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Bevacizumab Injection in Patients with Neovascular Age-Related Macular Degeneration Increases Angiogenic Biomarkers

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Purpose: To evaluate the expression of 19 angiogenic biomarkers in the aqueous humor before and after intravitreal bevacizumab injection (IVB) in eyes with neovascular age-related macular degeneration (AMD).

Design: Prospective, noncomparative, interventional case series.

Participants: Twenty-three eyes of 23 treatment-naïve patients with choroidal neovascularization (CNV) secondary to neovascular AMD.

Methods: Eyes were diagnosed with CNV secondary to neovascular AMD and were treated with 3 monthly IVBs. Aqueous humor samples were obtained by anterior chamber paracentesis at baseline and immediately before each intravitreal bevacizumab injection.

Main Outcome Measures: Aqueous humor levels of 19 angiogenic biomarkers (angiopoietin 2, bone morphogenetic protein 9 [BMP-9], epidermal growth factor [EGF], endoglin, endothelin 1, fibroblast growth factor [FGF]-1 and FGF-2, follistatin, granulocyte colony-stimulating factor [GCSF], heparin-binding EGF-like growth factor [HB-EGF], hepatocyte growth factor [HGF], interleukin 8, leptin, placental growth factor [PLGF], vascular endothelial growth factor [VEGF]-A, VEGF-C, VEGF-D, and tissue inhibitor of metalloproteinases [TIMP]-1 and TIMP-2) were measured. Best-corrected visual acuity (BCVA), spectral-domain OCT parameters, and intraocular pressure also were evaluated.

Results: Baseline aqueous VEGF-A expression was elevated in all study eyes before treatment initiation. A statistically significant decrease of VEGF-A was observed at the 1- and 2-month follow-ups. A statistically significant increased concentration was observed in 7 biomarkers: VEGF-C, angiopoietin 2, endothelin 1, follistatin, HB-EGF, HGF, and interleukin 8. The other 11 study biomarker levels (VEGF-D, BMP-9, EGF, endoglin, FGF-1, FGF-2, GCSF, leptin, PLGF, TIMP-1, and TIMP-2) did not show any significant difference during follow-up. The BCVA statistically improved significantly at 2 months. Spectral-domain OCT parameters improved significantly at all follow-ups. Mean intraocular pressure values were not statistically different during the study period.

Conclusions: Despite a decrease in VEGF-A, the aqueous levels of VEGF-C, angiopoietin 2, endothelin 1, follistatin, HB-EGF, HGF, and interleukin 8 increased significantly after intravitreal injection of bevacizumab. These upregulated angiogenic biomarkers may represent new therapeutic targets in exudative AMD. *Ophthalmology Retina 2017;■:1–7 © 2017 by the American Academy of Ophthalmology*

Angiogenesis is a fundamental physiologic process characterized by the development of new vessels from preexisting blood vessels and involves the stimulation of angiogenic growth factor receptors on vascular endothelial cells, proteolytic breakdown of the endothelial cell basal membrane, and endothelial cell proliferation and migration.[1] The vasculogenesis process is controlled by a dynamic balance between positive angiogenesis regulator factors (vascular endothelial growth factor [VEGF], hepatocyte growth factor [HGF], interleukin 8, transforming growth factors α and β, connective tissue growth factor, fibroblast growth factor [FGF], and angiopoietin) and negative angiogenesis regulator factors (pigment epithelium–derived factor, endostatin, and vasostatin). Angiogenesis occurs in the setting of imbalance of such factors that leads to overactivity of the proangiogenic cytokines.[2]

Neovascular age-related macular degeneration (AMD) is the main cause of severe visual loss among individuals older than 55 years in developed countries, and inhibition of angiogenesis is pivotal to the prevention and treatment of choroidal neovascularization (CNV) associated with neovascular AMD.[3] Vascular endothelial growth factor is a potent cytokine modulator of angiogenesis, promotes the growth of both retinal and choroidal new vessels, and is

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Published by Elsevier Inc.

http://dx.doi.org/10.1016/j.oret.2017.04.004
ISSN 2468-6530/17

considered critical for CNV development in neovascular AMD eyes.[4] The establishment of VEGF as an important regulator of angiogenesis in AMD revolutionized the field by stimulating the development of anti-VEGF agents that inhibit angiogenesis. Bevacizumab, ranibizumab, and aflibercept are commercially available immunoglobulin antibodies that bind and inhibit the biological activity of VEGF isoforms, and their intravitreal use has been associated with visual acuity improvement in neovascular AMD.[5]

Despite the success of anti-VEGF therapy, an incomplete response to anti-VEGF agents is observed in many neovascular AMD patients. Therefore, other biomarkers may be implicated in the pathogenesis of CNV resulting from AMD. The purpose of this study was to measure the expression of 19 vasogenic biomarkers, including VEGF, in the aqueous humor after 2 monthly intravitreal bevacizumab injections (IVBs) in treatment-naïve eyes with neovascular AMD. Additionally, mean vasogenic biomarkers levels, best-corrected visual acuity (BCVA), and spectral-domain (SD) OCT parameters were compared. We hypothesized that alternative biomarker pathways may maintain angiogenic stimulus in eyes with neovascular AMD despite anti-VEGF blockage with bevacizumab intravitreal injection.

