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Aqueous Levels of Angiopoietin-like 4 and Semaphorin 3E Correlate with Nonperfusion Area and Macular Volume in Diabetic Retinopathy

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Objective: To investigate the aqueous levels of angiopoietin-like 4 (ANGPTL4), semaphorin 3E (Sema3E), and vascular endothelial growth factor (VEGF) in patients with diabetic retinopathy and to ascertain their association with diabetic retinopathy phenotypes.

Design: Prospective, nonrandomized, comparative case series.

Participants: Of all 104 consecutive patients (104 eyes) who had intravitreal anti-VEGF injections from April 2012 through April 2013 for diabetic macular edema (DME), 51 had severe nonproliferative diabetic retinopathy (NPDR) and 53 had proliferative diabetic retinopathy (PDR). The controls were 54 consecutive nondiabetic patients who had undergone cataract surgery (54 eyes) during the same period.

Methods: The ANGPTL4, Sema3E, and VEGF levels in aqueous humor samples obtained before intravitreal injections were measured by enzyme-linked immunosorbent assay. Capillary nonperfusion area (NPA) was calculated from encircled angiography using the 7 standard field images described in the Early Treatment Diabetic Retinopathy Study protocol. Total macular volume (TMV) was measured by spectral-domain optical coherence tomography.

Main Outcome Measures: Aqueous ANGPTL4, Sema3E, and VEGF levels in severe NPDR, PDR, and control groups and their correlations with each other, NPA, and TMV.

Results: The severe NPDR and PDR groups had higher aqueous levels of ANGPTL4 and VEGF than the control group (all $P < 0.001$). The PDR group had higher ANGPTL4 and VEGF levels than the severe NPDR group (both $P < 0.001$). The aqueous ANGPTL4 levels of all diabetic retinopathy patients correlated positively with NPA ($r = 0.820$, $P = 0.003$) and TMV ($r = 0.824$, $P < 0.001$). The control group had higher aqueous Sema3E levels than the NPDR and PDR groups (both $P < 0.001$). Aqueous Sema3E levels correlated negatively with VEGF levels in all subjects ($r = -0.57$, $P = 0.025$).

Conclusions: The ANGPTL4 may be a candidate target in DME treatment and a biomarker of ischemic-induced retinopathy, including diabetic retinopathy. *Ophthalmology 2015;■:1–8 © 2015 by the American Academy of Ophthalmology.*

Macular edema in patients with ischemic retinopathies, including diabetic macular edema (DME), remains the leading cause of vision loss in working-age populations.[1] In ischemic retinopathies, sustained hypoxia exacerbates extraretinal vascular outgrowth, which can cause vision-impairing hemorrhage and retinal detachment.[2,3] A variety of proangiogenic factors are implicated in the progression of DME and retinal neovascularization. Thus, it is believed that to maintain the normal vasculature in the retina, proangiogenic and antiangiogenic factors should be balanced appropriately.[4–6]

Proangiogenic factors include vascular endothelial growth factor (VEGF), which is a potent inducer of vasopermeability and macular edema.[7] It has been shown recently that intravitreal injection of anti-VEGF agents often improves the visual acuity of patients with DME. However, some patients show only a partial response to this therapy, with persistent DME, poor visual acuity, or both.[8,9] This emphasizes the need to identify alternative therapeutic targets.

Previous studies showed that several factors modulate vascular permeability, angiogenesis, and inflammatory signaling.[10,11] These factors include angiopoietin-like 4 (ANGPTL4), which, like VEGF, plays an important role in angiogenesis and promoting vasopermeability. Angiopoietin-like 4 is a secreted glycoprotein that is induced by hypoxia[12] and interacts with proteoglycans from the extracellular matrix.[13] Perdiguero et al[14] reported that ANGPTL4 is expressed by retinal endothelial cells and that knockout of *angptl4* in mice strongly inhibits neovascularization in oxygen-induced retinopathy.

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The roles that antiangiogenic factors, including pigment epithelium-derived factor, play in retinal neovascularization have been studied.[15,16] Recently, semaphorins were shown to play pivotal roles in tumor-associated angiogenesis.[17] Moreover, Fukushima et al[18] reported that binding of the semaphorin 3E (Sema3E) ligand to the transmembrane PlexinD1 receptor initiates a signaling pathway that normalizes angiogenic directionality in both developing retinas and the ischemic retinopathy.

Although ANGPTL4 and Sema3E are proangiogenic and antiangiogenic factors, respectively, their roles in diabetic retinopathy remain poorly understood. In this study, the aqueous levels of these factors, as well as that of VEGF, in patients with diabetic retinopathy were measured. In addition, whether these factors correlate with the degree of vascular hyperpermeability or retinal ischemia in eyes with DME was assessed.

