·Basic Research·

Gene expression of transforming growth factor-β2 in retina of diabetic rats

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Abstract

• AIM: To detect the gene expression of TGF-β 2 in retinas of diabetic rats at different stages, to observe and analyze the effect of TGF-β2 on the retinas of diabetic rats, to explore the role of TGF-β2 in pathogenesis of diabetic retinopathy (DR), and to provide experiment data and experience for further clinic studies.

• METHODS: Sprague-Dawley (SD) rats were used and retinas were dissected. The total RNA was isolated from which the first strand of cDNA was prepared. Diabetes mellitus was induced by a single intraperitoneal injection of 60mg/kg streptozotocin (STZ) and the rats were held without insulin treatment until sacrifice. Besides, age-matched rats treated with saline were used as controls. Tail vein blood glucose was measured after 2 days and rats were considered hyperglycemic if blood glucose reading > 16.7mmol/L. Animals with blood glucose level <16.7mmol/L were excluded from the study. The rats were killed at the 4th, 8th, 12th, 16th, 20th and 24th week respectively after hyperglycemic models were established. The retinas were separated and preserved in liquid nitrogen. The expressions of TGF-β2 gene mRNA were detected by reverse transcription PCR (RT-PCR).

• RESULTS: The RNA of rat retina was integrative enough to be used to further carry out PCR analysis. Compared with control groups, the expression of TGF-β2 mRNA in retinas of diabetic rats was up-regulated at the 4th week, but there was no statistical difference (P >0.05); it was down-regulated at the 8th week, and there was statistical difference (P<0.05); it was also down-regulated at the 12th week, and there was statistical difference (P<0.05); at the 16th week there was no statistical difference (P >0.05); it was up-regulated at the 20th week, but there was no statistical difference (P>0.05); it continued to be up-regulated at the 24th week, and there was statistical difference(P <0.05).

• CONCLUSION: Since the expression of TGF-β2 mRNA in retinas of diabetic rats was down-regulated at the 8th week and 12th week statistically, up-regulated at the 24th week statistically, it has obviously shown that TGF-β2 was down- and up-regulated through the period of DR. That is, its changes are diphasic with time. It may confirm that TGF-β2, with the characteristic of diphasic regulation, played an important role in DR. It is necessary to study it furthermore.

• KEYWORDS: transforming growth factor-β2; gene expression; retina; diabetic rat; RT-PCR

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INTRODUCTION

Diabetic retinopathy (DR) is one of severe complications of diabetes mellitus (DM), and also is one of the most serious diseases in ophthalmology. It remains a leading cause of vision loss and new-onset blindness. Due to the lack of safe and effective medicine, the management of patients with DR has always been a tough job for ophthalmologists. With the onset of diabetes, cellular and biochemical alterations within the retina are set into motion. Recent studies have shown that cytokines may play an important role in the occurrence of DR[1,2]. It seems very likely that cytokines including TGF-β are involved in DR pathological process, although the exact mechanism is unknown yet[3,4].

Transforming growth factor-β (TGF-β) is a multifunctional cytokine, whose numerous cell and tissue activities include cell cycle control, regulation of early development, differentiation, proliferation, migration, extracellular matrix formation, hematopoiesis, angiogenesis, chemotaxis, immune functions, and induction of apoptosis [5]. In mammals, three isoforms of TGF-β have been identified: TGF-β1, TGF-β2, and TGF-β3. Their biological functions seem to be different according to a study using knockout mice[6].

In this study, we investigated the expression of TGF-β2 in retinas of diabetic rats at different stages with real-time reverse transcription polymerase chain reaction (RT-PCR), and discussed the possible functions of TGF-β2 in the pathogenesis of diabetic retinopathy.

MATERIALS AND METHODS

Experimental Animals A total of 80 adult male Sprague-Dawley rats(200±25)g were provided by Shanghai Laboratory Animal Center. The animals were housed in stainless steel cages and fed standard rat chow and tap water. They were kept in a room on a 12-hour light-dark cycle with an ambient temperature of 22℃±1℃.

Establishment of Diabetic Rat Models Rats were randomly divided into two groups: 36 in the normal control (CON) group and 44 in the diabetes mellitus (DM) group.

With the citrate buffer (pH 4.0) comprising 0.1mol/L sodium citrate and 0.2mol/L sodium phosphate, strepto-zotocin (STZ) was dissolved for the preparation of 10g/L STZ solution. DM was induced by streptozotocin (STZ) intraperitoneal injection with a dosage of 60mg/kg body weight. At 48 and 72 hours after the injection, the tail blood was collected to determine the level of blood glucose. If the blood glucose level >16.7mmol/L, the rat then was considered as a diabetic rat. Four rats in the DM group died, and three rats recovered to normal blood glucose level and were used for observation of drug toxicity. The blood glucose level and body weight were determined regularly after that. During the experimental period, 37 rats were successfully induced DM. And 6 rats in DM group were killed at the 4th, 8th, 12th, 16th, 20th and 24th week respectively.

