagents and materials

TLR4 antibody, Trizol, DEPC and RT-PCR kit were purchased from Sigma-Aldrich and agarose, ethidium bromide, chloroform, isoamyl alcohol, ethanol absolute and isopropyl alcohol were provided by the laboratory. Instruments included the ultra-low temperature freezer (Midea Group), ultraviolet spectrophotometer (Shanghai Jing Hong Laboratory Instrument Co., Ltd.), iCycler PCR amplification instrument (Bio-Rad; USA) and DC2000 gel image analyzer (Bio-Rad; USA).

2.3. Modeling of DN rats and group division

Rats were divided into the modeling group and control group after 1 week of breeding, including 30 rats in each group. Modeling of DN rats was as follows: (1) Firstly, 2.1 g citric acid was added to 100 mL distilled water as solution A and then 2.49 g trisodium citrate was added to 100 mL distilled water as solution B. Solution A was mixed with B by the ratio of 1:1.32 to get 0.1 mmol/L citric acid-trisodium citrate buffer solution. pH level of the buffer solution was adjusted to 4.5 by hydrochloric acid and sodium hydroxide; (2) A total of 600 mg streptozocin (STZ) was added to 60 mL citric acid-trisodium citrate buffer solution and rats were injected with STZ injection within 5min after the preparation; (3) All rats were fasted for one night, where the modeling rats received the intraperitoneal injection of STZ solution with the dosage of 65 mg/kg and the control ones received the injection of citric acid-trisodium citrate buffer solution with the dosage of 65 mg/kg; (4) Fasting blood glucose level of modeling rats after 1 week was detected. If it was higher than 16.7 mmol/L and the urine glucose was positive for 3 d (+++), then the modeling succeeded, otherwise it failed and would be given up. Six rats were killed in each group respectively at 2 w, 4 w, 6 w, 8 w and 12 w after the success of modeling. All rats accepted the free feeding without the insulin during the experiment and under the standard laboratory environment.

2.4. Samples collection and treatment

One day before killing, their urine was collected by metabolic cage for 24 h and storeed in the refrigerator under -80 ℃ for 24 h protein in urine test. Rats accepted one-night fasting before being killed for the weighing. Blood was collected from the caudal vein to measure the fasting blood glucose (blood glucose meter). After the ether inhalation anesthesia, the blood was collected from the abdominal aorta. After standing for 2 h, the blood was centrifuged at 3 000 r/min for 10 min. Supernatant was taken and stored in the refrigerator under -20 ℃ to detect the serum creatinine (by the automatic biochemical analyzer), urea nitrogen (by the automatic biochemical analyzer) and C-reactive protein (CRP) (by the scattering immunoturbidimetric assay). Kidneys were removed at both sides and the left one was weighted. Part of tissues was stored in the liquid nitrogen and detected in the laboratory together. The total RNA was extracted by Trizol method and then underwent the cDNA synthesis and polymerase chain reaction (PCR), with the upstream primer of TLR4, 5-GATTGCTCAGACATGGCAGT-3 and downstream primer, 5-CCCACTCGAGGTAGGTGTTT-3. Agarose gel electrophoresis was performed for products of RCR. It was analyzed by DC2000 gel image analyzer and the fragment size of TLR4 was 137 bp. Part of them were fixed by the neutral formalin and wrapped by the paraffin. The pathological examination and immunohistochemical analysis were performed on kidney tissues.

2.5. Pathological analysis of kidney tissues and detection of glomerular extracellular matrix

PAS staining was taken after the section of paraffin to observe the pathological change of kidney and measure the cross-section of glomerulus. The mean was regarded as the cross-section area of glomerulus for each sample [mean glomerular area (MGA)] and the mean glomerular volume (MGV) was calculated by the calculation formula of MGV=1.25×(MGA)3/2.

2.6. Detection of TLR4 expression

The immunohistochemical staining was taken for detection of TLR4 expression in kidney tissues (rabbit anti-rat TLR4 polyclonal antibody, sigma and Evision kit for the staining). mRNA expression of TLR4 in kidney tissues of rats was detected by reverse transcription-polymerase chain reaction

(RT-PCR).

2.7. Statistical analysis

It was analyzed by SPSS 16.0. Data was expressed as mean standard deviation. *t* test and LSD method were used for the group comparison and Pearson correlation for the multiple factor analysis. $P<0.05$ was considered as significant difference.

3. Results

3.1. Kidney weights and body weight

Modeling results showed that two groups had the symptoms of listlessness, fluff binding and decrease of self-cleaning activity, and significant polydipsia, polyphagia, polyuria and weight loss after the modeling. Compared to the control group, the kidney/body weight ratio in the modeling group was significantly higher after the modeling, with the statistical difference ($P<0.05$).