Methods

This prospective study was performed at the Department of Ophthalmology, Kyungpook National University, Daegu, South Korea. The study protocol was approved by the Institutional Review Board of Kyungpook National University Hospital and was conducted in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects after the research purpose was explained.

Participants

The study group consisted of all consecutive patients who underwent intravitreal bevacizumab injection for DME or senile cataract surgery between April 2012 and April 2013 in the Department of Ophthalmology of Kyungpook National University.

The following inclusion criteria were used for patients with severe nonproliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR) at first visit: presence of severe NPDR or PDR, as determined by the modified Early Treatment Diabetic Retinopathy Study (ETDRS) grade[19]; a best-corrected visual acuity of between 20/200 and 20/40; and central subfield macular thickness of 300 μm or more on spectral-domain optical coherence tomography (Spectralis OCT; Heidelberg Engineering, Heidelberg, Germany). Patients with severe NPDR or PDR were excluded if they met any of the following criteria: presence of vitreous hemorrhage; previous treatments, including anti-angiogenic medications such as bevacizumab, ranibizumab, pegaptanib, or laser photocoagulation; previous vitrectomy; or a recent myocardial infarction or cerebral vascular accident. In cases with bilateral lesions, the first eye that was diagnosed served as the affected eye. Control subjects were included if they were scheduled to have cataract surgery, did not have a history of diabetes mellitus, and had no retinal vascular diseases, as determined by comprehensive ophthalmic examinations.

Collection of Aqueous Humor

Before starting the intravitreal bevacizumab injection or cataract surgery, undiluted samples of aqueous humor (0.1−0.2 ml) were aspirated by limbal paracentesis using a 30-gauge needle attached to a tuberculin microsyringe. The samples were placed immediately into sterile tubes and stored at −80°C in a deep freezer until they were assayed.

Analysis of Angiopoietin-like 4, Semaphorin 3E, and Vascular Endothelial Growth Factor

The aqueous levels of ANGPTL4, Sema3E, and VEGF were measured by enzyme-linked immunosorbent assays using the commercially available ANGPTL4 ELISA kit (R&D Systems, Minneapolis, MN), the Sema3E ELISA kit (USCN Life Science & Technology Inc, Houston, TX), and the human VEGF immunoassay (Biosource; Invitrogen, Carlsbad, CA). The assays were performed according to the manufacturers' instructions.

For the ANGPTL4 assay, 2-fold diluted aqueous samples were added to the wells of plates that were coated with the anti-hANGPTL4 polyclonal goat immunoglobulin G antibody (DY3485; R&D Systems). After a 2-hour incubation and washing with a wash buffer, the solution was incubated for an additional 2 hours with 100 μl of detection antibodies. A working dilution of streptavidin-horseradish peroxidase and substrate solution was added to each well, and the plates were incubated for 20 minutes at room temperature. The intensity of color in the reaction mixture was measured at 450 nm by using a multilate reader.

For the Sema3E assay, 2-fold diluted aqueous samples were added to each well of a plate that was precoated with a Sema3E-specific antibody and incubated for 2 hours. Thereafter, avidin conjugated to horseradish peroxidase was added to each microplate well and incubated for 1 hour. The wells then were incubated for 20 minutes with tetramethylbenzidine (TMB) substrate solution. Only the wells that contained Sema3E, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a blue color. This color change was detected by spectrophotometry at a wavelength of 450 nm.

For the VEGF assay, 2-fold diluted aqueous samples were added to each well of a plate that was coated with anti-mouse VEGF polyclonal antibody and incubated for 2 hours. After washing with a wash buffer, the solution was incubated for an additional 2 hours with 100 μl of enzyme-linked polyclonal antibodies. A substrate solution was added and the plate was incubated for 30 minutes at room temperature. The intensity of color in the reaction mixture was measured at 450 nm by using a multilate reader.

Ophthalmic Examinations

All subjects underwent ophthalmic examinations at baseline, including best-corrected visual acuity measurement using the Snellen chart and dilated fundus examination with slit-lamp biomicroscopy. In addition, the subjects with severe NPDR and PDR underwent optical coherence tomography and fluorescein angiography.

Automated total macular volume (TMV) measurements were obtained by using spectral-domain optical coherence tomography. The 6×6-mm area of the macular region centered on the fovea was examined. Each consisted of 1024 A-scans per line. A macular profile of the central zone was obtained by using the fast macular volume preset, which consists of a 25-line horizontal raster scan that covers 20 × 20° and that is centered on the fovea. Scans were obtained in the high speed mode with the automated real-time feature enabled and set to 9 frames.

The eye-tracking system of the device was used to ensure that the scanning was performed in the correct position. The measurements were recorded by a well-trained technician who was masked to patient information. Only images with a quality score of more than 16 dB were selected as high-quality images.