Preparations of Total RNA in Retina and the First Strand of cDNA The eyeglobes of the experimental animals were enucleated after the determination of their body weights and fasting blood glucose levels on the respective dates (4th, 8th, 12th, 16th, 20th and 24th week). The anterior capsule of the eyeglobe and lens were removed. The retinas were peeled off and immediately placed into the liquid nitrogen for preservation. With Trizol reagent (Invitrogen Corporation, USA), total RNA was extracted. Then its concentration and purity were determined through ultraviolet and visible spectro-photometer. Its integrity was determined by 10g/L formalde-hyde agarose gel electrophoresis. The total RNA was isolated from which the first strand of cDNA was prepared by reverse transcriptase SuperScript II(Invitrogen Corporation,USA).

Primer Design On the basis of the principle of standard PCR primer design, we used PC gene software to design the primers of TGF-β2 and β-actin. To avoid the contamination of genome DNA, we put upstream and downstream primers at various exons. The sequence of the primer synthesis is as follows: TGF-β2 upstream primer: 5'-TGGTGTTGTACAG GCTGAGG-3'; downstream primer:5'-CACAAAACAGGAA CCTGG-3'; β-actin upstream primer: 5'-CAGCCATGTACG TAGCCATC-3'; downstream primer:5'-AGAGTACTTGCGC TCAGGAG-3'.

Gene Expression of TGF-β2 and Analysis The length of TGF-β2 PCR product was 472bp, while β-actin PCR product was 626bp. 20μL PCR amplification product was added into 20g/L sepharose gel which contained 0.5g/L ethidium bromide (EB), followed by electrophoresis for 45 minutes (5V/cm). It was observed under ultraviolet lamp and taken photos by gel imaging apparatus. Then PCR strap was analyzed by automatic gel image analysis system(SX image software). The relative value of TGF-β2/β-actin mRNA expression level was calculated. Results were expressed in the form of $\bar{x}\pm s$ and two-tail t-test was employed for the comparision of values between groups.

RESULTS

Observation of the Animal General Conditions The blood glucose levels of all the diabetic rats were higher than 16.7mmol/L, with the symptoms of polydipsia, polyphagia, polyuria, gradual loss of body weight. Compared with the rats in control group, the diabetic rats had characteristics of bradykinesia,acedia,non-aggressive and susceptible to infections.

Changes in Blood Glucose Level of Rats in Both Groups at Different Stages At various time points, the blood glucose levels of diabetic rats fluctuated and were maintained at a high level throughout the experimental period, and they were significantly higher than those of the normal control group (P<0.01,Table 1).

Expressions of TGF-β2 mRNA in Rat Retinas of Both Groups at Different Stages The value of A260/280 was 1.8-2.0 via spectrophotometry and the actual concentration was 0.4-1.0g/L, which showed that the extracted RNA was pure enough for the present study. The RNA of rat retina was integrative enough to be used for further PCR analysis. Compared with control groups, the expressions of TGF-β2 mRNA in retinas of diabetic rats were up-regulated at the 4th week, but there was no statistical difference (P>0.05). Expression level was significantly down-regulated at the 8th week and at the 12th week (P<0.05), while at the 16th week there was no statistical difference (P>0.05). It was up-regulated at the 20th week, although without statistical signifi-

Table 1 The blood sugar levels of normal rats and diabetic rats at different stages $\bar{x}\pm s$

Group	4wk	8wk	12wk	16wk	20wk	24wk
Normal	4.62±0.12	4.62±0.25	4.62±0.12	4.57±0.23	4.58±0.19	4.57±0.22
Diabetic	21.1±2.54	21.83±2.24	22.8±2.2	23.07±2.78	21.77±2.98	22.63±1.72
P value	8.72E-06	3.26E-06	2.62E-06	7.19E-06	1.53E-05	6.36E-07

Table 2 Expressions of TGF-β2 mRNA in retinas of normal and diabetic rats at different stages $\bar{x}\pm s$

Group	4wk	8wk	12wk	16wk	20wk	24wk
Normal	0.655±0.066	0.753±0.05	0.69±0.146	0.645±0.129	0.678±0.077	0.636±0.056
Diabetic	0.750±0.194	0.456±0.203	0.496±0.068	0.680±0.128	0.762±0.125	0.739±0.104
t	0.926	3.204	2.436	0.421	1.191	1.983
P	0.201	0.041	0.029	0.361	0.136	0.047

cance ($P>0.05$). Until the 24th week the expression level was continually up-regulated and there was statistically significant difference ($P<0.05$) (Table 2, Figure 1, Figure 2A-F).

