

## Brain-derived Neurotrophic Factor in the Aqueous Humor of Glaucoma Patients

Tsutomu Igarashi<sup>1</sup>, Kenji Nakamoto<sup>1</sup>, Maika Kobayashi<sup>1</sup>, Hisaharu Suzuki<sup>1</sup>,  
Takeshi Arima<sup>1</sup>, Yutaro Tobita<sup>1</sup>, Kazuhiro Takao<sup>1</sup>, Toru Igarashi<sup>2</sup>,  
Takahisa Okuda<sup>3</sup>, Takashi Okada<sup>4</sup> and Hiroshi Takahashi<sup>1</sup>

<sup>1</sup>Department of Ophthalmology, Nippon Medical School, Tokyo, Japan

<sup>2</sup>Department of Pediatrics, Nippon Medical School, Tokyo, Japan

<sup>3</sup>Division of Legal Medicine, Department of Social Medicine, Nihon University School of Medicine, Tokyo, Japan

<sup>4</sup>Division of Molecular and Medical Genetics, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

**Background:** Brain-derived neurotrophic factor (BDNF) may be involved in the pathogenesis of glaucoma. BDNF concentrations reported in previous studies have varied widely, and the concentration of BDNF in aqueous humor is unknown. In this study, BDNF concentrations in the aqueous humor of glaucoma patients and control patients were measured with ELISA kits.

**Methods:** This prospective, observational study examined BDNF levels in aqueous humor in 62 eyes of 43 patients who underwent cataract surgery or trabeculectomy (11 glaucoma patients and 32 non-glaucoma cataract patients as controls). BDNF concentrations were examined by 4 different enzyme-linked immunosorbent assay (ELISA) techniques.

**Results:** The mean  $\pm$  SD patient age was  $72.0 \pm 10.1$  (range 35 to 87) years. Two of the techniques detected no BDNF in aqueous humor in any samples ( $n=3$  and  $n=9$ , respectively); the average value was less than zero. An ultrasensitive ELISA kit did not yield reliable measurements. Finally, in an even more sensitive ELISA (Simoa-HD1), performed by an outside contractor, 25 (54.3%) eyes were below the detection limit, including 20 (55.6%) control and 5 (50%) glaucoma cases. For eyes with detectable BDNF, the overall BDNF concentration was 0.158 pg/mL ( $n=21$ ): 0.196 pg/mL ( $n=16$ ) in controls and 0.034 pg/mL ( $n=5$ ) in glaucoma cases.

**Conclusions:** BDNF level in aqueous humor varies widely. (J Nippon Med Sch 2021; 88: 128–132)

**Key words:** brain-derived neurotrophic factor, BDNF, glaucoma, aqueous humor, anterior chamber

### Introduction

Glaucoma is a group of eye diseases that result in damage to the optic nerve, with progressive degeneration of retinal ganglion cells (RGCs)<sup>1</sup>. Risk factors for glaucoma include high intraocular pressure (IOP), and standard treatment focuses on reduction of IOP by medication or surgery. However, a subset of glaucoma, known as normotensive glaucoma (NTG), does not respond to such treatment<sup>2</sup>. Therefore, factors other than IOP may be involved in the pathogenesis of the disease<sup>3</sup>, and depletion of brain-derived neurotrophic factor (BDNF) is a candi-

date factor.

BDNF is a member of the neurotrophin family of growth factors, which are a critical component for building and preserving neurons<sup>4</sup>. BDNF expression was investigated in glaucoma models<sup>5-7</sup>. BDNF is transported retrogradely from the superior colliculus to the RGCs in the optic nerve, and this flow is inhibited by acute IOP elevation<sup>1</sup>. Blockade of axonal transport may cause deficits in BDNF and thus RGC death in glaucoma<sup>17</sup>. BDNF is vital in maintaining RGCs and had a potent protective effect in various models of experimental glaucoma<sup>8-12</sup>.

Correspondence to Tsutomu Igarashi, MD, PhD, Department of Ophthalmology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

E-mail: tutomu@nms.ac.jp

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Clinically, serum BDNF concentrations were lower in patients with primary open angle glaucoma and NTG than in normal patients<sup>13-18</sup>.

Three studies of BDNF in aqueous humor used enzyme-linked immunosorbent assays (ELISAs). Zhang et al. reported BDNF levels of 0.84 pg/mL in glaucoma patients and 0.89 pg/mL in controls ( $p=0.11$ )<sup>19</sup>, and Shpak et al. reported levels of 35.2 pg/mL in glaucoma patients and 54.6 pg/mL in controls ( $p<0.001$ )<sup>17</sup>. However, Uzel reported levels of 9.36 ng/mL in glaucoma patients and 12.05 ng/mL in controls ( $p=0.011$ )<sup>18</sup>, approximately 10,000 times higher than those reported by Zhang et al. and Shpak et al. Measured values vary widely, and the concentration of BDNF in aqueous humor is unknown. In this study, BDNF concentrations in the aqueous humor of glaucoma patients and control patients were measured with various ELISA kits.

