Neuronal-Driven Angiogenesis: Role of NGF in Retinal Neovascularization in an Oxygen-Induced Retinopathy Model

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PURPOSE. To evaluate the role of nerve growth factor (NGF) in retinal neovascularization in an oxygen-induced retinopathy (OIR) model.

METHODS. The OIR model was established in C57BL/6j mice. NGF mRNA expression in retina was measured by quantitative real-time PCR. NGF expression in protein levels was evaluated by ELISA and immunostaining with NGF antibody. The effects of NGF on retinal neovascularization were evaluated by intravitreal injections of exogenous NGF and TrkA receptor inhibitor K252a, respectively, in an OIR model. Retinal neovascularization was measured by counting neovascular cell nuclei above the internal limiting membrane and by image quantification analysis in flat-mounted retinas perfused with fluorescein dextran.

RESULTS. NGF mRNA in retina had significantly high expression at postnatal day (P)17 in the OIR model compared with normally developing mice. Similarly, ELISA and immunostaining assay showed significantly increased NGF expression in retina at P17 in OIR mice but no significant differences at P12 or P24 compared with normal controls. Exogenous NGF intraocular injection enhanced angiogenesis in the retina in the OIR model; however, injection with K252a, a high-affinity trkA receptor inhibitor, significantly decreased retinal neovascularization compared with that seen in the controls.

CONCLUSIONS. NGF contributed to retinal neovascularization in the OIR model. Intravitreal injection with K252a, the trkA receptor inhibitor, reduced neovascularization, showing the potential therapeutic efficacy of NGF receptor inhibitor in OIR mice. (Invest Ophthalmol Vis Sci. 2010;51:3749–3757) DOI: 10.1167/iovs.09-4226

Neovascularization is the main pathologic feature of severe retinopathy. Abnormal growth of blood vessels and associated vascular leakage in diabetic retinopathy, retinopathy of prematurity, exudative age-related macular degeneration, and vascular occlusions are major causes of vision loss. Neovascularization is like a final common pathway of retinal vascular disease that leads to vision loss, often in response to retinal ischemia. The mechanisms governing this aberrant neovascularization during ischemic retinopathy are not yet defined, though many cytokines have been reported to induce aberrant neovascularization.1–7

It has been postulated that diabetes-induced early changes in neurons and glia contribute to the later development of vascular lesions in diabetic retinopathy.8 Recent studies have shown that neurons and glial cells may interact with blood vessels to contribute to pathologic neovascularization by creating a particular cytokine milieu.9–10 There is increasing interest in understanding the process of neuronal-driven angiogenesis.11–14 It has been demonstrated that neurons secrete growth factors, such as platelet-derived growth factor and vascular endothelial growth factor (VEGF), to guide angiogenic sprouting, particularly in low-oxygen conditions.15–19 Neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor, are well known for their roles in regulating survival, growth, and functional maintenance of neuronal cells. However, NGF recently has been described as a pleiotropic molecule that is involved in a variety of peripheral actions.20–23 Neurotrophic factors such as NGF, alone or in combination with other biologically active endogenous molecules, have been found to exert angiogenic activity in vitro and in vivo.24–28 They have been identified as novel, potent angiogenic molecules that exert a variety of effects on endothelial cells and in the vascular system in general.20–21 Thus far, however, no study has demonstrated the angiogenic activity of neurotrophic factors in the retina. Given that the retina is a neuronal tissue composed of neurons and glia, which produce a great amount of neurotrophic factors, we think it is important to explore the potential function of these neurotrophic factors in retinal vasculopathy.