DISCUSSION

Diabetic retinopathy (DR) is one of the most common and severe complications of diabetic mellitus, and is primary cause of acquired blindness. DR is a progressive microangiopathy characterized by small vessel damage and occlusion. The earliest pathologic changes are thickening of the capillary endothelial basement membrane and reduction of pericytes. Retinal angiogenesis is characteristic of the most severe stage of DR, namely, proliferative diabetic retinopathy (PDR). A number of factors have been implicated in the mechanism of neovascularization, including specific growth factors, glucose control and the balance of the components of the fibrinolytic system. But the pathogenesis of DR is still not fully understood. The role of growth factors in the development and increasing severity of DR has been demonstrated by many researches. Recent studies have shown that cytokines are involved in the pathogenesis of DR in general. Proliferative diabetic retinopathy (PDR) is featured with neovascularization and fibrosis [7]. Along with the technological development of molecular biology, it has been discovered that the occurrence an development of DR is related to the abnormal regulation of cell proliferation, and that several cytokines and growth factors are closely associated with the growth and proliferation of retinal cells, among which TGF-β is an important factor. A higher concentration of TGF-β2 in the eyes of diabetic patients may result in the neovascularization or formation of preretinal proliferative membrane, which may play a role in the progression of diabetic retinopathy[8,9].

Transforming growth factor-β (TGF-β) is known to play a

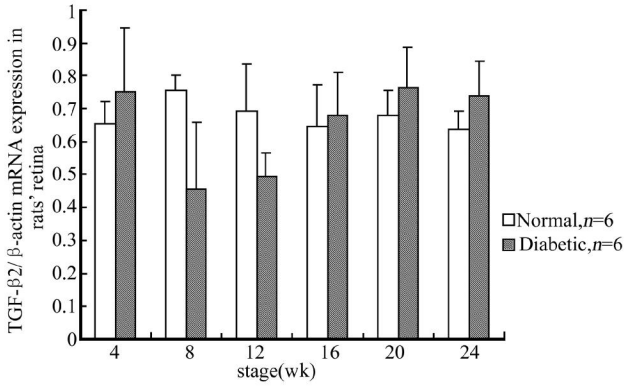

Figure 1 The TGF-β2 mRNA expression in retinas of normal and diabetic rats at different stages

pivotal role in the regulation of extracellular matrix (ECM) accumulation. Members of the transforming growth factor family (TGF-β) are multifunctional proteins that regulate cell growth, differentiation, migration, and extracellular matrix production and have crucial functions in embryonic development, wound healing, immune responses, and vascular development. Since DR is characterized by microangiopathy, the control of the neovascularization is believed to be important in the pathogenesis of the disease. Recently, TGF-β2 has been identified which may be associated with diabetic retinopathy. Diabetic retinopathy, in its development and progression, appears to involve the biological action of TGF-β2 to promote neovascularization as well as to increase extracellular matrix. Hirase *et al*[10] studied the concentration of TGF-β2 in the vitreous and reported that concentrations of both total and mature TGF-β2 in the vitreous collected during vitrectomy from patients with proliferative diabetic retinopathy were higher than those collected from the vitreous of patients with macular hole. Another report showed that the vitreous of eyes with intraocular fibrosis associated with proliferative vitreoretinopathy contained higher levels

