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Brief communication (Original)

The expression of opticin in vitreous body and retina of diabetes mellitus rats

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Background: Diabetic retinopathy is a common complication of diabetes mellitus. Opcitin is a glycoprotein present in the vitreous body. Its role in diabetic retinopathy needs to be further defined.
Objective: Investigate and compare the mRNA and protein levels of opticin in vitreous body and retina in normal and diabetic rats.
Methods: Twenty-four male Sprague-Dawley (SD) rats were randomly divided into two groups (12/group), a streptozocin-induced diabetes (STZ) group and a control group. In the STZ group, 1% sterile STZ solution was injected into the rats intraperitonally (60mg/kg). An equal volume of sodium citrate buffer solution was administrated in the rats from the control group. The rats were sacrificed one month after various treatments. The eye bodies of three rats from each group were removed and fixed with 4% paraformaldehydeion for the following pathological analysis. Meanwhile, the vitreous bodies and retina of the other nine rats from each group were removed for the real-time PCR, immunohistochemistry, and western blot assays.
Results: The mRNA level of opticin in the vitreous body of diabetes mellitus (DM) rats was 5.66% of that of the control ones (p <0.01). The expression of opticin mRNA in retina of DM rats was 9.28% of that of the control ones (p <0.01). In addition, opticin protein was expressed in the vitreous body and retina of the normal rats, whereas it was negative in the DM ones.
Conclusions: The opticin expression in vitreous body and retina of diabetes rats was significantly decreased or even disappeared, which may suggest a key role of opticin in the development of diabetic retinopathy.

Keywords: Diabetic retinopathy, opticin, rat

Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus (DM), and it is the fourth cause for the blindness followed by cataract, corneal disease, and glaucoma. With the increasing prevalence of DM, the number of DR patients is increasing, which can pose a significant burden to the society.

Opticin, a new glycoprotein found by Reardon et al. [1], belongs to the third category of leucine-rich repeats (LRR) proteoglycan family. It can stably exist in water solution with a dimmer of approximately 90kDa. Opticin is mainly present in the vitreous body and its mRNA can be determined in various tissues. Thus, Opticin plays an important role in maintaining vitreous body stability [2], linking the vitreous body [3] and the retina, and inhibiting angiogenesis [4-6].

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It was confirmed that Opticin could significantly suppress vascular endothelial growth factor opticin-A (VEGF-A) and fibroblast growth factor-2 (FGF-2) induced proliferation, metastasis, and budding of bovine vascular endothelial cells [5]. It might be due to vascular disappearance in the vitreous body in the development of eye or some proliferative diseases such as proliferative diabetic retinopathy (PDR) [2].

In the present study we produced streptozotocin (STZ) induced diabetic rat model. Then we assayed the mRNA and protein expressions of opticin in the vitreous body and retina. Based on these, we tried to clarify the possible role of opticin in the development of PDR.

Materials and methods

Main reagents

STZ and anti-Opticin antibody were purchased from Santa Cruz Biotech Co., Ltd. (CA, USA). First strand cDNA synthesis kit Fermentas and PCR kit

K0171 were obtained from MBI Biotech Co., Ltd. (USA).

Establishment of animal model and grouping

Twenty-four SPF Sprague-Dawley (SD) rats weighing 200 to 250g were provided by the Experimental Center of Chinese Center for Disease Control and Prevention (Beijing, China). All the experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals. This study was approved by the Ethnic Committee of Hubei Province.

The animals were raised in different cages (2/cage) and subjected to a 12-hour light/dark cycle. The animal room was maintained at 20±2°C and 30 to 60% relative humidity. The rats were given free access to standard food and water. The rats were randomly divided into STZ injection group and a control group (12/group). In the STZ injection group, 1% sterile STZ solution was injected into the rats intraperitonally (60mg/kg). An equal volume of 0.1mol/l sodium dihydrogen citrate buffer (6mg/kg) was injected into the rats from the control group.

Blood and urine sample were collected 72 hours after the injections. The diabetic model was confirmed to be successfully established when the blood glucose was equal to or more than 16.7mmol/l and urine glucose was positive. The blood glucose was measured using a ONE TOUCH blood glucose meter or standard glucose testing strips. In this part of the experiment, the weight and blood glucose were monitored every 2 weeks. After 1 month, the rats were sacrificed under an anesthesia by an intraperitonal injection of 10% ketamine (0.1 ml/100g). The eyeballs were removed from three rats in each group. The vitreous body and retina were collected from the residual nine rats in each group. The eyeballs were treated with 4% paraformaldehyde fixation for the following assay. The vitreous body and retina were stored in liquid nitrogen for later use.

