Aqueous Angiopoietin-Like 4 Levels Correlate With Nonperfusion Area and Macular Edema in Branch Retinal Vein Occlusion

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PURPOSE. To investigate whether macular edema (ME) due to branch retinal vein occlusion (BRVO) associates with retinal overexpression of angiopoietin-like 4 (ANGPTL4). The aqueous ANGPTL4 and vascular endothelial growth factor (VEGF) levels in patients with ME due to BRVO were measured, and the relationships between ANGPTL4 levels and the degree of retinal ischemia and edema were determined.

METHODS. The study and control groups consisted of all consecutive patients who were scheduled to undergo intravitreal bevacizumab injection for treatment-naïve BRVO with ME and senile cataract surgery, respectively. The study group was divided into the major BRVO and macular BRVO subgroups on the basis of the involved retinal area. The aqueous ANGPTL4 and VEGF levels were measured by enzyme-linked immunosorbent assay. In the patients with BRVO, capillary nonperfusion area by fluorescein angiography and central subfield macular thickness (CSMT) and total macular volume (TMV) by spectral-domain optical coherence tomography were determined.

RESULTS. Patients with ME due to BRVO (50 eyes) had higher aqueous ANGPTL4 and VEGF levels than the controls (61 eyes) (both \( P < 0.001 \)). The major BRVO had higher ANGPTL4 and VEGF levels than the macular BRVO (both \( P < 0.001 \)). The aqueous ANGPTL4 levels of all BRVO patients correlated positively with nonperfusion area (\( r = 0.901, P < 0.001 \)), CSMT (\( r = 0.574, P < 0.001 \)), and TMV (\( r = 0.453, P = 0.001 \)), even after adjustment for VEGF levels.

CONCLUSIONS. The aqueous ANGPTL4 levels correlated significantly with phenotypes of BRVO with ME. This suggests that ANGPTL4 may be a candidate biomarker and treatment target in ischemia-induced retinopathies, including BRVO.

Keywords: angiopoietin-like 4, branch retinal vein occlusion, central subfield macular thickness, nonperfusion area, total macular volume

Branch retinal vein occlusion (BRVO) is one of the most common retinal vascular disorders in elderly patients, and the resulting macular edema (ME) is the most frequent cause of visual impairment in BRVO. The pathogenesis of ME is very complex: It includes vascular dysfunction and breakdown of the blood-retinal barrier. Furthermore, obstruction of the retinal vein causes venostasis of the venule, which can lead to hypoxic damage to the retina. The retinal ischemia also stimulates the retinal expression of vascular endothelial growth factor (VEGF), which is a potent inducer of vasopermeability and ME.

To improve the visual acuity of patients with ME secondary to BRVO, injections with anti-VEGF agents, which decrease vasopermeability, are widely used. However, some patients respond only partially to this therapy, as they continue to display persistent ME and/or poor visual acuity. This indicates the need to identify alternative therapeutic targets.

Previous studies show that several factors modulate vascular permeability, angiogenesis, and inflammatory signaling. These factors include angiopoietin-like 4 (ANGPTL4), which, like VEGF, plays an important role in promoting vasopermeability and angiogenesis. Angiopoietin-like 4 is a secreted glycoprotein whose expression is induced by hypoxia. It interacts with proteoglycans from the extracellular matrix. Xin et al. reported that ANGPTL4 is expressed by hypoxic retinal Müller cells and that this promotes retinal vascular permeability both in vitro and in vivo.

In our recent study, we investigated the aqueous ANGPTL4 levels in patients with ME that was secondary to severe nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). We observed that the patients with diabetic ME had significantly higher aqueous ANGPTL4 levels than the control cataract group. Moreover, we found that the aqueous ANGPTL4 levels correlated positively with the severity of ME.

At present, the role that ANGPTL4 plays in ME secondary to BRVO remains poorly understood. To address this, we measured the aqueous levels of this factor and VEGF in patients who had ME due to BRVO. Whether these two factors correlate with the degree of retinal ischemia or vascular hyperpermeability in BRVO with ME was also assessed.

METHODS

This prospective cross-sectional study was performed at the Department of Ophthalmology of Kyungpook National Univers...
sity, 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 and control groups consisted of all consecutive patients who were scheduled to undergo intravitreal bevacizumab injection for treatment-naïve ME due to BRVO and senile cataract surgery, respectively, between October 2014 and March 2015 in the Department of Ophthalmology of Kyungpook National University.