The biological functions of NGF are mediated through two classes of cell surface receptors, a high-affinity tyrosine kinase receptor (trkA) and a low-affinity receptor p75.22–25 It has been demonstrated that the trkA receptor is expressed in vascular endothelial cells.26 The finding that neural guidance molecules influence blood vessel growth has suggested that neurotrophic molecules may regulate pathologic angiogenesis.27–29 We hypothesized that neuronal and glial cells from the retina may release specific neurotrophic factors such as NGF in response to ischemic injury resulting from hypoxia and that these may couple with other angiogenic factors to enhance endothelial cell activity mediated through a high-affinity trkA receptor, thus contributing to pathologic blood vessel alterations. In this study, we evaluated NGF expression in the retina of an oxygen-induced retinopathy (OIR) mouse model. We further studied the effects of NGF on neovascularization by performing intravitreal injection with exogenous NGF in OIR mice. To determine the significance of NGF and its receptor system (NGF/NGF-R) in the angiogenic process of neovascularization, we then administered K252a, a high-affinity NGF receptor (TrkA) inhibitor, to discover whether it could de-
crease the process of neovascularization.\textsuperscript{35,36} We also studied the tube-forming activity of retinal vascular endothelial cells in vitro after treatment with NGF.

**MATERIALS AND METHODS**

**Mouse Model of OIR**

C57BL/6j mice from Animal Laboratories of Zhongshan Ophthalmic Center (Guangzhou, China) were used. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center. OIR has been widely studied as an animal model of retinal neovascularization disease. According to a previously described method of Smith et al.,\textsuperscript{37} we used the following procedures to produce the OIR model. On postnatal day (P)7, the mouse pups and nursing mothers were placed in an airtight incubator (own production) ventilated by a mixture of oxygen and air to a final oxygen fraction of 75\% \pm 2\%. Oxygen levels were checked at least three times a day. OIR mice were returned to room air at P12.

One hundred thirty-six animals were divided randomly into two groups. Sixty-eight mice underwent the OIR procedure, and 68 untreated mice were used as normal controls to evaluate NGF expression in the retina. Thirty mice in each group were to undergo RNA isolation and real-time PCR, 30 in each group were to undergo protein extraction (ELISA), and 8 in each group were to undergo immunohistochemical staining. Forty additional mice underwent the OIR procedure for intraocular injection study.

**RNA Isolation and Quantitative Real-Time PCR**

Mice were killed and the retinas dissected from 30 OIR mice and 30 normal controls for RNA isolation at P12, P17, and P24 days (n = 10 for each time point). Retinas were collected and pooled together from at least two mice (four eyes) in the same group and were immediately frozen in liquid nitrogen. Frozen retinas were then pulverized, resuspended in reagent (Trizol; Life Technologies Ltd., Paisley, UK), and homogenized (Qiashredder; Qiagen Ltd., Crawley, UK). RNA was extracted according to the manufacturer’s protocol and treated with DNase I. The extracted total RNA was then reverse-transcribed with reverse transcriptase (Superscript II; Life Technologies Ltd.) according to the manufacturer’s instructions. The cDNA was diluted fivefold before PCR amplification.

Real-time PCR reactions were performed in a 50-μL mixture containing 5 μL cDNA preparation, 5× PCR mix (iQ SYBR Green mix; Bio-Rad, Hercules, CA), and 10 pmol each primer in a thermocycler (ABI 7500 Real-Time PCR system; Applied Biosystems, Foster City, CA). The following PCR parameters were used: 93°C for 3 minutes followed by 30 cycles at 93°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. The fluorescence threshold (Ct) was calculated with the system software. The absence of nonspecific products was confirmed by both the analysis of the melting point agarose curves and by electrophoresis in 3\% gels. Mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) served as an internal standard of mRNA expression. Transcriptional activity of Ngf and Gapdh genes was evaluated on the basis of mRNA copy number per 1 μg total RNA by the use of the real-time quantitative RT-PCR technique, which was performed in triplicate for each of 20 samples. Gene-specific primers were used, and the specificities were tested under normal PCR conditions. The following oligonucleotide sequences were used: mouse-Ngf α(102 bp), sense, 5′-TGT GCA GGA GAT GTG GTT-3′; antisense, 5′-AGG GCC CCA TGA TGT CAT AC-3′; mouse-Gapdh(73 bp), sense, 5′-CTG GTT CCT ACC CCC AAT GT-3′; antisense, 5′-TGT CATCATA TGG CAG GTT TCT-3′.

