Biomaterials 35 (2014) 3729–3735

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Monitoring the VEGF level in aqueous humor of patients with ophthalmologically relevant diseases via ultrahigh sensitive paper-based ELISA

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A R T I C L E I N F O

Article history:
Received 10 December 2013
Accepted 10 January 2014
Available online 28 January 2014

Keywords:
Paper-based ELISA
VEGF
Diagnosis
Ophthalmology
Diabetic disease

A B S T R A C T

The vascular endothelial growth factor (VEGF) level in aqueous humor has been used as an indicator to monitor specific diseases in the retinal ischemic condition. For clinical diagnosis, only about 200 μL of aqueous humor can be collected from the anterior chamber before the threat of anterior chamber collapse. It is necessary to develop an inexpensive diagnostic approach with the characteristics of highly sensitive, short operation duration, and requires small clinical sample quantities. To achieve the main objective of this study, we first prepared bevacizumab to be conjugated with HRP. We then deposited 2 μL aqueous humor from patients with different diseases onto each test zone of paper-based 96-well plates. After the colorimetric results were performed via ELISA protocol, the output signals were recorded using a commercial desktop scanner for analysis. In this study, only 2 μL from the aqueous humor of each patient was required for paper-based ELISA. The mean aqueous VEGF level was 14.4 pg/mL from thirteen patients ($N = 13$) with senile cataract as the control. However, the mean aqueous VEGF level from other patients with proliferative diabetic retinopathy ($N = 14$), age-related macular degeneration ($N = 17$), and retinal vein occlusion ($N = 10$) showed VEGF increases to 740.1 pg/mL, 383 pg/mL, and 219.4 pg/mL, respectively.

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1. Introduction

This paper describes the development of an inexpensive but robust and easy-to-handle diagnostic approach that uses a piece of filter paper to monitor the activity of three different diseases—proliferative diabetic retinopathy (PDR), age-related macular degeneration (AMD), and retinal vein occlusion (RVO)—by evaluating the level of vascular endothelial growth factor (VEGF) in aqueous humor. The VEGF level in aqueous humor has been used as an indicator to monitor the activity of specific diseases in the retinal ischemic condition [1–3]. Such diseases include diabetes, which induces microvascular occlusion, resulting in retinal ischemia. The ischemic retina secretes VEGF, an endothelial cell mitogen and an angiogenic inducer, into the vitreous cavity. VEGF increases vascular permeability and induces the formation of new vessels arising from the plane of the retina, resulting in PDR [1]. Inhibiting VEGF secretion, when coupled with ocular drug therapy, can both decrease vascular permeability and prevent iris and retinal neovascularization in PDR, providing PDR patients with better visual outcomes via regression of iris and retinal neovascularization and reduced leakage [4,5]. In both AMD and RVO, VEGF plays a similar role in the proliferation of abnormal vessels in the retina [2,6]. Because these abnormal vessels cause severe vision loss, anti-VEGF therapies have been widely used to treat and reverse abnormal vessel developments for patients with PDR, AMD, and RVO. However, routinely monitoring VEGF concentration within the eye is hampered by insufficient sample sizes and difficult sampling techniques. There are currently two approaches for clinical VEGF

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0142-9612/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2014.01.030

sampling, from either vitreous or aqueous humor, to evaluate VEGF level within the eye. Both approaches are difficult to accomplish without invasive procedures, and resulting samples are small. Small sample quantities limit in-vitro diagnostics significantly, especially in regards to conventional enzyme-linked immunosorbent assay (ELISA) techniques (antigen–antibody recognition). For clinical diagnosis, only about 200 μL of aqueous humor can be collected from the anterior chamber before the threat of anterior chamber collapse. However, the clinical value of samples is especially high as VEGF is a proven biomarker of response to drug therapy [7–9]. It is necessary, therefore, to develop an inexpensive but robust and easy-to-handle diagnostic approach that is highly sensitive and specific (to specific diseases), has a short operation duration (i.e., a rapid diagnostic approach), and requires small clinical sample quantities. Such an approach would be invaluable for diagnosing specific diseases in the ophthalmology community and could possibly assist in the diagnosis of other infectious disease.

Multiple bioengineering-based approaches have been developed to diagnose retinal-ischemic-based diseases such as PDR or AMD using small clinical samples including the following. First, commercial ELISA sets, which have been used to i) measure both VEGF and IL-6 (interleukin-6) levels in aqueous and vitreous samples from the same eye in order to understand whether the level of these two substances in aqueous humor is related to the severity and activity of diabetic retinopathy [7], ii) evaluate the level of VEGF, IL-6, IL-1β (interleukin-1β), and TNF-α (tumor necrosis factor-α) for potential molecular targets and pharmacokinetic analysis in aqueous samples from patients participating in drug studies or patients receiving standard care with either one or two injections of ranibizumab (lucentis®, Genentech, Inc., San Francisco, CA, USA) [4], and iii) study the VEGF concentration in aqueous samples before and after intravitreal injection of bevacizumab (Avastin®; a fully-length humanized monoclonal antibody, against all isoforms of VEGF-A) in eyes with PDR [4,5]. Second, an evidence investigator cytokine and growth factor biochip has been developed to quantitatively detect multiple analytes in aqueous humor from patients in order to determine the influence of intravitreal bevacizumab injection on the level of these cytokines and growth factors in clinically significant macular edema [10,11]. Third, Luminex xMAP suspension array technology has been used to investigate the level of 29 different growth factors and inflammatory cytokines (including VEGF and platelet-derived growth factor) in eyes with neovascular age-related macular degeneration before and during therapy with intravitreal ranibizumab injection [12].