Patients with ME due to BRVO were included in the study group if they met the following criteria at the first visit to the department: clinically detectable diffuse ME or cystoid ME that had been present for more than 1 month; a best-corrected visual acuity of between 20/400 and 20/40; and central subfield macular thickness (CSMT) of 300 \( \mu \)m or greater on spectral-domain optical coherence tomography (OCT) (Spectralis OCT; Heidelberg Engineering, Heidelberg, Germany). Study patients were excluded if they met any of the following criteria: previous treatment for ME, including with antiangiogenic medications such as bevacizumab, ranibizumab, or pegaptanib, laser photocoagulation, or vitrectomy; patients with diabetes mellitus and diabetic retinopathy; patients with iris ruberosis; a history of ocular inflammation and vitreoretinal diseases; and/or a recent myocardial infarction, cerebral vascular accident, or malignant hypertension.

The BRVO group was divided into the major BRVO and macular BRVO subgroups on the basis of classification criteria described previously.16,17 Major BRVO was defined as BRVO of one of the major branch retinal veins (usually near the optic disc) that affected the entire segment of the retina drained by the vein. Macular BRVO was defined as BRVO of one of the macular venules that thus affected only a segment of the macular retina.

Control subjects were included if they were scheduled to have cataract surgery, did not have a history of hypertension or diabetes mellitus, and had no retinal vascular diseases, as determined by comprehensive ophthalmic examinations by retina specialists.

Collection of Aqueous Humor

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

Analysis of ANGPTL4 and VEGF Levels

The aqueous levels of ANGPTL4 and VEGF were measured by enzyme-linked immunosorbent assays (ELISAs), namely, the commercially available ANGPTL4 ELISA kit (R&D Systems, Minneapolis, MN, USA) and the human VEGF immunoassay (Biosource; Invitrogen, Carlsbad, CA, USA). The assays were performed according to the manufacturers’ instructions as follows.

For the ANGPTL4 assay, 2-fold diluted aqueous samples were added to the wells of plates that were coated with the anti-human ANGPTL4 polyclonal goat IgG antibody (Dy3485; R&D Systems). After a 2-hour incubation followed by washing with a wash buffer, the solution was incubated for an additional 2 hours with 100 \( \mu \)L detection antibodies. A working dilution of streptavidin-horseradish peroxidase and substrate solution was then 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 multiplate reader (FLUOstar Omega; BMG Labtech, Ortenberg, Germany).

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 \( \mu \)L 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 the multiplate reader.

Ophthalmic Examinations

All subjects underwent ophthalmic examinations at the initial visit, including best-corrected visual acuity measurement using the Snellen chart and dilated fundus examination with slit-lamp biomicroscopy. In addition, the subjects with BRVO underwent fluorescein angiography and OCT before the intravitreal bevacizumab injection.

Automated CSMT and total macular volume (TMV) measurements were obtained by using spectral-domain OCT. The 6-\( \times \)6-mm area of the macular region centered on the fovea was examined. Each scan 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 \( \times \)20° and is centered on the fovea. Scans were obtained in the high-speed mode with the automated real-time feature enabled and set to nine frames. The CSMT was calculated automatically as the average retinal thickness within a circle of a 1000-\( \mu \)m diameter centered on the fovea (the center circle of the Early Treatment Diabetic Retinopathy Study grid; software version 5.4.6.0). 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 (i.e., high-quality images) were selected. Two retinal specialists checked for correct alignment of the retinal layers while using the Spectralis OCT software.

Measurement of Capillary Nonperfusion Area by Using Fluorescein Angiography

At the venous phase (between 45 seconds and 2 minutes), fluorescein angiography (HRA-2; Heidelberg Engineering) images were obtained. After digital capture, the images were transferred to Adobe software (Adobe Systems, Inc., San Jose, CA, USA). The capillary nonperfusion area was defined as dropout of the retinal capillary bed. The nonperfusion areas were determined and outlined manually with Adobe software. The foveal avascular zone and fluorescent areas were excluded from the nonperfusion area. The boundaries of the nonperfusion area were encircled by using the area measurement function (Fig. 1). Areas of fluorescence blockage due to retinal hemorrhage were excluded from the nonperfusion area by comparing the fluorescein angiography image with retinal photographs as described previously.18 The total image area was calculated in pixels and then converted into a disc area (DA) for the purpose of clinical interpretation; for this, a scale factor based on the assumption that one DA was 2.7 mm² was used as described previously.19,20 The nonperfusion area of the
angiograph was evaluated and calculated by two independent masked retina specialists.