**ELISA and Immunohistochemical Staining**

As described for the RNA isolation-PCR study, retinas were dissected and collected from another 50 OIR mice at P12, P13, P14, P17, and P24 and from 50 normal controls for protein extraction (n = 10 for each time point). Retinal specimens from two mice (four eyes) were pooled. The freshly dissected, unfixed retinal tissues immersed in modified RIPA buffer with a 1:100 protease inhibitor cocktail (Sigma, St. Louis, MO) were homogenized, and lysates were centrifuged at 13,000g for 15 minutes at 4°C. Supernatants were collected, and total protein was quantified by bicinchoninic acid assay (Pierce, Rockford, IL) according to the manufacturer’s protocol. Supernatants were then assayed without dilution using highly sensitive, commercially available mouse b-NGF (Promega, Madison, WI) and mouse VEGF (R&D Systems, Minneapolis, MN) sandwich enzyme-linked immunosorbent assay (ELISA) kits. All procedures were performed according to the manufacturers’ instructions. Each sample (100 μL) was run in duplicate and compared with a standard curve.

Another eight mice from each group were used for immunohistochemical staining. On P17, the animals were killed, and both eyes were enucleated and fixed for 2 to 6 hours in 4\% buffered formaldehyde. According to a previously described method,\textsuperscript{37-39} more than 50 serial 6-μm paraffin-embedded axial sections were obtained, starting at the optic nerve head. Immunohistochemistry was performed in 6-μm retina sections. Endogenous peroxidases were quenched with 0.3\% H2O2, and background staining was blocked by incubation in 5\% normal bovine serum. The diluted 1:100 primary antibody for mouse b-NGF (Santa Cruz Biotechnology, Santa Cruz, CA) incubations were carried out in a humidified chamber overnight at 4°C, and the tissues were subsequently incubated with a biotinylated secondary antibody with an avidin-biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 45 minutes at room temperature and then reacted with 3,3′-diaminobenzidine/H2O2.

**Immunofluorescence Staining**

Eyes were enucleated and embedded in OCT compound (Tissue-Tek; Sakura Fine Technical, Torrance, CA) overnight for cryosectioning, and then 10-μm serial sections were cut. Frozen sections of mouse eyes were dried at room temperature and postfixed in cold acetone for 10 minutes. Sections were washed with phosphate-buffered saline (PBS) and blocked with 5\% BSA in PBS for 30 minutes at room temperature. Slides were incubated with primary antibodies (1:100 mouse monoclonal anti-glial fibrillary acidic protein [GFAP] antibody [GA5; Cell Signaling Technology, Beverly, MA] and 1:100 rabbit anti–NGF [BioWorld, Dublin, OH]) antibody overnight at 4°C. Then the slides were rinsed three times with PBS/0.05% Tween-20 and incubated with fluorescein-labeled secondary antibodies (1:200 Alexa 555-conjugated donkey anti-mouse/Alexa 488-conjugated donkey anti-rabbit; Invitrogen, Carlsbad, CA) at room temperature for 1 hour In addition, some slides were used only for 1:50 isoelectric staining (Invitrogen). Slides were thoroughly washed with PBS/Tween-20 and counterstained for 8 minutes at room temperature with the nuclear dye 1:2000 Hoechst 33258 (Invitrogen). After repeated washing with PBS/Tween-20, the slides were mounted and analyzed by a confocal microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany). Fluorescence pictures were taken with identical exposure settings. For negative control, slides stained without primary antibodies showed no signals.

**Intraocular Injections**

Another 40 mice underwent the OIR procedure for intraocular injections. On P12, when these OIR mice were returned to room air, they were divided randomly into two groups to receive intravitreal injections of NGF, or K252a (TrkA receptor inhibitor) in the right eye, and control vehicle (PBS with 0.025% BSA) in the contralateral eye. Mice were anesthetized with subcutaneous administration of 50 mg/kg ketamine and 5 mg/kg xylazine and were fixed under a microsurgical microscope. One drop of local anesthetic was administered before injection. Intravitreal injections were performed using a 5-μL Hamilton syringe with a 52-gauge needle, according to a previously described method.\textsuperscript{5,49} The eye was punctured at the upper nasal limbus, and 1 vol of 0.5 μL reagent solution or control vehicle was injected in one
eye of each mouse. Because reflux of a certain amount of intraocular fluid is unavoidable when the pipette is removed from the injection site, the pipette was kept in place for 10 seconds to allow diffusion of the solution. The reagent was diluted in sterile PBS. The volume and concentration of reagent were as follows: NGF (2 μg/0.5 μL; Peprotech, Rocky Hill, NJ) and K252a (4 μg/0.5 μL; Biosource, Camarillo, CA) or neutralizing anti-NGF antibody (0.5 μg/μL; Abcam, Cambridge, UK).38 Repeat injections were performed through a previously unmanipulated section of limbus 2 days later. In literature reports, NGF intraocular injection has usually been conducted in an animal model to study its neuroprotective function.42,43 In this study, we increased the dose of NGF intraocular injection to study its angiogenic effect in the retina. Technical considerations in these neonatal mice precluded testing of more concentrated solutions or more frequent administration.