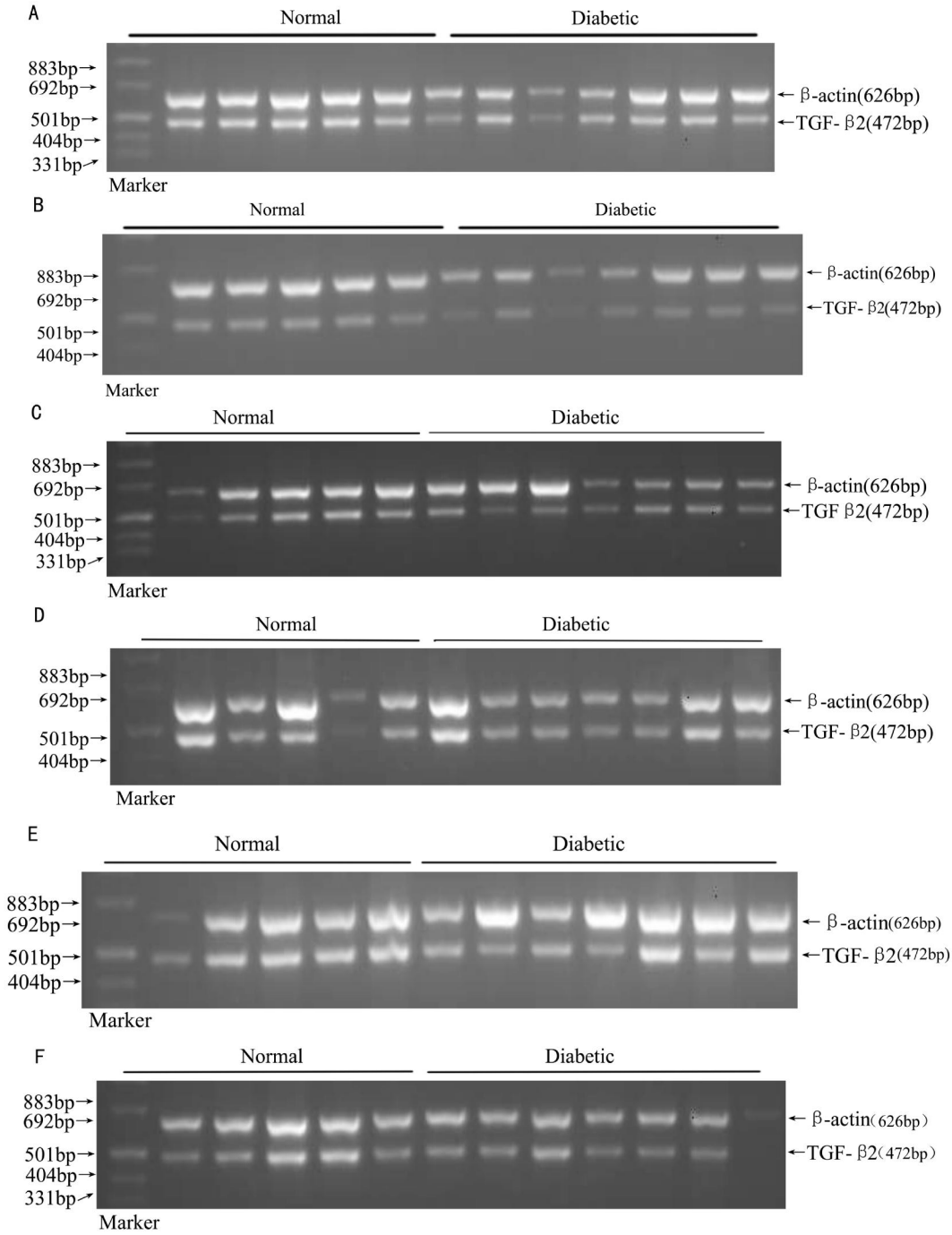

Figure 2 A:4th week TGF-β2/β-actin PCR product gel electrophoresis;B:8th week TGF-β2/β-actin PCR product gel electrophoresis;C:12th week TGF-β2/β-actin PCR product gel electrophoresis;D:16th week TGF-β2/β-actin PCR product gel electrophoresis;E:20th week TGF-β2/β-actin PCR product gel electrophoresis;F:24th week TGF-β2/β-actin PCR product gel electrophoresis

of TGF-β2 than the vitreous from eyes with uncomplicated retinal detachment without intraocular fibrosis. These findings suggest that TGF-β2 plays a role in the formation of preretinal proliferative membranes.

In our study, the mean concentration of total TGF-β2 was significantly higher in the retinas of diabetic rats compared with the control at the 24th week. But at the 8th week and 12th week, it was down-regulated obviously. So TGF-β2 may play a diphasic effect in diabetic retinopathy. At the 8th week and 12th week, the pathological changes of DR were thickening of the capillary endothelial basement membrane and reduction of pericytes. It clarified that at early stage the down-regulated expression level of TGF-β2 may be caused by the reduction of pericytes. Then the decrease of TGF-β2 finally resulted in the loss of inhibition to capillary endothelial cells. The retinopathy came to the stage of microvessel proliferation. At the 24th week TGF-β2 was significantly higher in the retinas of diabetic rats, which may be related to

the progressive microvascular damage, and monocytes and platelets produced TGF-β. Under the effects of TGF-β and other cytokines, retinal pigment epithelial cells and retinal glial cells migrated under the vitreous and retina, and the macrophages and fibroblasts were stimulated to produce collagen, fibronectins (FN), etc. which might make the capillary basement membrane thickened and promote the formation of capillary lumen. Ultimately it led to the proliferative diabetic retinopathy (PDR).

Since the expression of TGF-β2 mRNA in retinas of diabetic rats were down-regulated at the 8th and 12th week statistically, up-regulated at the 24th week statistically, it has obviously shown that TGF-β2 was down- and up-regulated through the period of DR. That is, TGF-β2 has diphasic regulating effects with the time. It may be confirmed that TGF-β2 plays an important role in DR. It was necessary to study it further in order to prevent the development of DR at the early stage.

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