**Statistical Analyses**

Statistical analysis was performed by using SPSS V18.0 for Windows (SPSS, Inc., Chicago, IL, USA). The ANGPTL4, VEGF, nonperfusion area, CSMT, and TMV results are expressed as mean ± standard deviation (SD). Independent t-test and χ² tests were used to compare the BRVO and control groups. To assess the relationship between aqueous biomarker concentrations and BRVO phenotypes (namely, nonperfusion area, CSMT, and TMV), Pearson’s correlation coefficients and 95% confidence intervals were calculated. Multiple linear regression analysis was used to adjust for the aqueous VEGF concentration. P values of less than 0.05 were considered to indicate statistical significance.

**RESULTS**

In total, 61 consecutive control subjects and 60 consecutive patients were scheduled to undergo senile cataract surgery and intravitreal bevacizumab injection for BRVO with ME, respectively, during the study period. Ten patients with BRVO were excluded because they had previous treatments for ME, including anti-VEGF or laser photocoagulation. Thus, the study consisted of 61 control subjects and 50 patients with ME due to BRVO.

The clinical characteristics of the two groups are summarized in the Table. The control and BRVO groups had mean ages of 61.0 ± 5.9 (range, 49–76) years and 61.0 ± 10.6 (range, 42–84) years, respectively, and 55.7% (34/61) and 46.0% (23/50) were male, respectively. Age was normally distributed in both groups. The two groups did not differ significantly in terms of age or sex distribution. In the BRVO group, the mean symptom duration was 3.7 ± 2.5 months. The 50 BRVO group patients were divided into two subgroups on the basis of the size of the occluded area: 34 (68%) had major BRVO and 16 (32%) had macular BRVO.

**Relationship Between BRVO and Aqueous ANGPTL4 and VEGF Levels**

In the control and BRVO groups, the mean aqueous ANGPTL4 levels were 1727.0 ± 818.7 and 16,767.5 ± 11,995.0 pg/mL, respectively (Fig. 2A). The ANGPTL4 levels in the BRVO group were significantly higher than those in the control group (P < 0.001). The major BRVO subgroup also had significantly higher ANGPTL4 levels than the macular BRVO subgroup (23,270.8 ± 9449.1 vs. 3854.6 ± 1552.4 pg/mL, P < 0.001).

In the control and BRVO groups, the mean aqueous VEGF levels were 36.9 ± 17.6 and 105.1 ± 70.7 pg/mL, respectively (Fig. 2B). The BRVO group had significantly higher VEGF levels than the control group (P < 0.001). The major BRVO subgroup also had significantly higher VEGF levels than the macular BRVO subgroup (132.2 ± 74.2 vs. 54.3 ± 11.4 pg/mL, P < 0.001).

There was no correlation between the duration of ME and the aqueous ANGPTL4 levels (r = 0.040, P = 0.805) in the BRVO group. There was no correlation between age and the

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**TABLE. Clinical Characteristics of the Patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control, n = 61</th>
<th>BRVO, n = 50</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
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<td>61.0 ± 10.6</td>
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</tr>
<tr>
<td>Male sex, n (%)</td>
<td>34 (55.7)</td>
<td>23 (46.0)</td>
<td>0.307†</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>21 (42.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptom duration, mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRVO classification, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major BRVO</td>
<td>34 (68.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macular BRVO</td>
<td>16 (32.0%)</td>
<td></td>
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</tr>
</tbody>
</table>

* Values are mean ± standard deviation unless otherwise indicated.
† Independent t-test.
‡ χ² test.

**FIGURE 1.** Representative examples of fluorescein angiography in eyes with macular edema that is due to branch retinal vein occlusion (BRVO). The areas of retinal capillary nonperfusion are outlined in red. The extent of the nonperfusion area in the major BRVO (A) and macular BRVO (B) cases is 30.7 and 0.44 disc area, respectively.
aqueous ANGPTL4 levels in the control group ($r = -0.077, P = 0.566$).