Quantification of Neovascular Cell Nuclei Anterior to the Internal Limiting Membrane

Twenty OIR mice that had received intravitreal injections (n = 10 for each group) were used for quantitation of neovascular cell nuclei above the internal limiting membrane, according to a previously described method.37–39,44 On P17, the animals were killed, and both eyes were enucleated and fixed for 2 to 6 hours in 4% buffered formaldehyde. More than 50 serial 6-μm paraffin-embedded axial sections were obtained starting at the optic nerve head. After staining with periodic acid/Schiff reagent and hematoxylin, 10 intact sections of equal length, each 30 μm apart, were evaluated for a span of 300 μm. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section using a fully masked protocol. The mean of all 10 counted sections yielded average neovascular cell nuclei per 6-μm section per eye. No vascular cell nuclei anterior to the internal limiting membrane were observed in normal, unmanipulated animals.

Quantification of Retinal Neovascularization in the Flat-Mounted Retinas

The other 20 OIR mice that had received intravitreal injections (n = 10 for each group) were used for imaging quantification of retinal neovascularization. On P17, the animals were killed by cardiac perfusion with a solution of 50 mg/mL fluorescein-labeled dextran in sodium chloride, as described previously.37 Both eyes were enucleated and fixed for 2 to 6 hours in 4% buffered formaldehyde. Images were obtained using a high-resolution charge-coupled device (CCD) camera (DP30BP; Olympus) with a computerized system. Acquisition of images focused on the preretinal plane and selecting tufts based on pixel intensities. Selected regions were then summed to generate the total area of neovascularization and calculating the total area using imaging software (Image-Pro Plus 5.1; Media Cybernetics). Student’s t test was used to statistically compare the different experimental groups.

Statistical Analysis

Data are reported as mean ± SEM. Statistical analysis was performed with statistical software (SAS Institute Inc., Cary, NC). Differences among the groups were assessed by ANOVA to compare the level of NGF expression. For the study of intravitreal injection, data were analyzed with the use of a t test to compare the difference between two groups. All reported P values were two-sided. P < 0.05 was accepted as statistically significant.

RESULTS

Increased NGF mRNA Expression in at P17 OIR Model

Results of real-time PCR data are represented as Ct values, where Ct is the threshold cycle at which the amplified product is initially detected. To correct differences of transcriptional activity of NGF between samples for quantification analysis, the target gene was normalized to the internal standard GAPDH gene. We used the comparative Ct method and analyzed RNA expression in samples relative to that of the control in each experiment. The comparative transcriptional activity of the NGF gene is summarized in Figure 1. There was no significant difference of NGF mRNA expression in the retina after oxygen induction at P12 (0.0175 ± 0.00180 vs. 0.0257 ± 0.0018). However, a significantly large increase in NGF mRNA expression occurred at P17 in the OIR model compared with normal controls (0.1833 ± 0.0172 vs. 0.0385 ± 0.0073). At P24, NGF mRNA expression decreased in the OIR group (0.0507 ± 0.0134 vs. 0.0396 ± 0.0070). *P < 0.01.

