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Research article

Method development to quantify Bv8 expression in circulating CD11b+ cells in patients with neovascular age-related macular degeneration (nvAMD) exhibiting Anti-VEGF refractoriness

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ABSTRACT

A subset of neovascular age-related macular degeneration (nvAMD) subjects appears to be refractory to the effects of anti-VEGF treatment and require frequent intravitreal injections. Prokineticin-2 (Bv8) expression in CD11b$^+$ cells has been linked to anti-VEGF response. We have developed a reproducible method to quantify gene expression in circulating CD11b + cells. Utilizing this method we tested the hypothesis that high Bv8 expression in circulating CD11b$^+$ cells is associated with anti-VEGF refractoriness in nvAMD patients. Two groups of nvAMD subjects undergoing treatment with anti-VEGF agents were recruited and classified as refractory or non-refractory to anti-VEGF treatment (n = 33 for each group). Two blood draws were obtained from each subject 1–9 months apart. Peripheral blood mononuclear cells (PBMCs) were isolated and CD11b$^+$ cells were purified via magnetic bead separation. RNA was purified, and relative expression of Bv8 among the subjects was compared via quantitative PCR analysis. Utilizing this approach no significant difference was detected in the mean LogRQ values between the first and second blood draws (*t*-test, p = 0.826) indicating low intra-patient variability and demonstrating good reproducibility of the assay. There was no significant difference in Bv8 expression between nvAMD subjects classified as refractory versus non-refractory. We were unable to find a correlation between Bv8 expression in CD11b + cells and anti-VEGF refractoriness in human nvAMD subjects. Relatively high expression in Bv8 in these subjects did not correlate with clinical treatment history, as measured by the frequency of injections. Utilizing this well characterized technique, studies are underway to examine alternative gene expression profiles in various circulating cell populations that may contribute to anti-VEGF refractoriness.

1. Introduction

The estimated prevalence of age-related macular degeneration (AMD) in the US population aged 40 years and older is 6.5% (Liu et al., 2011). As the median age of the US population increases, the socioeconomic costs of this disease will increase (Friedman et al., 2004). Neovascular AMD (nvAMD), characterized by the growth of pathologic blood vessels under the retina termed choroidal neovascularization (CNV), accounts for 10–15% of cases of AMD, but is responsible for more than 80% of severe vision loss and legal blindness attributable to AMD (Wong et al., 2008). The exact pathogenic mechanisms underlying CNV development are still poorly understood, but it is clear that vascular endothelial growth factor (VEGF) is involved (Miller et al., 2013). Because of this, intravitreal injections of anti-VEGF agents have become the mainstay of therapy for nvAMD. However, despite the improvement in vision seen in most nvAMD patients undergoing anti-VEGF treatment (Brown et al., 2006; Group et al., 2011; Heier et al., 2012; Rosenfeld et al., 2006), the requirement for repeat anti-VEGF injections to achieve this visual increase is highly variable (Rofagha et al., 2013). Studies that have utilized pro re nata (PRN) regiments for treatment with anti-VEGF agents have found a mean of 5.6–6.3 injections/y were required, with a large spread of injections around the mean (Comparison of Age-related Macular

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Degeneration Treatments Trials Research et al., 2012; Ho et al., 2014). Some subjects required considerably more injections, up to 12 injections/y, to maintain the same level of improvement from baseline visual acuity seen in subjects receiving fewer injections (Ho et al., 2014). Thus there appears to be a subset of nvAMD subjects who are refractory to the effects of anti-VEGF treatment, and require more frequent injections (Fung et al., 2007; Lalwani et al., 2009).

The underlying reasons for this variability in response to anti-VEGF treatment remain speculative. Various explanations have been proposed, including upregulation of alternative pro-angiogenic signaling pathways, development of anti-therapeutic antibodies targeted towards the anti-VEGF agents, and protection of the aberrant blood vessels in choroidal neovascularization (CNV) by pericytes (Azam et al., 2010; Bergers and Hanahan, 2008). One possibility is the expression of alternate angiogenic proteins such as prokineticin-2 (Bv8) in circulating cells such as CD11b + cells that may render the CNV refractory to anti-VEGF therapy. The CD11b + circulating cell population primarily consists of neutrophils, lymphocytes and mononuclear cells. Tumors in mice that were refractory to systemic anti-VEGF treatment were found to have increased infiltration of circulating $CD11b^+$ cells relative to anti-VEGF sensitive tumors (Shojaei et al., 2007a). Bv8, a protein that has been linked to proliferation of endothelial cells and stimulation of angiogenic responses, was strongly expressed in the CD11b + population (Shojaei et al., 2007b). Treatment with neutralizing antibodies targeting Bv8 resulted in tumor suppression and increased the effectiveness of anti-VEGF treatment in refractory tumors. Similar mechanisms may be involved in nvAMD. It has been demonstrated that neutrophils and macrophages are abundant in CNV lesions in nvAMD (Zhou et al., 2005).