**Correlation Between Aqueous ANGPTL4 and VEGF Levels and Capillary Nonperfusion Area**

The BRVO group had a mean nonperfusion area size of $29.4 \pm 26.7$ DA. The major BRVO subgroup had a significantly larger nonperfusion area than the macular BRVO subgroup ($42.0 \pm 23.4$ vs. $27.7 \pm 2.3$ DA, $P < 0.001$). In the total BRVO group, nonperfusion area size correlated significantly with both aqueous ANGPTL4 levels ($r = 0.901$, $P < 0.001$) (Fig. 3A) and aqueous VEGF levels ($r = 0.726$, $P < 0.001$) (Fig. 3B). Even after adjustment for aqueous VEGF levels, aqueous ANGPTL4 levels continued to correlate significantly with nonperfusion area size ($\beta = 0.822$, $R^2 = 0.790$, $P < 0.001$). The mean excluded areas of intraretinal blood (% of total analyzed nonperfusion areas) in the BRVO group was $9.2 \pm 5.4\%$.

**Correlation Between Aqueous ANGPTL4 and VEGF Levels and CSMT**

The BRVO group had a mean CSMT of $579.1 \pm 139.7$ µm. The major BRVO subgroup had significantly greater CSMT than the macular BRVO subgroup ($625.2 \pm 134.4$ vs. $477.7 \pm 90.7$ µm, $P = 0.002$). In the total BRVO group, CSMT correlated

**FIGURE 2.** Bar graphs showing the mean aqueous levels of (A) angiopoietin-like 4 (ANGPTL4) and (B) vascular endothelial growth factor (VEGF) in the control and branch retinal vein occlusion (BRVO) groups. The two groups were compared in terms of the aqueous levels of each biomarker. All concentrations were standardized to picograms per milliliter. The $P$ values were obtained by using independent $t$-tests. The error bars indicate the standard error of the mean.

**FIGURE 3.** Correlation between aqueous levels of (A) angiopoietin-like 4 (ANGPTL4) and nonperfusion area, (B) vascular endothelial growth factor (VEGF) and nonperfusion area, (C) Angiopoietin-like 4 and central subfield macular thickness (CSMT), and (D) VEGF and CSMT in the branch retinal vein occlusion (BRVO) group. Angiopoietin-like 4 correlated positively with both nonperfusion area ($r = 0.901, P < 0.001$) and CSMT ($r = 0.574, P < 0.001$). Vascular endothelial growth factor also correlated positively with both nonperfusion area ($r = 0.726, P < 0.001$) and CSMT ($r = 0.401, P = 0.006$). The nonperfusion area size was expressed as disc area (DA).
positively with both the aqueous ANGPTL4 levels \( (r = 0.574, P < 0.001) \) (Fig. 3C) and the aqueous VEGF levels \( (r = 0.401, P = 0.006) \) (Fig. 3D). Even after adjusting for the aqueous VEGF level, the aqueous ANGPTL4 level continued to correlate significantly with CSMT \( (\beta = 0.606, R^2 = 0.304, P = 0.005). \)

**Correlation Between Aqueous ANGPTL4 and VEGF Levels and TMV**

The BRVO group had a mean TMV of 12.4 \pm 1.9 mm\(^3\). The major BRVO subgroup had larger TMVs than the macular BRVO subgroup \( (15.0 \pm 1.9 \text{ vs. } 11.3 \pm 1.3 \text{ mm}^3, P = 0.006) \). In the total BRVO group, TMV correlated positively with both the aqueous ANGPTL4 levels \( (r = 0.453, P = 0.001) \) and the aqueous VEGF levels \( (r = 0.356, P = 0.022) \). Even after adjusting for the aqueous VEGF level, the aqueous ANGPTL4 level continued to correlate significantly with TMV \( (\beta = 0.453, R^2 = 0.193, P = 0.055). \)

**DISCUSSION**

Vascular endothelial growth factor is a potent hyperpermeability factor that has been demonstrated to play a key role in the pathogenesis of ME. This explains why anti-VEGF injections substantially improve the visual acuity of many patients with ME. However, additional therapies are needed for those patients who respond only partially to anti-VEGF injections. Thus, there is ongoing interest in other potential treatment targets. Several recent studies reported that the secretory protein ANGPTL4 mediates the development of vasopermeability and angiogenesis in hypoxic conditions.\(^{14,21,22}\) In particular, hypoxia-inducible factor (HIF)-1\(^{\alpha}\) upregulates the production of ANGPTL4 by both hypoxic retinal Müller cells and the ischemic inner retina in the oxygen-induced retinopathy model.\(^{14}\) These observations may be relevant to BRVO because BRVO, which preferentially induces the inner breakdown of the blood–retinal barrier, thereby resulting in ME,\(^{25}\) also causes capillary damage in the affected region, which leads to nonperfusion and ischemia. These observations led us to measure the ANGPTL4 levels in aqueous samples from patients with BRVO with ME.