Increased NGF and VEGF Protein Expression in OIR Model

Similar to mRNA expression, the data from ELISA showed significantly increased NGF expression in the retinas of OIR mice beginning at P14 (180.45 ± 35.16 vs. 110.35 ± 16.28; P < 0.01) and reaching the highest peak at P17 (288.58 ± 80.19 vs. 120.78 ± 21.53; P < 0.01) but showed no significant difference at P12 or P24, respectively, compared with normal controls. NGF levels in the retinas of OIR mice are shown in Figure 2. Figure 3 shows representative pictures of retinal slides from the OIR model and normal controls with immuno-
neovascular cell nuclei above the internal limiting membrane among different groups. The number of neovascular cell nuclei in the NGF group is significantly increased compared with the counterpart control. Interestingly, however, the number of neovascular cell nuclei is significantly decreased in the K252a injection or anti-NGF blocking antibody (data not shown) group compared with PBS control.

Measurement of Retinopathy Neovascularization in the Flat-Mounted Retinas

We successfully established an oxygen-induction ischemic retina model. The retina showed the typical appearance of ischemic retinopathy at P12 and P17 (Figs. 7A, 7B). Figures 7C to 7F illustrate the fluorescein dextran–perfused retinas from 17-day-old mice that had been exposed to hyperoxia from P7 to P12 and had received intravitreal injections. Supplementary Figure S1, http://www.iovs.org/cgi/content/full/51/7/3749/DC1, shows how we defined the neovascularization area in a high-resolution image. According to the profile of vasculature node, node area, and tube length, as well as blood vessel tortuosity, the neovascularization (tufts) area was carefully delineated with a red line by using confocal images focused at the preretinal plane and selecting tufts based on pixel intensities. Selected regions were then summed to generate the total area of neovascularization, and the total area was calculated with imaging software (Image-Pro Plus 5.1; Media Cybernetics) based on the defined area. The data showed that retinal neovascularization was significantly enhanced after injection with NGF; however, a mild decrease in neovascularization was found in K252a-injected mice compared with the counterpart control (Fig. 8). A limitation of the study was the use of only fluorescein injection, which could have underestimated non-perfused tufts; fluorescein injection plus isoelectric staining may be used in neovascularization quantitative analysis to provide more accurate evaluation of the neovascularization.

DISCUSSION

Studies have shown that ischemia induces significant VEGF upregulation and have suggested that VEGF may be the dominant angiogenic stimulus in retinal neovascularization; however, roles for other proangiogenic and angiogenic factors, such as platelet-derived growth factor, erythropoietin, stromal cell-derived factor-1, and basic fibroblast growth factor, cannot be ruled out. In this study, significantly increased expression of NGF, either at the mRNA or the protein level, was
found in the OIR model, implicating a potential role of NGF in retinal neovascularization.

NGF abundantly produced and used by retinal ganglion cells (RGCs), bipolar neurons, and glial cells is well known for its role in regulating survival, growth, and functional maintenance of RGCs, photoreceptors, and other retinal neurons. Consistent with this, our data showed that the activated astrocytes and neurons localized in GCL, INL, and OPL produced NGF markedly in ischemic retina. The protective function of NGF intraocular administration has been clearly demonstrated in experimental models of ischemic, traumatic, hypertensive injury, and NGF was recently reported to exert neuroprotective effects by inhibiting the apoptosis of RGCs. Interestingly, evidence of NGF acting as an angiogenic agent has emerged from preclinical and clinical studies. NGF, alone or in combination with other biologically active molecules, can have an effect on endothelial cells and on angiogenic activity. It has been documented that the application of NGF enhanced blood vessel growth and angiogenesis markedly in vitro and in vivo. Neurotrophic factors, including NGF, may play a functional role in reparative neovascularization. Our finding that NGF enhanced retinal vascular endothelial cell activity with increased tube forming in culture (Matrigel; BD Biosciences, Bedford, MA; Supplementary Fig. S2) provides evidence to support that NGF may contribute to retinal neovascularization by acting on endothelial cells.

NGF, as a new potent angiogenic factor, has been shown to enhance VEGF production, although Colafrancesco et al. have presented the controversial suggestion that NGF may reduce the synthesis and release of VEGF. Both NGF and VEGF have recently attracted special interest because they are critically involved in the survival and protection of brain and retinal cells and because they have reciprocal angiogenic and neurotrophic effects on blood vessels and neurons. We found significantly increased expression of VEGF at P17 and at P24 in the OIR model compared with the normal control. Although the increased VEGF expression was not significantly correlated with NGF upregulation by a linear regression correlation analysis in our study, it is possible that the enhanced expression of retinal VEGF might be linked to the local upregulation of NGF. Park et al. have reported that VEGF levels increase as diabetic retinopathy advances, which is associated with NGF upregulation.