Although these bioengineering-based approaches have allowed us to obtain promising clinically relevant information with various diseases in ophthalmology, multiple issues still need to be thoroughly addressed such as 1) the requirement of both high-cost equipment and well-trained specialists to perform either the biochip or multiplex assays, and 2) the availability of only small clinical samples obtainable only via invasive procedures. We have attempted to address the small sample volume issue with the development of P-ELISA, which only requires 2 μL of aqueous humor and has ~fg/mL-level sensitivity. We also have developed a procedure to modify bevacizumab. This provides three distinct advantages: 1) it allows us to directly measure the VEGF level in aqueous humor using only a tiny amount of the modified bevacizumab as both the labeling antibody (against VEGF in aqueous humor) and the reporter (displaying the colorimetric-based output signal) in our P-ELISA; 2) it significantly increases the sensitivity of our P-ELISA, which was one of the main disadvantages of our previous study [13] and a general concern when using antibody-antigen recognition diagnostics; and, 3) it has the potential to advance research toward the co-development of drugs and diagnostic tools to evaluate disease activity of specific diseases, i.e., the development of diagnostic approaches using modified bevacizumab to monitor the activity of three diseases (PDR, AMD, and RVO in this study). Although some investigators have proposed optical coherence tomography (OCT) imaging on an "as-needed" basis [14], P-ELISA using aqueous humor samples can provide another promising and reliable diagnostic tool. In addition, while designing the experiments in this study, we took U.S. FDA regulations into account (i.e., analytical performance and clinical validation), especially in regards to our in-vitro diagnostic approach (in-vitro diagnostic devices [IVD]) and performance evaluation with appropriate data analysis (potentially moving toward the co-development of a drug and diagnostic tool for a specific disease and approaching our ultimate goal of translational medicine). In this way, we hoped to advance an additional step from laboratory-based study to clinically relevant application, and further demonstrate strength and suitability as a transformative application in the real world that would provide impactful benefit to not just academia, but ameliorate real healthcare concerns.

2. Materials and methods

2.1. Antibodies and antigens

To achieve the main objective of this study, we first prepared bevacizumab (Avastin®, Genentech, Inc., San Francisco, CA, USA), which is a monoclonal antibody for VEGF, to be conjugated with HRP according to the protocol of EasyLink HRP Conjugation Kit as follows: 1) dilute Avastin-antibody with HEPES (Sigma Aldrich, St. Louis, MO, USA) to the concentration of 0.8 mg/mL; 2) add EasyLink-modifier and EasyLink-HRP for 3 h at room temperature to modify the end of the Avastin-antibody; 3) add EasyLink-Quencher to end the reaction. After this process, we can use this HRP-conjugated Avastin for VEGF detection using P-ELISA. Commercial human recombinant vascular endothelial growth factor (VEGF expressed in *Escherichia coli*, Sigma–Aldrich, St. Louis, MO, USA) as a model antigen and HRP-conjugated Avastin as target antibody were used to establish a calibration curve before using human aqueous humors.

2.2. Aqueous humor from patients

With approval from the Taichung Veteran General Hospital Institutional Review Board, we obtained aqueous samples during cataract operation for measurement of VEGF for retinal diseases (IRB No. CF11213). VEGF has been implicated in the pathogenesis of AMD, PDR, and macular edema due to RVO. The experiments in this study were conducted from 14 aqueous samples collected from patients with PDR, 17 samples from patients with AMD, 10 samples from patients with RVO and 13 samples from patients with senile cataract as the control group. All of the 54 samplings were well tolerated by the patients with no adverse events.

2.3. Color fundus photographs and fluorescein angiography

All color fundus photographs and fluorescein angiographs were performed with a confocal scanning laser ophthalmoscope, Heidelberg Retina Angiograph 2 (HRA2, Heidelberg Engineering, Germany).

2.4. P-ELISA for detection of VEGF level from patients

We built a multiple-step procedure to carry out P-ELISA using paper-based 96-well plates made by the wax printing method as follows [15,16]: 1) we first deposited 2 μL aqueous humor from patients with different diseases onto each test zone and allowed for 10 min of drying; 2) we aliquoted 2 μL 1% BSA blocking buffer on each test zone; 3) after 10 min of drying, we then aliquoted 5 μL HRP-conjugated Avistin with a concentration of 0.8 mg/mL to conjugate with VEGF proteins; 4) we added 2.5 μL streptavidin onto the paper-based test zones to enhance the signal at room temperature until the test zones dried; and, 5) after carrying out the washing step again with washing buffer, we placed 2 μL of a solution of the enzyme substrate (a mixture of 3,3′,5,5′-tetramethylbenzidine and H_2O_2) onto our paper-based test wells to obtain colorimetric-based output signals (from colorless to blue), which were recorded using a commercial desktop scanner (EPSON; No.:GT-10000 +) that cost ~100.00 U.S. dollars.

2.5. Quantifying the intensity of test zone through a scanner

The results from P-ELISA were recorded at 0, 1, 2, 4, 6, and 7 min with a handheld cellphone camera (from HTC Inc., Taiwan). The distance between cellphone and test zones, as well as exposure parameters was consistent at 0, 1, 2, 4, 6, and 7 min. The recorded color signal of each test zone after scanning was switched to grayscale at 8 bit and 600 dpi and analyzed using the commercial image-processing package software, Photoshop® (Adobe, Photoshop CS5), in order to obtain the grayscale-

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