Methods

In this prospective study, the aqueous levels of 19 angiogenic biomarkers were measured in eyes with neovascular AMD treated with IVB at the Retina Department of Public Service Hospital of São Paulo (IAMSPE), São Paulo, Brazil. The institutional review board of the Federal University of São Paulo (reference number, 215195) and the Public Service Hospital of São Paulo (reference number, 0115/10) approved the off-label use of bevacizumab and collection of aqueous humor in the current study. All patients provided informed consent before treatment.

Participants

Aqueous humor samples were obtained before each IVB from 23 eyes of 23 consecutive patients (10 men, 13 women; mean age±standard deviation, 76.4±9.4 years) with active CNV resulting from neovascular AMD. All study patients underwent 3 monthly IVBs (1.25 mg/0.05 ml; Avastin; Genentech, South San Francisco, CA). Active CNV resulting from AMD was confirmed by fluorescein angiography and SD OCT. None of the study participants had been treated previously for neovascular AMD.

Ophthalmologic Examination

Patients underwent a comprehensive ophthalmologic examination (at baseline and 1 and 2 months after IVB) that included BCVA, biomicroscopic examination, and intraocular pressure measurement. Color fundus photography and fluorescein angiography were performed with a Topcon TRC-50IA fundus camera (Tokyo Optical Co. Ltd, Tokyo, Japan).

Three OCT parameters (central retinal thickness [CRT], macular volume [MV], and macular thickness [MT]) were measured using the SD OCT Cirrus 4000 (Carl Zeiss Meditec, Dublin, CA) at the baseline, 1-month, and 2-month follow-up examinations. Central retinal thickness, MV, and MT were calculated after acquiring a sequence of 128 horizontal sections recorded in the high-resolution mode (27 000 A-scans per second). Macular cube 512 × 128 and 5-line raster scans were performed.

Sample Collection

Aqueous humor samples (volume, 0.1 ml) were obtained from the anterior chamber using a 30-gauge needle paracentesis before each IVB (at baseline and 1 and 2 months after IVB, but before the third injection). Topical anesthesia was induced before the injection with instillation of tetracaine 1% eye drops. Povidone—iodine was applied to the eyelid margins, and a lid speculum was inserted after application of a sterile drape. The IVBs were performed via the pars plana using a 30-gauge needle. Undiluted aqueous humor (0.1 ml) was collected in sterile tubes and stored immediately at −80° C until analysis.

Measurement of Biomarkers

The concentrations of 19 vasogenic biomarkers (angiopoietin 2, bone morphogenetic protein 9 [BMP-9], epidermal growth factor [EGF], endoglin, endothelin 1, FGF-1, FGF-2, follistatin, granulocyte colony-stimulating factor [GCSF], heparin-binding EGF-like growth factor [HB-EGF], HGF, interleukin 8, leptin, placental growth factor [PLGF], VEGF-A, VEGF-C, VEGF-D, and tissue inhibitor of metalloproteinases [TIMP] 1 and TIMP-2) were measured in the aqueous humor using an enzyme-linked immunometric assay (Luminex Merk Millipore; Merck KGaA, Darmstadt, Germany).

The assay manufacturer reported minimum detectable doses of 0.50, 0.080, 0.13, 0.74, 0.56, 3.17, 7.61, 0.15, 2.46, 0.13, 4.37, 0.085, 14.37, 0.068, 0.81, 0.77, 0.37, 7.64, and 20.66 pg/ml for angiopoietin 2, BMP-9, EGF, endoglin, endothelin 1, FGF-1, FGF-2, follistatin, GCSF, HB-EGF, HGF, interleukin 8, leptin, PLGF, VEGF-A, VEGF-C, VEGF-D, TIMP-1, and TIMP-2, respectively. The minimum detectable dose was confirmed by adding 2 standard deviations to the mean optical density value of standard replicates and calculating the corresponding concentration. Standard curves for each biomarker level were generated using the reference standard supplied with the kit.

Statistical Analysis

Data were analyzed using commercially available R software (R Development Core Team, 2016; https://www.r-project.org/), version 3.2.2. Linear mixed models with a random intercept for subject were used to compare the mean vasogenic biomarkers levels, BCVA, and SD OCT measurements at each time point (baseline and 1-month and 2-month follow-ups), as illustrated in Figure 1 and Table 1. We treated time as a categorical variable, and thus estimated means for the outcomes separately at each time point. P values less than 0.05 were considered statistically significant.

Pathway Analysis

A protein—protein interaction network associated with the 8 statistically significant angiogenic biomarkers (VEGF-A, VEGF-C, angiopoietin 2, endothelin 1, follistatin, HB-EGF, HGF, and interleukin 8) was created using Qiagen's Ingenuity Pathway Analysis (Qiagen, Redwood City, CA). Ingenuity Pathway Analysis is a commercial software package that makes use of computational algorithms and statistical tests to identify the most highly connected and significant protein interactions. Figure 2 was adapted from Ingenuity Pathway Analysis results.

Results

In total, 69 anterior chamber biopsies were collected from 23 eyes, and there were no complications, such as uveitis, lens

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