Coordinated actions of NGF and VEGF have been demonstrated in different tissues under various physiological conditions. The functional relationship between NGF and VEGF,
however, is unclear because both NGF and VEGF are pleiotropic factors with many physiologic and pathologic roles. Thus, based on the available data, we propose that there is a dual role for these angiogenic and neural factors. Ischemia triggers abundant NGF/VEGF release by neuronal tissue. Early release of these growth factors in response to retinal ischemia might be a protective reaction to maintain neuron survival, but this beneficial response may have the secondary effect of inducing excessive angiogenesis in the retina. NGF is likely to be a “double-edged sword” in ischemia-induced retinopathy. It should be noted that vascularization also plays a pivotal role in neuronal protection.

Angiogenesis is a complex process regulated by many growth factors. The multiple regulatory components interact and modulate their individual effects and are further regulated by physiological stimuli. Some factors have demonstrated seemingly environmentally dependent roles in this complex process. The multilayered regulatory system makes it difficult to accurately predict the effect of any single factor or the relationship among these angiogenic factors. Our data suggest that VEGF is not the sole major mediator of OIR-associated pathologic neovascularization but that NGF also contributes to the process. The increased release of NGF, similar to that of

![Figure 7](image-url) - Representative pictures of the retinal flat-mount with fluorescein-dextran perfusion. (A, B) Typical appearance of ischemic retinopathy at P12 and P17 of the OIR model. (A) Central avascular area is a typical feature of the OIR model at P12. (B) Blood vessel tufts, presumed extraretinal neovascularization, and blood vessel tortuosity are shown obviously at P17. (C–F) Fluorescein dextran–perfused retinas from P17 OIR mice that had received intravitreal injections at P12. (C) Retina of NGF-treated eye, showing markedly angiogenic features with extensive presumed extraretinal neovascularization, blood vessel tufts, and tortuosity. (D) Retina of the control eye with PBS-BSA injection. (E) Retina of K252a-treated eye has comparatively lower angiogenesis with obvious avascular area compared with the control eye. (F) Retina of the contralateral control eye with PBS injection.

![Figure 8](image-url) - Average retinal neovascularization area. There is a significantly enhanced neovascularization after injection with NGF (1.98 ± 0.43 vs. 0.58 ± 0.27); however, a mild decrease in neovascularization is seen after injection with K252a (0.39 ± 0.07 vs. 0.67 ± 0.12) compared with the counterpart controls. *P < 0.05.
NGF, is also a protective response to ischemia. The present study provides a basis for further studies on the role of NGF and other neurotrophic factors (such as brain-derived neurotrophic factor and glial-derived neurotrophic factor) in ischemic vasculopathies and their interactions with other angiogenic factors.

The major goal of this study was to explore the secondary effect of NGF as an enhancer of angiogenesis. Periarterial angiogenesis and intraretinal angiogenesis (Supplementary Fig. S5) were enhanced in the OIR mouse after treatment with NGF, although statistical analysis regarding intraretinal angiogenesis could not be performed because of a limited number of samples. The finding of significantly higher efficiency of tube formation of retinal vascular endothelial cells in culture after treatment with NGF revealed the potential mechanism of angiogenic action of NGF. We also investigated the role of NGF to understand the process of neuron-driven angiogenesis in retinopathy. Feit-Leichman et al.8 postulated that diabetes-induced early changes in neurons and Müller glia contribute critically to the later development of vascular lesions in diabetic retinopathy. Ali58 reported a twofold increase in NGF expression in diabetic rats. Recently, serum and tear NGF levels were found to be higher in patients with proliferative diabetic retinopathy than in nondiabetic control patients.70 Abundant NGF produced by neurons and glia has been demonstrated to be a potent stimulus for new vessel growth in our study and in other studies reported in the literature. Our results suggest that NGF contributes to retinal neovascularization either by acting on endothelial cells or by upregulating other angiogenic factors, such as VEGF. Our study also provides a potential explanation for the unusual neovascularization that occurs in the retinal vasculopathy complications of diabetes mellitus because the vasculopathies that affect the ischemic myocardium and limbs in this disease are, paradoxically, characterized by an insufficient angiogenic response.72–74