3.2. Biochemical indices

Rats in the modeling group had higher the blood glucose, 24 h urine protein and blood urea nitrogen after the modeling, with the statistical difference in the increase of serum creatinine, kidney/body weight ratio, CRP and serum TNF-α after 4 w of modeling compared to the control group ($P<0.05$) (Table 1).

3.3. Extracellular matrix proliferation of DN rats at different time points

There was no significant change in kidney tissues of the control group. But the volume of glomerulus increased at 4 w, the visible mesangial region widened at 12 w, extracellular matrix assembled and chromatin reconstitution of PAS increased in the modeling group. The cross-section area and mean volume of glomerulus in the modeling group at 4 w, 6 w, 8 w and 12 w were significantly higher than ones in the control group, with the statistically significant difference ($P<0.05$) (Table 2).

3.4. Protein expression of TLR4

The expression of TLR4 at each time point in the control group was relatively low. Rats in the modeling group had the high expression of TLR4 in kidney's glomerular basement membrane, proximal convoluted tubule and renal interstitial area since 2 w, with the significant difference compared to

Table 1

Biochemical indices at different time points.

Group		Blood glucose (mmol/L)	Urine protein (mg/24h)	Serum creatinine (μmol/L)	Blood urea nitrogen (mmol/L)	Kidney/body weight ratio (mg/g)	CRP (μg/L)	TNF-α (μg/L)
Control group		4.4±0.3	1.2±0.7	55.6±8.9	5.5±2.3	4.2±0.6	4.3±2.1	1.1±0.3
Modeling group	2 w	22.3±3.4*	10.1±6.3*	58.3±10.2	9.3±2.2*	4.6±0.8	4.3±2.4	1.1±0.2
	4 w	23.2±3.2*	11.8±7.1*	63.6±7.8	8.9±3.1*	5.6±1.0*	5.2±3.5*	1.6±0.3*
	6 w	19.6±4.1*	11.2±6.9*	75.5±11.3*	11.5±4.7*	6.3±1.2*	10.6±6.3*	1.9±0.4*
	8 w	25.6±2.8*	10.1±7.6*	82.6±11.5*	16.2±4.8*	6.3±0.8*	15.5±4.1*	2.1±0.5*
	12 w	26.1±2.0*	11.3±7.7*	101.7±15.8*	13.6±4.0*	6.1±1.3*	21.2±6.4*	2.6±0.4*

*: Compared to the control group, $P<0.05$.

Table 2

Extracellular matrix proliferation of DN rats at different time points.

Group	Control group	Modeling group at 2 w	Modeling group at 4 w	Modeling group at 6 w	Modeling group at 8 w	Modeling group at 12 w
Cross-section area of glomerulus (μm^2)	6 321.4±326.2	6 464.3±356.1	7 135.2±413.7*	7 069.5±408.3*	7 432.6±511.6*	8 013.7±623.5*
Mean volume of glomerulus ($10^3 \mu m^3$)	631.8±47.6	672.6±51.3	786.4±58.6*	713.2±60.1*	826.4±65.3*	925.6±46.7*

*: Compared to the control group, $P<0.05$.

Table 3

Protein expression of TLR4 (%).

Group	2 w	4 w	6 w	8 w	12 w
Modeling group	7.65±1.74*	8.31±2.06*	8.58±2.42*	10.53±3.71*	11.86±4.12*
Control group	3.24±0.68	3.26±0.65	3.20±0.64	3.22±0.66	3.28±0.64

*: Compared to the control group, $P<0.05$.

the control group (P<0.05) (Table 3).

3.5. TLR4 mRNA expression in rat's kidney

The expression in rats of the modeling group was higher than the one of the control group since the 2nd week. As the time flied, its expression increased, with the statistically significant difference between two groups (P<0.05). TLR4 expression at each time point in the control group was relatively low (Figure 1).

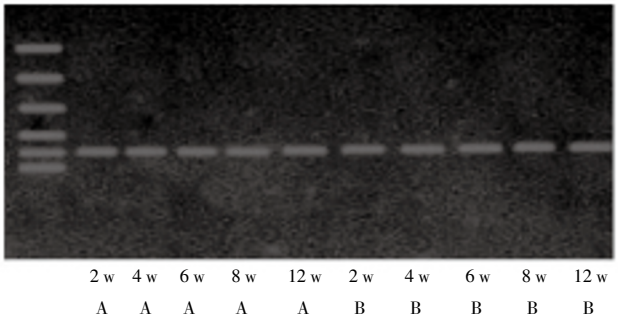

Figure 1. TLR4 mRNA expression in rat's kidney.
A: Control group; B: Modeling group

3.6. Protein expression of TLR4 and multiple factors correlation analysis

There was the certain correlation between the protein expression of TLR4 and the increased serum titer of 24-hour urine protein excretion, serum creatinine, CRP and TNF-α (Table 4).

