

Expression of Netrin-1 in Diabetic Rat Retina

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Abstract

Purpose: To detect the expression of Netrin-1 in the retinas of diabetic rats and evaluate the relationship between Netrin-1 mRNA and diabetic retinopathy (DR).

Methods: Twenty healthy adult male Sprague-Dawley (SD) rats were randomly divided into a diabetic model group (DM) and a normal control group (NC), each group was composed of ten rats. Streptozocin (STZ) was administered intraperitoneally at a single dose of 60 mg/kg to diabetic group. The control rats were injected only with citrate buffer. Collection of serum at 72 h after STZ treatment and measuring blood glucose levels confirmed the development of diabetes. The rats with blood glucose level 16.67 mmol/L or higher were considered to be diabetic and were used in the experiment. Retinal tissues were harvested and expression of netrin-1 mRNA and protein in the retina tissues was examined by reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis, respectively.

Results: Diabetic rats showed classic symptoms of diabetes mellitus, such as polydipsia, polyphagia, and polyuria. Cataract was seen in the DM group at 3 months after administration of STZ. Both netrin-1 mRNA and protein levels retina were dramatically increased in the DM rats compared to the NC group ($P < 0.05$).

Conclusion: Diabetic rats can be successfully established by intraperitoneal injection of streptozotocin (60 mg/kg). Netrin-1 may play an important role during the development of diabetic retinopathy. (*Eye Science 2013; 28: 148-152*)

Keywords: netrin-1; diabetic retinopathy; streptozotocin

Introduction

The improved economic development and changes in lifestyle and diet have resulted in increased mor-

bidity of diabetes mellitus (DM) worldwide. DM can induce many severe complications, such as vasculopathy, renal failure, acra gangrene, and blindness. In fact, twenty years after the onset of diabetes, almost all patients with type 1 diabetes and over 60% of patients with type 2 diabetes will have some degree of retinopathy¹. Diabetic retinopathy comprises of both microangiopathy and neuropathy, which starts at a very early stage²⁻⁴. Early diabetic retinopathy, both in humans and in experimental animal models, is characterized by increased vascular permeability and capillary closure⁵⁻⁶. It is a progressive vascular disease that can eventually lead to blindness. Thus, timely prevention and treatment of diabetic retinopathy is important.

Long-time duration of high blood glucose induces a slowing down of blood flow, degradation of vascular pericytes, and abnormal vascular exudation, which causes a relatively ischemic and hypoxic environment around the retina. Many cytokines such as VEGF are increased in compensation, and these play an important role in the progression of diabetic retinopathy, and especially neovascularization⁷.

Netrin-1 is a diffusible axon-guiding molecule that governs axonal outgrowth and guidance^{8,9}. It can guide the retinal ganglion cells to concentrate fiber nerves and exit the developmental eyeball. Recent studies have found that netrin-1 can promote developmental and pathological angiogenesis in vitro and in vivo. Here, we examined the expression of netrin-1 in the retinas of diabetic and normal control rats to elaborate whether netrin-1 takes part in the pathogenesis of diabetic retinopathy.

Materials and methods

STZ-induced diabetic models

Twenty male Sprague-Dawley (SD) rats were pur-

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chased from the Animal Experiment Department of Central South University, Changsha, China and were housed at a temperature of $24\pm 1.0^{\circ}\text{C}$ and relative humidity of 40–50% in a clean environment under 12:12 h light and dark cycle. The animals had free access to food and purified water was made available randomly. The research protocol of all animals was strict conformed to the ARVO Statement for the Use of Animal in Ophthalmic and Vision Research. The rats were randomly divided into diabetic model group (DM) and normal control group (NC), ten rats per group. The rats were fasted overnight (12 h) and not limited to drinking water. About 1% Streptozotocin in freshly prepared sodium citrate buffer (pH 4.5) was injected into the peritoneal cavity once in a dose of 60 mg/kg to induce diabetes. Rats in the NC group were injected with the same volume of sodium citrate buffer (approximately 0.5 mL). Diabetes was confirmed after 72 h of STZ injection and again on weekly basis during the experiment, and only the animals with glucose levels higher than 16.67 mmol/L were considered diabetic. The mRNA and protein level of Netrin-1 in retinas was measured using RT-PCR and western blot analysis 3 months following STZ injection.

