Original Article
Neuron-derived netrin-1 and netrin-4 proteins are additional effective targets in diabetic retinopathy beyond VEGF

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Abstract: Vascular endothelial growth factor (VEGF) is the typical representative factor of diabetic retinopathy (DR) and is considered to be a key inducer of retinal vascular permeability in DR. Anti-VEGF has been widely used in clinical treatment but every patient is effective, therefore, it is necessary to find other effective factors that participate in the pathology of DR. We provide evidence from both human and animal experiments for the considerable roles of classical neuronal guidance factors, netrin-1 and netrin-4, in indicating and amending the pathology of DR. We reveal that levels of both netrin-1 and netrin-4 are reduced while VEGF increases in DR patients and animal models. We demonstrate through different experimental methods that augmenting netrin-1 and netrin-4 can alleviate vasculopathy and neuropathy which appear in DR. Our findings offer additional effect targets besides VEGF for DR and suggest we should increase the focus on neurovascular crosstalk as DR is a neurovascular disease.

Keywords: Netrin-1, netrin-4, VEGF, diabetic retinopathy, neurovascular disease

Introduction

Diabetic retinopathy (DR) is the most common and serious complication of diabetes. It is characterized by the appearance of neurodegeneration and microangioma in the early stage and the development of neovascularization, vitreous hemorrhage, and retinal detachment in the advanced stage. Although there are numerous therapies, DR remains a major cause of visual loss in working adults in developed countries [1]. Recently, an increasing number of studies have postulated that neurodegeneration is an early change in DR, which includes neuronal apoptosis and reactive gliosis. It is also related to glutamate excitotoxicity, reduced neuroprotective factors and impairment of the neurovascular coupling [2-5]. Astrocytes and Muller cells support neurons and blood vessels (Stone et al., 1987). Their synapses are involved in forming the blood-retinal barrier (BRB) and when they have a dysfunction, the BRB leaks. Above all, DR should be considered as a neurovascular complication.

Various hypothetical mechanisms have presented for DR: (1) biochemical and molecular changes [6, 7]: increase of polyol-inositol metabolism, advanced glycation end products, protein kinase C activation and oxidative stress; (2) inflammatory mechanisms: abnormal increase expression of ICAM in vascular endothelial cells leading to leukocytic aggregation and neutrophil infiltration [8], representative inflammatory factors such as tumor necrosis factor-a, IL (interleukin)-6, and IL-1β are increased in retinas of DR patients or rats [9]; and (3) excessive expression of vascular endothelial growth factors (VEGF): VEGF involves in the formation of retinal neurodegeneration in the development of the early microvascular changes which occur in DR, such as the breakdown of the blood-retinal barrier (BRB) [10, 11], vasoregression [12], and the impairment of neurovascular coupling [13, 14]. It is considered to be a key inducer of retinal vascular permeability [15]. This mechanism has revolutionary significance in the therapy of DR and anti-VEGF agents have had sound clinical value.
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in recent years. All the above mechanisms lead to two pathological phenomena: neural and vascular damage.

Although different mechanisms have been elucidated in the formation of neurodegeneration or neovascularization [4, 16], considerably less is understood about the pathological processes of common pathways that lead to vasculopathy and neuropathy. Current studies on the molecular mechanisms that cause DR have mostly focused on VEGF. Anti-VEGF has been widely used in clinical treatment all over the world, but some patients receive little or no effect from it. Other effective therapies include intravitreal corticosteroid, laser therapy and vitrectomy, but none of them can reverse the progress of DR. Moreover, all the above cures present some level of side effects. These include increased intraocular pressure and cataract formation, the occurrence of thromboembolic events [17] and potential neuronal toxicity [18]. Even the most mature and applicable treatment of panretinal photocoagulation can sometimes result in the formation of reduced visual fields and scotomas. The limitations of current therapies indicate the urgent need for new interventions.