Quantitative PCR assay of Opticin mRNA in vitreous body and retina

Vitreous body and retina were homogenized in a glass homogenizer and then 1.5 ml Trizol (Invitrogen, USA) was added. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, USA) in accordance with the manufacturer's instructions.

After DNase I treatment, 2 μg of RNA was reversely transcribed using a ReverTra Ace-TM kit (Toyobo, Japan). Then the following primers were used: Opticin (150-bp product), forward (5'-GCCACTTAATTTGCATTTCGC-3') and reverse (5'-ATCCCTTTCTTTCAT GGTTCTCC-3'); β-actin (149-bp product), forward (52 -GAAAAGATGACC CAGATCATG-32) and reverse (52 -ATCTTCATGA GGTAGTCCGTC-32). The primers were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

25 μl of standard reaction system included 12.5 μl of Real time PCR Master Mix SYBR GreenI, 0.5 μl of primer forward (10 μmol/l), 0.5 μl of primer reverse (10 μmol/l), 1 μl of cDNA, and 10.5 μl of ddH_2O. The following reactions were performed for 40 cycles. The annealing temperature was maintained at 55°C; the rest of the conditions included denaturation at 94°C for five seconds followed by extension at 72°C for 10 seconds. The data were analyzed using IQ5 software of Gene express module (Bio-Rad, USA). Three replicate reactions were performed and values were normalized to the housekeeping gene GAPDH. C_T values were determined by using the 7500 System SDS Software (version 1.2.3; Applied Biosystems, USA). Expression ratios were finally calculated in accordance with $2^{-\Delta\Delta CT}$ method.

Immunohistochemistry analysis

The eyeballs were fixed in 4% paraformaldehyde for 24 hours. Then they were processed using degrease, rehydration in graded alcohols, and further processed using the streptavidin immunoperoxidase method. In brief, the sections were submitted for antigen retrieval at 92 to 95°C for 10 minutes in citrate buffer (0.01mol/l, pH6.0). Subsequently, the slides were incubated in 10% normal serum for 30 minutes, followed by incubation overnight at 4°C with the diluted rabbit anti-rat Opticin polyclonal antibody (Santa Cruz, USA) antibody (1:100). The slides were then incubated with biotinylated anti-rabbit immunoglobulins for 30 minutes at 37°C, followed by streptavidin peroxidase complex for 15 minutes at 37°C. PBS (pH7.4) was selected as a negative control.

Western blot assay

The total tissue protein was extracted using a total protein extract kit in accordance with the manufacturer's instructions. The concentration of the total protein was quantified by Bradford method.

Thirty micrograms of the proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE), and then the proteins were transferred onto a sheet of polyvinylidene fluoride (PVDF) transfer membrane. The transfer membrane was semi-dry at 300 mA for 30 minutes. Then, the membrane was blocked by 5% skim milk for four hours. The membrane was washed three times with tris buffered saline (TBS) for five minutes each. Subsequently, rabbit anti-rat opticin polyclonal antibody (Santa Cruz Biotechnology, USA) antibody (1:200) was added and incubated at 4°C overnight. Then HRP-conjugated goat anti- rabbit IgG (1:3000) (Zhongshan Golden Bridge Biotechnology Co., Beijing, China) was added and incubated at room temperature for another two hours. The membrane was stained by enhanced chemiluminescence (ECL) reagent (Pierce, USA) and imaged on X-ray film (Fuji film, Japan) by autoradiography. Quantity one Imagine System and analysis software (Bio-Rad, USA) were used to analyze the specific straps quantitatively. β-actin was selected as an internal control. The relative protein level is expressed as a ratio between the densities of opticin and β-actin.

Statistics and presentation of data

All data were expressed as mean□standard deviation. Student's t-test was used to compare the difference between groups. p <0.05 was defined as statistically significant.

Results

The expressions of opticin mRNA is down-regulated in vitreous body and retina in the diabetes rats

The mRNA levels of opticin in the vitreous body and retina of DM rats was significantly reduced compared to control. The opticin in the vitreous body and retina of DM rats was only 5.66% and 9.28% of the control respectively (p <0.01) as shown in **Figure 1**.

The expression and location of opticin protein in vitreous body and retina in the diabetes rats

Western blot result revealed that opticin protein clearly appeared at a site of about 45 kDa. However, there was no strap in the vitreous body and retina of diabetic rats. It showed that compared with the normal rats the expression of opticin protein was significantly decreased or disappeared in the vitreous body and retina of diabetic animals.

Meanwhile, the opticin protein in the normal rats was mainly distributed in the vitreous body and retina. In some sections it was expressed in iris, ciliary body, and sclera. The brown staining was present in the vitreous body. The staining was darker at the closer surrounding region. In addition, the brown staining was also observed in the retina. However, no positive staining was seen in the vitreous body and retina of the DM rats (**Figure 2)**.