## Materials and Methods

### Study Population

This prospective, observational study adhered to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the Nippon Medical School Hospital (approval number: 227026). This study was registered in the Japanese UMIN Clinical Trials Registry (clinical trial identifier: UMIN000021304) before patient enrollment. Written informed consent was obtained from all participants before any clinical evaluations were performed.

From September 2016 to December 2019, BDNF levels in aqueous humor were examined in 62 eyes of 43 patients who underwent cataract surgery or trabeculectomy (11 glaucoma patients and 32 non-glaucoma cataract patients as controls) in the Department of Ophthalmology of Nippon Medical School in Tokyo. Eight patients were excluded in accordance with the exclusion criteria. The mean  $\pm$  SD patient age was  $72.0 \pm 10.1$  years (range 35 to 87 years). Glaucoma was diagnosed by evaluating IOP, gonioscopy, optic nerve head change, and presence of a visual field defect (Humphrey Field Analyzer, Zeiss, Oberkochen, Germany).

Exclusion criteria included severe ophthalmic disease, such as corneal dystrophy, degenerative retinal disease, and uveitis, and any ophthalmic surgery within 3 months. Moreover, patients with diseases that could affect BDNF concentration, such as depression, epilepsy, and Alzheimer disease, were excluded.

### Preparation of BDNF from the Anterior Chamber

Anterior aqueous humor (100  $\mu$ L or more) was aspirated from the anterior chamber. Samples were frozen on

dry ice within 1 minute and stored at  $-80^{\circ}\text{C}$  until use in experiments 1 and 2. In experiments 3 and 4, after 100  $\mu$ L of the sample was dispensed, and 33.3  $\mu$ L of the protein-stabilizing cocktail solution (#89806, Thermo Fisher Scientific K.K., Tokyo, Japan) was added, samples were immediately frozen on dry ice and stored at  $-80^{\circ}\text{C}$ .

### Experiment 1

BDNF levels were measured with a human BDNF Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's protocol ( $n=3$ ). The standard range is 62.5 to 4,000 pg/mL, and sensitivity is 20 pg/mL. If the sample was sufficient, two replicates were used.

### Experiment 2

BDNF levels were measured with an ultrasensitive human BDNF ELISA kit (Aviscera Bioscience, Inc., Santa Clara, CA, USA) in accordance with the manufacturer's protocol ( $n=9$ ). The standard range is 11 to 750 pg/mL, and sensitivity is 3 pg/mL. If the sample was sufficient, two replicates were used.

### Experiment 3

After the protein-stabilizing cocktail solution was added and stored within 1 minute, BDNF levels were measured with an ultrasensitive human BDNF ELISA kit (Aviscera Bioscience) in accordance with the manufacturer's protocol ( $n=5$ ). If the sample was sufficient, two replicates were used.

### Experiment 4

After the protein-stabilizing cocktail solution was added and stored within 1 minute, the samples were sent to SCRUM Inc (Tokyo, Japan), a company specializing in life sciences. The company measured BDNF levels with an in-house digital ELISA on the Simoa-HD1 Platform (Quanterix, Lexington, MA, USA), which measures low concentrations with precision<sup>20</sup>. The lower limit of detection (LLoD) is 0.008 pg/mL, and the lower limit of quantitation is 0.027 pg/mL. If the sample was sufficient, two replicates were used ( $n=41$ ).

### Statistical Analysis

The mean values of measurements were calculated for each group, and comparisons between groups were made with the unpaired *t*-test (Excel; Microsoft, Tokyo, Japan). A *p* value of  $<0.05$  was considered significant.