Many molecular mechanisms have been revealed in vasculopathy with DR, and there is sufficient evidence for a mutual pathway between nerves and blood vessels, which is called neurovascular crosstalk [19]. We have recently revealed that netrin-1 and netrin-4, two members of the netrins family, which is a classical axonal guidance cue, also play a role in microangiopathy. Netrins is a soluble protein family from floor plate cells in rodents that was found by Tessier-Lavigne and colleagues [20-22]. A growing body of research has shown that netrins have a dual effect on angiogenesis [23-26], and they mediate attractive and repulsive responses through different receptors (DCC, UNC-5B, neogenin) and local concentrations. Recent studies have reported that low concentrations of netrin-1 can stimulate pro-angiogenic CD146, which is the co-receptor of the VEGF endothelial receptor that acts on VEGF signaling pathways and then mediates angiogenesis, while an elevated dose of netrin-1 inhibits angiogenesis [25]. Netrin-4 is expressed in all ocular tissue and contributes to angiogenesis in the retina [27, 28]. It acts as a negative regulator of vascular branching [27] and plays a role on VSMC and EC-pericyte interactions and the formation of the basement membrane (BM) [24, 29, 30]. Although evidence has shown that netrin-1 and netrin-4 both work in vascular disease, the function areas are different, and the role of netrin-1 and netrin-4 in mediating the VEGF level and blood retinal barrier in DR has not been determined to date.

In this article, we show that netrins play an important role in mitigating microvascular lesions in DR. We discovered in both DR patients and animal models that netrin-1 and netrin-4 significantly decrease in early and late stages of DR. Increasing the level of netrin-1 and netrin-4 may provide a novel therapy, other than anti-VEGF, against neurovascular disease in DR.

Materials and methods

Clinical samples

Clinical subjects were recruited from patients who visited the Hospital of Qingdao University, Ophthalmology Department. This study was subject to approval by the Ethics Committee of The Affiliated Hospital of Qingdao University and followed the Declaration of Helsinki guidelines. Each patient signed an informed consent form. Fifty proliferative diabetic retinopathy (PDR) patients (the average age was 64-year-old) with tractional detachment and 30 patients with idiopathic macular epiretinal membrane (IMEP) (the average age was 60-year-old), who served as the control group, were recruited. Serum samples were obtained before surgery and fibrovascular membranes and undiluted vitreous were collected during vitrectomy. All patients in the present study underwent an ophthalmoscope examination and fluorescein angiography. Their vitrectomy surgery was performed at one hospital and by one doctor. Samples from IMEP patients were also collected as controls.

Animal model

Eighty adult male Sprague-Dawley rats (180-200 g) were randomly divided into the experimental group and control group. All protocols followed the Institutional Animal Care guidelines. Rats were housed under a constant temperature and humidity and were exposed to a 12:12-h light-dark cycle. Animals had free access to food and water.
The diabetes model was induced by intraperitoneal injection of STZ (60 mg/kg dissolved in sterile saline) and was confirmed 48 h after injection. The rats were subsequently monitored by measuring the glucose concentration in tail blood samples using an Accu-Chek Glucometer (Roche Diagnostic, USA). The control group was injected with sterile saline (n=10), and rats were treated with timed-release insulin at a dose of 1 unit whenever the blood glucose increased above 500 mg/dl. Diabetes model induction followed the procedures of the Animal Models of Diabetic Complication Consortium (AMDCC) [31-33]. Blood glucose and weight were measured in both groups every week until 12 weeks after the success of model building.

We continued to feed the remaining rats of the two groups until 24 weeks, then divided the experimental group into the netrin-1 (2 µl, 100 µg/ml) intravitreal injection group, netrin-4 (2 µl, 100 µg/ml) intravitreal injection group and placebo (2 µl sterile saline) intravitreal injection group. Normal glucose rats were regarded as the control group. After 1 month of treatment, the rats were all put to death (n=15 in each group with a total of 45 remaining DM rats).