Indeed, patients with ME due to BRVO had significantly higher aqueous ANGPTL4 levels than the control subjects with cataracts. Moreover, the ANGPTL4 levels in patients with BRVO with ME correlated positively with both CSMT and TMV. Both CSMT and TMV are indicators of retinal edema in the macula, and are increased by vascular hyperpermeability. Our recent study showed that the ANGPTL4 levels of patients with ME due to severe NPDR or PDR also correlate positively with TMV.\(^{14}\) Thus, our recent and current observations suggest that ANGPTL4 overexpression may occur in ischemic retinopathies in general and that this overexpression promotes the development of ME.

The current study showed that the patients with BRVO also had significantly higher aqueous VEGF levels than the control subjects. This is consistent with the observations of several previous studies.\(^{24-26}\) Since VEGF levels correlate positively with ME severity, it is possible that this correlation was responsible for the association between ANGPTL4 and ME. However, the present study showed that aqueous ANGPTL4 levels continued to correlate positively with CSMT and TMV after adjusting for aqueous VEGF levels. Notably, Babapoor-Farrokhan et al.\(^{25}\) showed recently that the aqueous ANGPTL4 levels in individual patients with diabetic ME do not show a consistent relationship with VEGF levels. They also showed that when the expression of both ANGPTL4 and VEGF by hypoxic Müller cells was inhibited, the two inhibitions acted in an additive fashion to reduce the angiogenic potential of the Müller cells. Thus, it seems likely that ANGPTL4 promotes ME via a mechanism that is independent of intraocular VEGF levels.

Given that Xin et al.\(^{14}\) showed that the hypoxic inner retina of an animal model expresses high levels of ANGPTL4, we measured the nonperfusion area by fluorescein angiography and investigated its correlation with aqueous ANGPTL4 levels. Indeed, the aqueous ANGPTL4 levels correlated positively and significantly with nonperfusion area size, including after adjustment for aqueous VEGF levels. We also observed this correlation in patients with diabetic ME previously.\(^{15}\) Hence, our present findings show that in BRVO with ME, there is a potential correlation between retina ischemia and high levels of ANGPTL4.

Hayreh et al.\(^{16,17}\) classified BRVO, on the basis of the size of the occluded area, into two groups, namely, major BRVO and macular BRVO. We used these classification criteria to divide our BRVO with ME group into major and macular BRVO subgroups. Analysis of these two phenotypes revealed that the major BRVO subgroup had greater nonperfusion area, CSMT, and TMV than the macular BRVO subgroup. Furthermore, the major BRVO subgroup had higher aqueous ANGPTL4 levels than the macular BRVO subgroup. Similarly, we showed previously that patients with PDR had both greater nonperfusion area and higher ANGPTL4 levels than patients with severe NPDR.\(^{15}\) These observations suggest that patients with a greater area of hypoxic retina express ANGPTL4 at higher levels than patients with smaller hypoxic retina areas. They also suggest that this molecule could serve as a biomarker of the ME that arises from ischemic retinopathies, including BRVO and diabetic retinopathy.

There are several limitations in this study. First, nonperfusion area was measured by using the HRA-2 confocal laser-scanning system for fluorescein angiography. This system does not cover the far peripheral retina, unlike ultra-widefield fluorescein angiography. Thus, studies using ultra-widefield fluorescein angiography will be necessary to confirm whether aqueous VEGF or ANGPTL4 levels still positively correlate with the nonperfused areas.

A second study limitation is that the nonperfusion area could be underestimated, even though retinal hemorrhage-mediated blockage of the fluorescence can be differentiated from nonperfusion by comparing the fluorescein angiographs and photographs. Longer follow-up of the changes in nonperfusion area and aqueous ANGPTL4 levels after resorption of retinal hemorrhage would demonstrate the relationship between these two factors with greater accuracy.

In conclusion, the present study has shown an association of ANGPTL4 with ME due to BRVO with clinical evidence. Patients with BRVO with ME had higher aqueous ANGPTL4 levels than the control cataract patients; moreover, the aqueous ANGPTL4 levels in patients with BRVO with ME correlated positively with their nonperfusion area size, CSMT, and TMV. Thus, ANGPTL4 may be a potential biomarker for the severity of retinal ischemia and vascular hyperpermeability in patients with ME due to BRVO.

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