RNA isolation and determination of netrin-1 mRNA expression

All animals were sacrificed at 3 month after diabetes was induced. The rats were killed with a 10% chloral hydrate solution (0.4 ml/100g) anesthetic, and then the eyes were enucleated rapidly. The retinas were bluntly separated under a dissecting microscope and then placed into EP tubes containing Trizol reagent (Invitrogen, USA) immediately after the removal of impurities. Total RNA was extracted from the retinal samples in accordance with the Trizol reagent instructions. A 3–5 μg sample of total RNA was reverse transcribed according to the reverse transcription (RT) kit (Fermentas, USA) instructions. The RT conditions were $65^{\circ}\text{C} \times 5 \text{ min} \rightarrow 37^{\circ}\text{C} \times 5 \text{ min} \rightarrow 42^{\circ}\text{C} \times 60 \text{ min} \rightarrow 70^{\circ}\text{C} \times 5 \text{ min}$. The RT product (1 μL) was then amplified by PCR. The specific primers were designed on a computer with Primer Premier ver.5.0. The primers for Netrin-1 were 5'-GCACAACGTTACGCTCAC TC-3' (sense) and 5'-GTACATTTTGCGGCACTGCG-3' (antisense),

and the size of the amplified fragment was 169 bp. PCR products for GAPDH were used as a positive control and internal standard. The primers for GAPDH were 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5' -TCCACCACCCTGTTGCTGTA-3' (antisense). The size of the amplified fragment was 452 bp. Amplification conditions included an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 40 s, an extension at 72°C for 40 s, and a final extension at 72°C for 10 min. PCR products were electrophoretically separated on 1.5% agarose gel in $0.5 \times \text{TBE}$ buffer. The optical densities of Netrin-1 were determined by BandScan (version 5.0). The densitometric values were normalized against GAPDH values.

Western blot analysis

The murine retinas were collected and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% NP-40) containing protease inhibitors (Boehringer Mannheim, Germany). Total protein was resolved by SDS-polyacrylamide gel electrophoresis, and was then transferred onto a nitrocellulose membrane. The membrane was incubated with chicken anti-rat polyclonal netrin-1 (1:100 dilution; CH23002, Neuromics, Inc., USA) and polyclonal anti-rat β -actin (1:10,000 dilution; Santa Cruz), Peroxidase conjugated secondary antibodies was used as secondary detection reagents with an enhanced chemiluminescence kit (KeyGEN, China). Chemiluminescent signals were visualized by exposure to X-ray film.

Statistical analysis

Quantitative data were expressed as mean \pm S.E. Statistical analysis was performed using SPSS (version 16.0) software. One-way ANOVA followed by Student's *t*-test was used to evaluate statistical significance and $P < 0.05$ was considered statistically significant.

Results

Establishment of the rat diabetic model

After onset of diabetes, serum glucose levels in the DM group increased 4–5-fold compared with the NC group (Figure 1A). The difference in weight between the DM group and the normal control group

were statistically significant (Figure 1B). STZ-induced diabetic rats showed apparently typical symptoms of diabetes compared with normal rats, such as polydipsia, polyphagia, and polyuria.

Upregulation of mRNA level of netrin-1 in diabetic retinas

The expression of Netrin-1 mRNA was evaluated by RT-PCR. Netrin-1 mRNA was expressed in both normal and diabetic retinas, but the level of netrin-1 mRNA was dramatically increased in diabetic retinas

at 3 months after onset of diabetes. The difference was statistically significant ($P < 0.05$) (Figures 2A and 2B).

Upregulation of protein level of netrin-1 in diabetic retinas

The protein level of netrin-1 in retinal tissues was determined by western blotting. Netrin-1 was expressed in both normal and diabetic rat retinas, but a significant increase was detected in the diabetic retinas at 3 months after the onset of diabetes ($P < 0.05$,