In the present study, we aimed to develop a reproducible method to assay gene expression in a subpopulation of circulating cells. We were able to validate a qPCR assay for a potential biomarker for anti-VEGF refractoriness using blood samples obtained from patients currently receiving anti-VEGF therapy for nvAMD. Our primary focus was examining not only inter-patient variability, but also variability within individual patients. As an initial candidate gene, we examined Bv8 as a potential biomarker, and tested the hypothesis that high Bv8 expression in circulating $CD11b^+$ cells is associated with anti-VEGF refractoriness in nvAMD patients.

2. Materials and methods

2.1. Subject recruitment

A total of 66 subjects were recruited for the study, 33 in each class of anti-VEGF refractory and non-refractory. Demographic data is outlined in Table 1. Subjects were classified as refractory or non-refractory to anti-VEGF treatment based on frequency of anti-VEGF injections in the preceding 12 months prior to recruitment. Refractory subjects had received 7 or more anti-VEGF injections in the preceding 12 months. Non-refractory subjects had received 4 or less anti-VEGF injections in the preceding 12 months. These numbers were based on recruiting nvAMD subjects that were above or below the average number of anti-VEGF injections/y as determined by the CATT studies (Comparison of Age-related Macular Degeneration Treatments Trials Research et al., 2012; Group et al., 2011). Subjects were recruited for each classification (refractory or non-refractory) until an N of 33 for each group was reached. The rationale for re-injections was determined by the individual treating physicians and was based on status of retinal fluid on optical coherence tomography (OCT), vision change or presence of retinal hemorrhage. All subjects received ranibizumab (Lucentis®), bevacizumab (Avastin®) or aflibercept (Eylea®) injections in their treatment history. Technical control samples were obtained from 5 healthy individuals. Informed consent was obtained from each subject before any blood draws were performed. The research adhered to the tenets of the Declaration of Helsinki. The research protocol was approved by the Institutional Review Board of Aspire IRB (Santee, CA 92071).

2.2. Peripheral blood mononuclear cell (PBMC) isolation

An initial blood draw of 50 mL was obtained from each subject. A second blood draw was obtained 1–9 months after the first from 60 of the 66 subjects. The time of the blood draw was noted for each collection. For technical controls, the two blood draws were obtained 4 h apart for each control subject. PBMCs were isolated with Vacutainer CPT tubes (Becton Dickinson, New Jersey, USA) following manufacturer's instructions. The number of PBMCs isolated was counted using a Countess automated cell counter (Invitrogen, Massachusetts, USA).

2.3. Sorting of CD11b-positive cells

PBMCs (1×10^7) were suspended in 80 μL of bead sorting buffer (PBS, 2 mM EDTA, 0.5% BSA) and 20 μL of CD11b microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the cell suspension. The cells were incubated for 15 min at 4^OC, and then washed with 1 mL of bead buffer. The cells were run through a single MS column (Miltenyi Biotec) according to manufacturer's instructions. Cell numbers in the positive and negative fractions were determined using the Countess cell counter.

2.4. Flow cytometry

Cells (5×10^6) from the unsorted PBMC suspension, CD11b-positive and negative fractions were suspended in 100 μL of bead sorting buffer. Ten μL of CD11b-FITC antibody (Miltenyi-Biotec) was added to the cell suspensions. The samples were incubated for 10 min at 4^OC in the dark. 500 μL of bead sorting buffer was added to wash the cells. The labeled samples were spun down and resuspended in 500 μL buffer. 125 μl of 4% Paraformaldehyde was added and the cells were fixed for 10 min at 4^OC. After fixation, the cells were spun down and resuspended in 500 μL buffer for analysis. The cell sorting was performed on a FACScan instrument. FlowJo v8.8.6 was used to analyze the results.

Table 1
Demographic data for recruited subjects. Descriptive statistics for refractory and non-refractory nvAMD subjects showing age, baseline VA (means and SD), gender and smoking status (n values).

Group	Age	Baseline VA (logMar)	Gender		Smoking status	
	Mean (SD)	Mean (SD)	Female (n)	Male (n)	Non-smoker (n)	Current/previous smoker (n)
Refractory	77.79 (6.97)	0.38 (0.24)	15	18	20	13
Non-refractory	80.7 (7.32)	0.43 (0.36)	23	10	26	7