Immunofluorescence staining

Fibrovascular membranes and IMEP from clinical samples were collected and eyeball specimens from the animal models were removed from the nucleus. All of them were fixed with 4% paraformaldehyde for 4 h at room temperature, incubated in 30% sucrose overnight and frozen in OCT compound. We then embedded the fibrovascular membranes, IMEP and the whole eyes and made frozen sections. We used normal sheep serum seal slices and washed off the sealing fluid. We added rat antibodies against netrin-1 (netrin-4, VEGF) in the monoclonal antibody mice (2 µg/ml) and Alexa Fluor 568 sheep antibodies against mice (10 µg/ml) on tissue sections as primary and second antibodies. Images were acquired with an inverted fluorescence microscope (Zeiss, Le Pecq, France).

Immunohistochemistry

We made paraffin sections and employed antigen repair (with 0.01 mol/L sodium citrate buffer) and incubation. The sections were then washed and blocked with a normal goat serum working fluid. Rabbit anti-human netrin-1 and anti-rat netrin-1 were used as the primary antibodies and goat anti-rabbit IgG antibodies were used as the secondary antibodies. Images were obtained with a microscope. Netrin-4, VEGF and Occludin were analyzed with the same procedure.

Assessment of netrin-1, netrin-4 and VEGF levels with an ELISA

Netrin-1 and -4 in the vitreous and VEGF in the vitreous of patient and rat retinas were measured with an ELISA according to the manufacturer’s instructions (Neo Scientific). Color changes were measured with a plate reader (Synergy 2. Bio Tek Inc.) at a wavelength of 450 nm. Concentrations were determined by comparing the O.D. of the samples to a standard curve. All assays were conducted in duplicate. The exact values of Netrin-1, netrin-4, VEGF in human vitreous are contained in the (Table S1).

RT-PCR

RT-PCR detected netrin-1, netrin-4, and VEGF mRNA expression in patient fibrovascular membranes, the epiretinal membrane and SD rat retinas, respectively. Total RNA of the clinical and retinal samples was isolated using Trizol reagent (Invitrogen, US). GAPDH was used as the internal gene. Netrin-1, netrin-4 and VEGF primers were subjected to double-stranded chimeric fluorescent staining (SYBR Green I) to amplify the corresponding gene. Netrin-1, netrin-4, VEGF and GAPDH upstream and downstream primer sequences are listed in the (Table S2). The annealing temperature was 60°C.

Evans blue permeation assay

Retinal EB permeation was performed following the description in Xu et al. (2001) with modifications. EB was injected at 45 mg/kg through intrajugular injection and circulated for 2 h before retinal extraction. Stretched preparation of the retina to observe vascular leakage was performed with fluorescence microscopy. Vascular permeation (EB permeation, EBP) was quantified in the fellow eye. Formamide 0.3 ml was added and incubated in 70°C for 18 h. Absorbance differences [A, old term: optical density (OD)] between 620 nm and 740 nm were analyzed. The difference value × 1,000=net value of A. EBP (measured in ng/mg) was calculated as (EB [mg]/dry retinal weight [g])/plasma EB per unit time [mg]/
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of PDR patients with tractional retinal detachment due to the role of neovascularization and fibrovascular membranes. There were no significant differences in gender, age, body mass index (BMI), waist hip ratio and hypertension history between PDR patients and controls. In 50 cases of PDR patients, 20 cases underwent whole retinal photocoagulation, all after more than four months. An ELISA was performed to detect the levels of netrin-1, netrin-4 and VEGF in the vitreous. The results showed that the concentrations of netrin-1 and netrin-4 in the vitreous of PDR patients were lower than the control group, and the difference had statistical significance (P = 0.027; P = 0.025). However, VEGF in the vitreous of PDR patients had an obviously higher concentration when compared to the control group (P < 0.001; Table S1; Figure 1A). To explore whether there was a relationship between the decrease in netrins and increase in VEGF, we carried out a correlation analysis. The results showed that the level of VEGF, netrin-1, and netrin-4 in the vitreous of PDR patients had a high negative correlation and the correlation had statistical significance (R = -0.904, F = 215.7, P < 0.0001; R = 0.933, F = 322.6, P < 0.0001; Figure 1B), while factors in the corresponding control group showed no correlation.