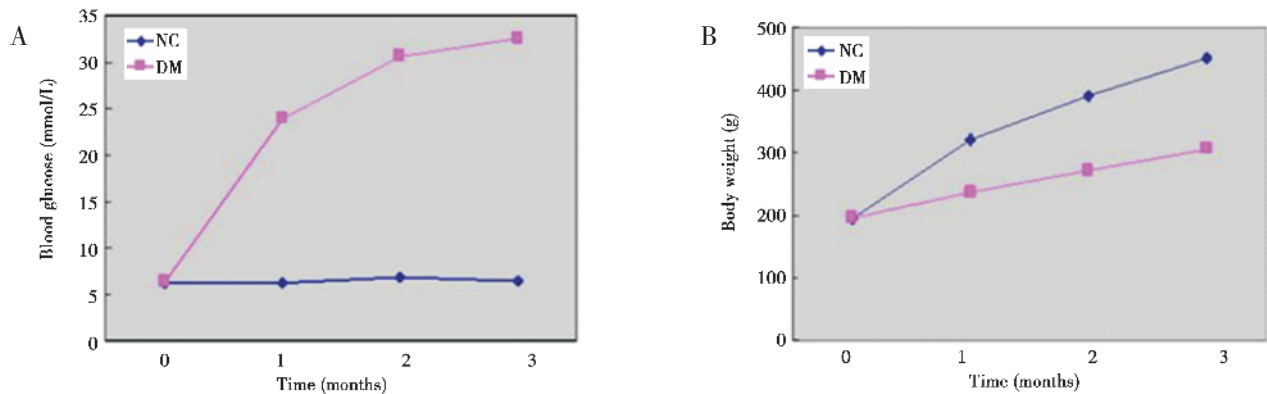


Figure 1 Serum glucose level and body weight of diabetic rats and normal control rats at one to three month after STZ injection. Data are expressed as the mean \pm SE.

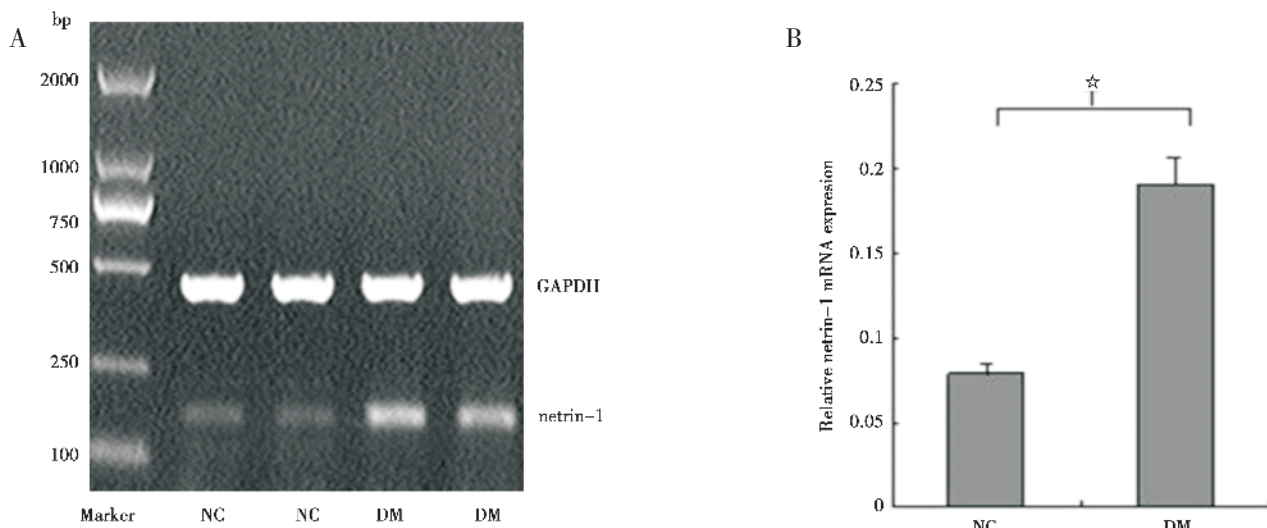


Figure 2 Expression of netrin-1 mRNA in the retinas of diabetic and normal control group. A; Total RNAs of each individual was isolated and netrin-1 or GAPDH transcripts were analyzed by RT-PCR. B; Relative netrin-1 mRNA quantification related to GAPDH mRNA. The level of netrin-1 mRNA was dramatically increased in the retinas of diabetic rats compared to the normal controls ($P < 0.05$)

showed in Figures 3A and 3B).