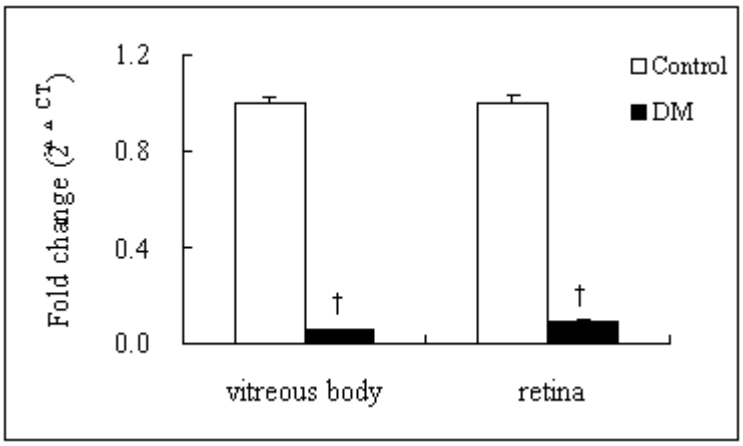

Figure 1. The expression of opticin mRNA in the vitreous body and retina in the normal and diabetic rats ($\bar{x}$±s, n = 3, 9). □p<0.01 versus Control

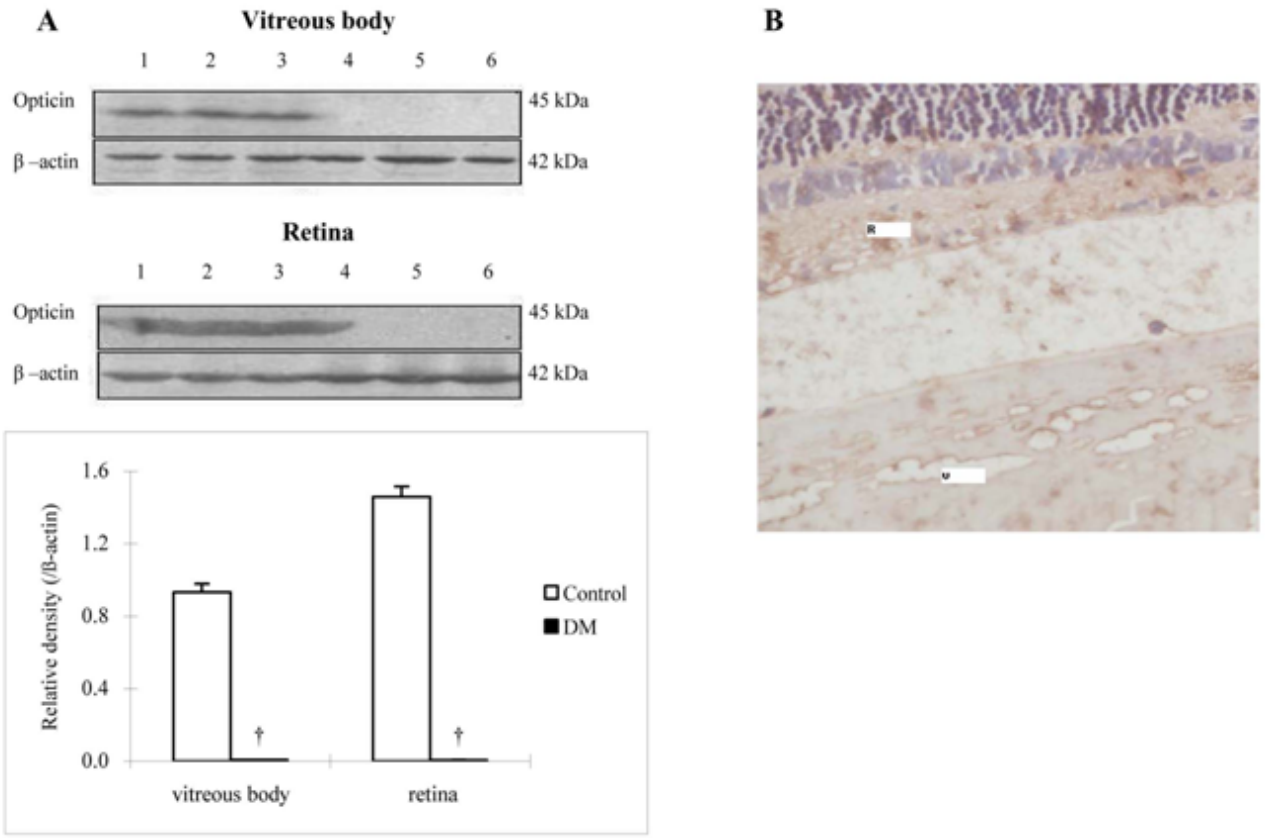

Figure 2. The expression of opticin protein in vitreous body and retina in the normal and DM rats ($\bar{x}\pm s$, n = 3, 9). **A:** Western blot assay of Opticin protein in vitreous body and retina. Lane 1-3: Control group; Lane 4-6: DM group. †p <0.01 versus control. **B:** The distribution of Opticin protein in the vitreous body and retina of diabetic rats. R = Retina, V = Vitreous body.