Netrin-1 and netrin-4 are weakly expressed in the fibrovascular membranes of PDR patients from the immunofluorescence staining and RT-PCR analysis

To further identify the expression of netrin-1 and netrin-4 in PDR patients, fibrovascular membranes in the experimental group and IMEP in the control group were collected. Consistent with the ELISA results in the vitreous, immunofluorescence staining-based detection of netrin-1 and netrin-4 showed weak expression in the fibrovascular membranes, while normal expression in the IMEP was observed. Fluorescent dots in fibrovascular membranes were difficult to observe, while the IMEP) had a certain degree of dot fluorescent dye. VEGF showed a significant increase in the fibrovascular membranes when compared to expression in the IMEP compared to the control group, who had obvious fluorescent tags in the PDR group and weak expression in the control group (Figure 2A). RT-PCR analysis of samples from these two group of patients corroborated data from the ELISA and immunofluorescence sta-
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Figure 2. A. Immunofluorescence staining results in human specimens. a, c, e. Represent the expression of netrin-1, 4 and VEGF in fibrovascular membranes, respectively; b, d, f. Represent their expression in the IMEP. These factors were assessed by immunofluorescence staining. B. RT-PCR gel electrophoresis results of netrin-1, 4 and VEGF in the two groups. Lanes 1 and 2 represent the IMEP group and PDR group, respectively. Netrin-1 and netrin-4 in the IMEP group had higher expression compared to the PDR group, while VEGF had a reverse result.
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Further examination revealed that netrin-1 and netrin-4 were roundly decreased in patients with late stage of DR. RT-PCR results are shown in Figure 2B: Lane 1 and 2 represents the IMEP group and the PDR group, respectively. For contrast, we tuned the internal reference GAPDH into the same brightness as the two sets of samples. It was evident that Lane 1 showed an obvious bright stripe compared to Lane 2 for netrin-1 and netrin-4, while a darker stripe was observed in Lane 1 compared to Lane 2 for VEGF. All the above data and correlations in human samples suggest the necessity to explore the role of netrin-1 and netrin-4 in diabetes-induced lesions.

**Body weight and blood glucose changes in streptozotocin-induced diabetic rats**

Before the implementation of the experiment, weights in the control and DM groups were 180 ± 3.0 g and 181 ± 3.1 g. Weights in control group gradually increased and the average weight increased to 489 ± 14 g at 12 weeks, while in the DM group, weight increased slowly in the first 4 weeks and then fell off. The middleweight was 189 ± 11 g at 12 weeks. During the experimental process, blood glucose levels in the control group were stable and the levels were all below 10 mmol/l, while before STZ pre-injection, blood glucose in the DM group was similar to those of the control group at 24 h after injection. Nonfasted blood sugar levels had a significant rise, which was all steadily above 16.7 mmol/L. The value was 29.2 ± 3.8 mmol/L after being induced for 12 weeks. The above results indicated that the model of diabetes mellitus was successfully established. The diabetic rats were maintained for 28 weeks and some of them were then executed at different points according to the experimental process. Weight changes and glycemic excursions after intraperitoneal injection of streptozotocin (STZ) are shown in (Figure S1).