Discussion

Retinal neovascularization is a major complication

of diabetic retinopathy and hypoxia plays an important role. Decreased blood flow was noted in the retinas of diabetic rats⁸. Moreover, chronic hyperglycemia can lead to oxidative injury, leukostasis,

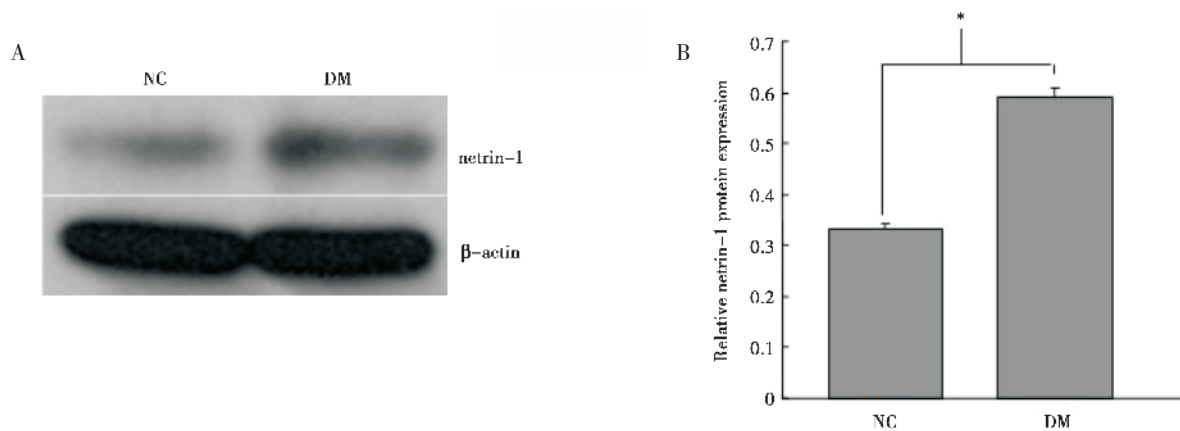


Figure 3 The protein level of netrin-1 in the retinas of diabetic and normal control group. Expression of netrin-1 in the diabetic rats retinas increased statistically compared to the normal controls ($P < 0.05$, marked as *)

microthrombus formation, and activation of cellular adhesion molecules⁹. These responses could give rise to apoptosis of vascular pericytes and endothelial cells and formation of acellular capillaries^{10,11}. The earliest clinical signs of DR are microaneurysms and hemorrhages followed by the formation of dilated, tortuous irregular veins and retinal nonperfusion. The STZ-induced diabetic rat displayed similar retinal morphologic and functional changes to those observed in the early stages of human diabetic retinopathy¹².

Netrin-1 has been confirmed to induce migration, proliferation, and tube formation in multiple endothelial cell lines in *in vivo* and *in vitro* assays with loss-of-function and gain-of-function strategies¹³. It is a potent mitogen and chemoattractant for both endothelial cells and vascular smooth muscle cells (VSMC) with a specific activity comparable to that of VEGF. Moreover, netrin-1 is capable of independently inducing sprout angiogenesis and augmenting the VEGF induced sprout angiogenesis in murine corneal limbus^{14–16}. Tian et al. reported that the mRNA and protein levels of netrin-1 were upregulated simultaneously with the increase of retinal neovascularization in their oxygen induced retinopathy mice model¹⁷. Yang Y et al. verified that netrin-1 promoted placental vascular growth in a matrigel plug assay and an *in vitro* rat aortic ring assay. They observed that netrin-1 was strongly stimulated during *in vivo* neovascularization in a mouse matrigel plug and induced sprouting of endothelial cells *in vivo* in rat aortic rings¹⁸. Here, we found that both mRNA and

protein levels of netrin-1 were upregulated in the rat retinas displaying diabetic retinopathy. We speculate that netrin-1 may act as in the same manner as VEGF or other angiogenic growth factors. Over-expression of netrin-1 in diabetic retinas may promote proliferation and differentiation of the endothelial cells and VSMC and even evoke new vessels in order to improve its blood supply and microirrigation in compensation.

So far, netrin-1 has been shown to promote angiogenesis and accelerate revascularization and reperfusion in a murine model of ischemia. However, under hypoxic conditions, the alpha subunit of hypoxia-inducible factor 1 (HIF-1) becomes stable and interacts with coactivators to regulate the expression of target genes that contain the hypoxia response element (HRE). Many genes involved in DR were found to be regulated by HIF-1. Examples include VEGF, VEGFR-1, PDGF-B, endothelin-1, EPO and iNOS. Netrin-1 was strongly induced in response to hypoxia by a mechanism dependent on HIF-1¹⁹. In this study, we found that expression of netrin-1 was significantly increased in diabetic retinas compared to the normal control. We speculate that HIF-1 could also promote expression of netrin-1 at early stage of DR, and may therefore participate in the pathogenesis of DR. However, further experiments are necessary to determine the exact role of netrin-1 in DR.

Disclosure statement

There is no conflict of interest to declare.

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