In several disease states, abnormal growth of blood vessels is associated with local neuronal degeneration. Although the relationship between neovascularization and neuron damage has received much attention,75 a direct relationship remains unclear. Other neurotrophic factors or neuronal-related factors have been reported to be specifically upregulated in vascular abnormalities or to act on vascular endothelial cells.30 Neuronal cells can be especially sensitive to oxidative damage, at least in part because of the stressful and naturally oxidative environment of the retina.75,76 Our study provides evidence for yet another mechanism of ischemic injury associated with neovascularization: NGF as a neurotrophic factor. In addition to its primary role in preventing neuronal cell death, it has a secondary effect of increasing angiogenesis. Ischemia leads to NGF release, which protects neurons but may exacerbate abnormal vessel growth. This supports a link between retinal neovascularization and neuronal damage through cytokines, such as NGF and VEGF, which pleiotropic factors that affect both vessels and neurons. Further studies are needed to better understand the role of cytokines in the link between retinal vascular abnormalities and neuronal tissue.

Intravitreal injection with either K252a or neutralizing anti-NGF antibodies reduced neovascularization in our OIR model. TrkA is responsible for the survival and growth properties of neurotrophins.33,77–79 and K252a is a high-affinity TrkA receptor inhibitor.55,56 It has been demonstrated that trkA receptor is present in endothelial cells and that NGF contributes to pathologic blood vessel alternations by enhancing vascular endothelial cell activity mediated mainly through a high-affinity trkA receptor. K252a can reduce NGF-induced vascular endothelial cell activity by blocking the Trk A receptor.72 Our finding that retinal neovascularization was significantly decreased in K252a-injected mice indicated that the angiogenic function of NGF can be blocked by inhibition of the TrkA receptor and that K252a is a potential therapeutic reagent for decreasing neovascularization.

Similarly, it has been previously shown that oral treatment with a tyrosine kinase inhibitor can reduce retinal neovascularization in the OIR mouse model.80 Other studies have reported that local injection of receptor tyrosine kinase inhibitor MAE 87 or PTK/ZK reduces retinal neovascularization in OIR mice. Unsoeld et al.81,82 believed that MAE 87 inhibits both the VEGF and the IGF-1 cascade to reduce retinal neovascularization.83,84 Considering all these findings together, we agree that the tyrosine kinase inhibitor might be a promising agent for local treatment of retinal neovascularization through the blocking of some angiogenic factors, including NGF. Further experiments are needed to determine whether a specific tyrosine kinase inhibitor, such as K252a, can inhibit the action of other cytokines. Future studies should also demonstrate the effects of different tyrosine kinase inhibitors in decreasing angiogenesis and the potential adverse effects of treatment with Trk receptor inhibitors.

In summary, we found increased NGF expression in the OIR mouse model, either in the mRNA level or the protein level. NGF treatment was found to enhance retinal vascular endothelial cell activity in culture; intravitreal injection with exogenous NGF enhanced retinal neovascularization in OIR mice. These results demonstrated that in the retina, a particular cytokine milieu containing NGF contributes to pathologic angiogenesis, representing a further and previously undiscovered effect of NGF on retina. The ability of intravitreal injections with K252a to reduce neovascularization demonstrates the potential therapeutic efficacy of this NGF receptor inhibitor. Our study provides new evidence that NGF, as a neurotrophic factor, potentially has angiogenic effects in ischemic retinopathy. Ischemia-induced retinopathy is an aberrant neovascularization process initially driven by neurotrophins, such as NGF, that are released from neuronal tissue to activate vascular endothelial cells or to upregulate other angiogenic factors. These results support the concept that NGF may induce neoangiogenesis, a process that to a degree is reparative but that, when excessive, leads to pathologic retinopathy. The finding with regard to the neuronal control of angiogenesis increases our opportunity to discover new therapeutic strategies to inhibit pathologic retinal neovascularization, which also suggests a new paradigm for considering the relationship between vasculature and associated retinal neuronal tissue.

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References