**Neuronal netrin-1 and netrin-4 were decreased in the early stage of streptozotocin-induced diabetic retinopathy**

Given the decreased levels of netrin-1 and netrin-4 and their correlation with VEGF in the vitreous and fibrovascular membranes in PDR patients, we decided to further detect the expression of these factors in the early stages of streptozotocin-induced DR models. We detected netrin-1 and netrin-4 at 6 months of DM through immunohistochemical staining (IHC) analysis. The results showed that netrin-1 and netrin-4 had a modest expression in the vehicle-injected control group and was mainly expressed in the ganglion cells and nerve fiber layer, while they had lower-expression in the DM group. The dyes in the corresponding parts were lighter. The immunohistochemical staining results of netrin-1 and netrin-4 in the two groups are presented in Figure 3A. As a previous study certificated, netrin-1 produced by retinal neurons [34] and netrin-4 can generate in all ocular tissues [27] which corresponded to our IHE results that netrin-1 and netrin-4 were present in the ganglion cell layer. To corroborate the drop of netrins and their relationship with netrins in the retina, RT-PCR was performed to further detect their RNA expression levels. We could detect the levels of netrins and VEGF as early as 4 weeks after induction of DM. After measuring their levels at 3 months and 6 months, we found that netrin-1 and netrin-4 decreased and VEGF was upregulated as time continued (Figure 3B).

**Exogenous injection of netrin-1 and netrin-4 have efficiently protective effects on reducing vascular permeability of DR**

After evaluating the expression of netrin-1 and netrin-4 in DR patients and DM animal models, we proceeded to upregulate their levels over 24 weeks of DM by intravitreal injection to investigate the potential therapeutic in DR (n=15 in each group). After injection for 4 weeks, we used a stretched preparation of the retina to observe retinal vascular permeability with fluorescence microscopy and quantitatively evaluate vascular leakage by Evans blue permeation (EBP). Without any pathological changes in the normal blood vessels, retinal microangiomas were observed and vascular leakage was significantly augmented in the DM group. Meaningfully, after injecting exogenous netrin-1 and netrin-4 into rat eyes, reduction of fluorescein leakage occurred (Figure 4A). Retinal permeability test results showed that the EBP in the normal control group, the DM group, netrin-1 injected group and netrin-4 injected group, respectively were (10.03 ± 2.04), (35.22 ± 4.89), (21.45 ± 3.16) and (22.14 ± 3.24) ng/mg (Figure 4B). EBP in the DM group was distinctly higher than in the normal group (P<0.001), and the permeation in the netrins
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Figure 3. A. Immunohistochemical staining of netrin-1, 4 in the two groups. a, c. Representative netrin-1 and netrin-4 in the control group with both mainly expressed in the ganglion cell layer; b, d. Representative netrin-1 and -4 at 6 months in the DM group, respectively. They had weaker expression in the corresponding location (n=3). The arrow notes the location of Occludin protein. B. 1, 2, 3, and 4 lanes were controls, DM at 1 month, at 3 months, and at 6 months, respectively (n=3 at each point). Expression of netrin-1 and netrin-4 gradually reduced with the passage of time, while VEGF expression increased simultaneously.

Concordantly, IA value of retina (IA represented the expression of occludin) in netrin-1 (3.04 ± 0.52; P<0.001), netrin-4 (3.02 ± 0.54; P<0.001) group, respectively, had a significantly higher level than in the DM group (1.32 ± 0.22), while the value in the normal group (4.85 ± 0.81) was higher than any of them (Figure 5B). Further evidence of netrin-1 and netrin-4 injected group was evidently reduced compared with the DM group (P_{nt1}<0.001; P_{nt4} <0.002).

Concordantly, IA value of retina (IA represented the expression of occludin) in netrin-1
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in mitigating diabetic angiopathies was obtained from IHE. In this respect, we detected the expression of Occludin protein, a tight junction protein that exists between endothelial cells and cell junctions. Consistent with the result of IA values, IHE showed decreased expression of Occludin around the blood vessels in the DM group, while there was trachychromatic staining in the netrin-1 and netrin-4 group compared to the DR group (Figure 5A). Notably, the observed increased levels of netrin-mediated reduced retinal vascular permeability were suggestive of an effect in protecting the barrier function.