Table 4
Protein expression of TLR4 and multiple factors correlation analysis

Indexes	Protein expression of TLR4	
	Correlation coefficient r	P value
24-hour urine protein excretion	0.899	0.039
Serum creatinine	0.941	0.016
CRP	0.858	0.021
TNF-α	0.843	0.018

4. Discussion

DM is a common metabolic endocrine disease characterized by the disturbance of glucose and fat metabolism and rise of glucose level in the plasma. Its pathologic and physiological characteristics are the absolute or relative insulin hyposecretion and increased activity of pancreatic glucagon which result in the metabolic disturbance. There are numerous patients with DM all around the world. According to statistics, the number of patients with DM was up to 239 million in 2010. Besides, with the change of lifestyle, the number of patients with DM tends to be increased. The DM can affect multiple systems of the whole body, along with many complications. Where, DN is the most important microvascular disease and it is also one of key factors to cause the end-stage rental failure. Presently, it is considered that the incidence of DN is influenced by many aspects, including the hemodynamic disorders, disorder of biochemical metabolism regarding the high glucose and genetic factor. Theories of inflammation and immunity have attracted much attention. Researches indicate that the occurrence and development of DM are closely related to the platelet activation and endothelial cell loss. TLRs are the only key transmembrane proteins in mammals to transfer the information of antigen recognition from outside to inside of the cell, as an important factor in the immune reaction. TLR is the main part of mediated immune and inflammatory reaction, while TLR4 plays an important role both in the recognition of lipopolysaccharide and signal transduction of mediated inflammatory reaction[6,7].

Presently, there are limited researches on the relationship between TLR4 and DN. The correlation between them is generally rooted in researches on complications of DM. Dasu *et al*[8] found that there is increased expression of TLR4 in the peripheral blood of patients with type 1 and type 2 DM, which is related to the inflammatory reaction. Meanwhile, ShiH et al indicated that rats with TLR4 defect would become fat after the high-fat diet, but it could partially prevent the insulin resistance induced by the high-fat diet, which showed that the activation of TLR4 pathway was related to the insulin resistance[9]. The insulin resistance is the key mechanism for the occurrence of type 2 DM. The insulin treatment can reduce the blood glucose and the expression of TLR4 protein[10]. Furthermore, researches also indicated that the up-regulated expression of TLR4 could cause the occurrence of diabetic peripheral neuropathy, diabetic retinopathy and atherosclerosis[11-13]. There are limited researches on TLR4 in the inflammatory activation pathway and expression change of DN. Based upon the observation of TLR4 expression in the glomerulus of rats with DN, this paper discusses the role of TLR4 in the occurrence and development of DN.

We adopt the intraperitoneal injection of STZ solution for the modeling of rats with DN and take the detection by methods of immunohistochemistry after the modeling. This research shows that the expression of TLR4 is mainly on the basement membrane and amniotic fluid and membrane of part vascular endothelial cell. It also shows that rats in the modeling group have the increased expression of TLR4 in glomerulus since 2 w and maintain the high level of blood glucose, 24-hour urine protein and blood urea nitrogen after the modeling. Then the level of serum creatinine, kidney/body weight ratio, CRP and serum TNF-α increases and

the increase tendency becomes significant as the course of disease prolongs. The correlation analysis shows that there is the high positive correlation between TLR4 expression and serum TNF-α and CRP. Abundant researches also prove the critical role of TGF-α 1 and CRP in the development of DN-induced renal fibrosis.

In this immunohistochemistry experiment, we found that the protein expression of TLR4 in renal tubule of rats with DN was significantly higher than the one in the control group and PCR results also showed that the expression of TLR4 in the kidney of rats in the modeling group was higher than the one in the control group since 2 w. The expression increases as the time flies with the statistical difference between two groups ($P<0.05$). It indicates that the expression of TLR4 in the renal tubular epithelium mediates the tubular substance leukocyte infiltration and tubular injury. The increased expression of TLR4 in the glomerulus can activate the immunocyte and renal parenchymal cell. It excretes abundant inflammatory mediator and cytokine and causes the continuous inflammatory reaction that promotes the tubular interstitial fibrosis and glomerular sclerosis.

In conclusion, this research shows that there is the high positive correlation between the expression of TLR4 and the degree of inflammatory reaction, kidney injury and tissue fibrosis, which indicates that TLR4 in the occurrence and development of DN may cause the continuous inflammatory reaction and activate the immune reaction of kidney tissue. The interference in the expression of TLR4 in kidney tissues may delay the occurrence and development of DN.

Conflict of interest statement

We declare that we have no conflict of interest.

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