Discussion

DM is a systemic disease with glucose metabolic disorder and the prevalence is increasing yearly. Diabetic retinopathy (DR) is a common serious complication and it is one important reason for blindness [10]. Generally, DR can be divided into two stages including background retinopathy and proliferative retinopathy. The formation of new blood vessels mainly in the vitreous body and retina is a sign for proliferative retinopathy. Actually, capillary abnormalities, angiogenesis, and blood-retinal barrier destruction are involved in the occurrence and development of DR. However, the pathogenesis has not been clearly elucidated.

In 2000, Reardon et al. first found a new glycoprotein named Opticin from vitreous body [1]. Opticin belongs to the leucine-rich repeats (LRR) proteoglycan family and it can stably exist in water solution with a dimmer of approximately 90kDa. The polymerization site for the two opticin monomers is likely to be its leucine-rich repeats (LRR) domain. This dimmer will be degenerated into two monomers after metformin hydrochloride extraction or electrophoresis.

Opticin is secreted by ciliary body. Hobby et al. found that opticin gene was located at 1q31 by radiation hybrid genetic positioning technology [11]. Opticin is highly expressed in eyeball perpetually and it is distributed in the vitreous body and retina. The Opticin mRNA can be measured in multiple tissues. The expression of Opticin protein was also found in human iris, ciliary body, and retina. In addition, opticin protein was expressed in mouse trabecular meshwork, iris, vitreous body, retina, optic papilla, and brain [6]. Friedman et al. confirmed that the opticin was widely expressed in human vitreous body, cornea, iris, ciliary body, and choroid using an N-terminal purified antibody [7]. A study showed that Opticin was relevant to the growth and development of vitreous body [9], which was helpful to maintain the stability of vitreous body

[2], link the vitreous body [3] and retina, and inhibit angiogenesis [4, 10, 12].

Meanwhile, Opticin is a ligand of integrin, which might be associated with the angiogenesis in vitreous body [4]. It was confirmed that Opticin could significantly suppress vascular endothelial growth factor opticin-A (VEGF-A) and fibroblast growth factor-2 (FGF-2) induced proliferation, metastasis, and budding of bovine vascular endothelial cells [5]. Matthew et al. found that Opticin could reduce 31% of endothelial cells migration, 37% of tubular formation, and 45% of colloid invasion. In addition, Opticin significantly attenuated the formation of chicken allantoic flocking membrane vessels.

Subsequent studies found that Opticin reduced FGF-2 induced MAPK phosphorylation which was crucial to the cell migration and the formation of tubular structure. Thus, Opticin may be a potential inhibitor to suppress the vascular invasion into vitreous body. It was confirmed that Opticin was an endogenous angiogenesis inhibitor that could interact with $\alpha 2\beta 1$ integrin [14]. Opticin displayed antiangiogenesis and antiproliferative properties in breast cancer cells, which was consistent with *in vitro* and *in vivo* experiments [15]. Study on retina proliferative diseases including proliferative diabetic retinopathy, central retinal vein occlusion, posterior vitreous membrane and internal retinal membrane revealed that even in the late stage of proliferative retinal diseases, ciliary epithelial cells would continue to express and release Opticin. Opticin interacts with Collagen type II and exists in the retina epiretinal membrane where it might act on the generation of the retina epiretinal membrane in the presence of proliferative diabetic retinopathy. Therefore, Opticin may be associated with PDR. On base of this, Opticin can be selected as a candidate gene to study the etiology of DR.

In the present study, we assayed the expression of opticin mRNA in rats' vitreous body and retina by real-time PCR. Western blot and immuno-histochemistry were used to measure the expression of opticin protein. The results suggested that opticin was highly expressed at both mRNA and protein levels in the normal rats. However, the expression of opticin mRNA in the vitreous body and retina from the diabetic rats was significantly downregulated and the levels were 5.66% and 9.28% of that of the normal rats respectively. Meanwhile, the Opticin protein was not determined after one month in the diabetic rats. These findings demonstrated that the expression of opticin was decreased at the late stage of PDR, which confirmed the speculation of Ramesh et al. that Opticin indeed played some role in the PDR [16]. However, the relationship between Opticin and PDR needs further investigation.

The authors have no conflicts of interest to declare.

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