Aggrandize concentrations of netrin-1 and netrin-4 can reduce VEGF levels in diabetic retinopathy

In light of the negative correlation between netrins and VEGF in the PDR vitreous, we sought to explore whether netrins have an impact on VEGF. IHE results demonstrated that after increases in the content of netrin-1 and netrin-4 in retina, VEGF had a meaningful regressive level, while there was an exorbitant level in the DR group at the same time point (Figure 6A). Further, we used an ELISA to detect VEGF levels in different groups, VEGF in

Figure 4. A. Retinal stretched preparation and vascular leakages. A stretched preparation of retina was observed and compared under the same fluorescence microscope with the same exposure time (n=3). An arrow notes the EB leakage area, and the arrowhead indicated the microangioma. B. Retina permeability test. A retina permeability test showed that the DM group had a significant vascular leakage compared with the control group, and N1 and N4 could decrease the leakage.
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Figure 5. A. Occludin localization in the retinas of different groups. IHE results showed that Occludin positive expression was around the vascular wall and inner nuclear layer in the control group and the color was deep brown, while in the DM group, the positive staining was lighter and the expression was weakened. The positive protein became deeply stained with the increase of N1 and N4 in retinas (n=3). (The arrow indicates the Occludin expression site). B. IA value of retina. IA value represents the content of Occludin protein. The DM group had a lower level of Occludin than the CON group, after injection of N1 and N4, Occludin levels were significantly increased. *, #, &P<0.05. (*, #, &represents the comparison between DM and each group, DM and N1 group, DM and N4 group, respectively).

Discussion

Diabetic retinopathy is a complex complication of diabetes, which results in severe visual loss. At present, there are many symptomatic treatments, such as anti-VEGF, laser therapy and vitrectomy, but none of them can reverse the progression of DR. This reminds us of the need to explore other therapeutic avenues. An increasing number of mechanisms revealed that DR is a neurovascular lesion that damages nerves and blood vessels of the retina [35-37]. Therefore, looking for a common pathway of nerves and blood vessels in the study of DR is of great significance. In the present study, we produced evidence that in a health retina, netrin-1 and netrin-4 are modestly expressed,
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while they have degraded levels during the development of DR. The level of netrin-1 and netrin-4 detected in fibrovascular membranes and IMEP were valuable data and had great significance because the two clinical specimens are hard to collect and intuitively reflected the level of tissue disease. The discoveries and studies of netrins have been important for other diseases besides DR for more than 20 years [20-22]. We demonstrated that overexpression of netrin-1 and netrin-4 levels have a protective role in vascular damage and can decrease the expression of VEGF, a vascular destructive factor.

As a neuronal guidance factor, the netrin family exerts itself as a candidate for curing neovascular diseases [25, 28]. There is evidence that an exogenous netrin injection has a protective effect on neuronal apoptosis and anti-angiogenic formation [27]. These make netrins a novel therapeutic target in DR, which is a neurovascular disease. Among the netrin family, netrin-1 and netrin-4 seem to be the most com-

Figure 6. A. IHE staining for VEGF. VEGF mainly showed on the vascular wall in ganglion cell layer in control group, the color was brown (as indicated by the black arrows); the expression was obviously increased and the expressive ranges were wider, from ganglion cell layer to inner nuclear layer in DM group (as indicated by the black arrows); After injected N1 and N4, the expression of VEGF return to normal position and content in retinal vessels (as indicated by the black arrows) (n=3). B. The concentration of VEGF in each group. ELISA results showed that the content of VEGF in DM group was obviously higher than control group, while the expressions were down-regulated after N1 and N4 injection. *, #, &P<0.05. (n=3; *, #, &represents the comparison between DM and each group, DM and N1 group, DM and N4 group, respectively).
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Netrin-1 and netrin-4 can be produced in retinal ganglion cells and other layers of the retina, which is consistent with our IHE results suggesting that netrin-1 and netrin-4 are mainly expressed in the ganglion cell layer in normal retinas, while both have a pale tint in the corresponding position of DR. All the above results reveal that netrin-1 and netrin-4 have some relationships with the development of DR.

To further verify the role of netrin-1 and netrin-4, we injected exogenous abundant netrin-1 and netrin-4 into the vitreous of diabetic rats, and the results showed both of them can reduce retinal vascular permeability. All our findings were consistent with those of several studies that suggested that the guidance cue of netrin-1 is associated with the processing of DR [38]. It indicates that full-length netrin-1 has a potential role in vascular repair and preferentially preserves the remaining normal vasculature to supply ischemia tissue during retinopathy [34]. Other similar study have reported that elevated doses of netrin-1 can inhibit angiogenesis [25] and netrin-4 acts as an antiangiogenic factor through the regulation of both endothelial and perivascular cells in retina [39]. Given these research results and our data that neural-derived netrin-1 and netrin-4 have decreased levels in the development of DR, increasing their concentrations can be an efficient way in the treatment of this disease.

VEGF is a damaging factor that takes part in neurodegeneration in the early phase of DR and it prompts the retina to produce neovascularizations in advanced DR, and finally results in the formation of proliferative membrane and vitreous hemorrhage [10, 11]. In the present study, an increased level of VEGF in the vitreous can be detected in every DR patient and experimental animal, and this illustrates that VEGF plays a promoting role in the pathogenesis of DR. While a completely opposite result in our present study showed that netrin-1 and netrin-4 had decreased levels compared with VEGF in the DM group. We then performed a regression analysis in which we found that netrins have negative relationships with VEGF. This means that netrin-1 and netrin-4 play opposite roles as VEGF in the pathological progress of DR. Further, we verified our speculation that although netrins had a different molecular avenue than VEGF, netrins may have an impact on VEGF. We then observed the expression of VEGF by injecting netrin-1 and netrin-4 in the vitreous of DR rats. The results showed that netrin-1 and netrin-4 indeed decreased the significant augmentation of VEGF in DR rat retinas. Significantly, our results are consistent with a series of recent reported studies that low concentrations of netrin-1 can acting on VEGF signaling pathways and then mediate angiogenesis, while elevated dose of netrin-1 can inhibit angiogenesis [25], and a high expression level of netrin-4 can neutralize the increase of VEGF in oxygen-induced retinopathy [40]. Based on the above views, they further validated our hypothesis that netrin-1 and netrin-4 both have interactions with VEGF.

Similar results were described for other neovascular diseases in that hypersecretion of netrin-1 or netrin-4 by tumor cells puts off the formation of tumor angiogenesis in different animal models [39]. All the above studies suggested that injecting high doses of netrins can prevent the progression of neovascular disease, including diabetic nephropathy, tumors and so on. However, there are still several studies that have shown a totally opposite result in that netrin-1 and netrin-4 are increased in diabetes or diabetic retinopathy [28, 41]. These results may be associated with different testing times and the compensatory mechanisms of the damaged tissue.

By studying the levels of neural-derived factors netrin-1 and netrin-4, and their interaction with VEGF in DR, we obtained a deeper insight into the role of netrins in the participation of neurovascular crosstalk of pathological changes in DR. We provided a novel idea that increased netrin-1 or netrin-4 can be another therapeutic pathway apart from anti-VEGF.

Acknowledgements

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Disclosure of conflict of interest

None.

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References


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**Table S1.** ELISA results of netrin-1, 4, and VEGF in human vitreous

<table>
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<th>Factors (pg/mg)</th>
<th>PDR group (n=50)</th>
<th>Control group (n=30)</th>
<th>P value</th>
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<tr>
<td>Netrin-4</td>
<td>1580.23 ± 200.08</td>
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<td>VEGF</td>
<td>3022.33 ± 125.94</td>
<td>602.18 ± 157.36</td>
<td>P&lt;0.001</td>
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The concentrations of netrin-1, netrin-4 and VEGF in human vitreous were detected by ELISA (n=50 in PDR group; n=30 in CON group). All values in table presented as mean ± SD; Statistical significance: P<0.05, determined by student test.

**Table S2.** Upstream and downstream primer sequence of netrin-1, netrin-4, VEGF and GAPDH

<table>
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<th>Detectable gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Amplified fragment</th>
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Figure S1. The trend of weight and blood glucose. Body weight (A) and blood glucose (B) were determined in the first 3 months in DM and control group after the forming of diabetes rats. The red line represented the control group, the blue line represented DM group.