## Results

### Experiment 1

BDNF could not be detected in aqueous humor in any of the samples ( $n=3$ ). The average value was less than

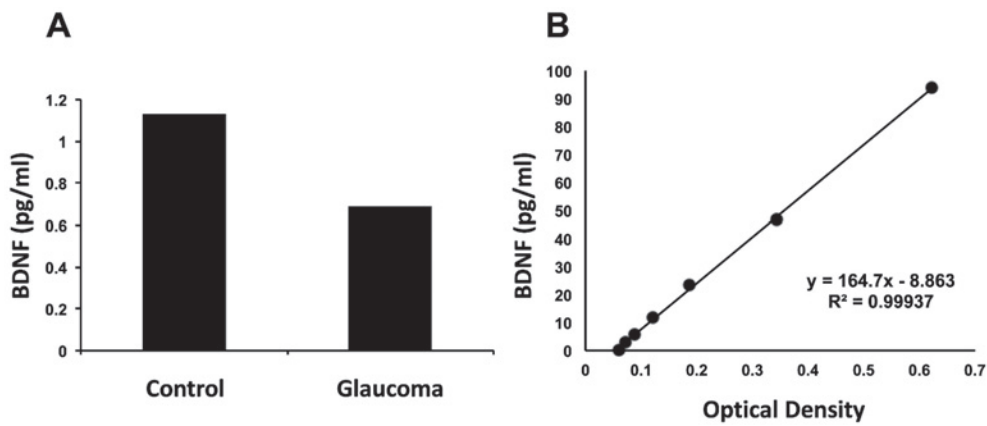


Fig. 1 BDNF concentrations in aqueous humor as determined with an ultrasensitive human BDNF ELISA kit (Aviscera Bioscience) and protein-stabilizing cocktail solution (A) BDNF in control was 1.13 pg/mL (n=3) and BDNF in glaucoma was 0.69 pg/mL. The standard range of this ultrasensitive human BDNF ELISA kit (Aviscera Bioscience) is 11 to 750 pg/mL, and sensitivity is 3 pg/mL. (B) Each value was determined after drawing a linear approximation curve.

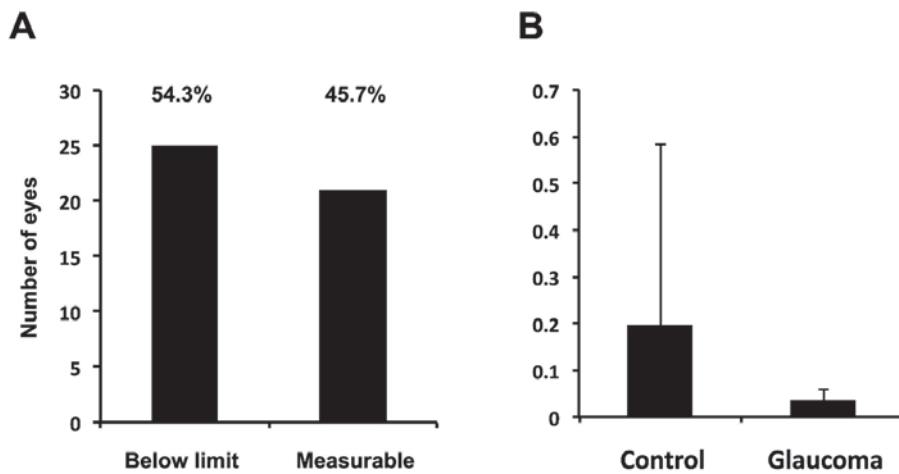


Fig. 2 BDNF concentrations in aqueous humor as determined with an in-house digital ELISA on the Simoa-HD1 Platform and protein-stabilizing cocktail solution (A) The number of eyes below the detection limit was 25 (54.3%): 20 (55.6%) in the controls and 5 (50%) in glaucoma patients. (B) In examined eyes with detectable BDNF, mean BDNF was 0.158 pg/mL overall, 0.196 pg/mL in controls, and 0.034 pg/mL in glaucoma patients.

zero.

#### Experiment 2

BDNF could not be detected in aqueous humor in any of the samples (n=9). The average value was less than zero.

#### Experiment 3

The average BDNF concentration in aqueous humor was 0.95 pg/mL (n=5). BDNF concentration was 1.13 pg/mL (n=3) in controls and 0.69 pg/mL (n=2; Fig. 1A) in glaucoma cases. The standard range of this ultrasensitive human BDNF ELISA kit (Aviscera Bioscience) is 11 to 750 pg/mL, and sensitivity is 3 pg/mL. Each value was

determined by drawing a linear approximation curve (Fig. 1B). Because ultrapure water that contained no BDNF showed a BDNF concentration of 1.1 pg/mL, and the concentrations of four samples were less than 1.1 pg/mL, these results were deemed unreliable.

#### Experiment 4

A total of 46 eyes were examined: 36 control eyes and 10 glaucoma eyes. In experiment 4, BDNF was measured with an in-house digital ELISA on the Simoa-HD1 Platform. Because the LLoD was 0.0081 pg/mL, the detection limit was less than 0.008 pg/mL. Twenty-five (54.3%, Fig. 2A) eyes were below the detection limit, including 20

(55.6%) control eyes and 5 (50%) glaucoma cases. After examining eyes with detectable BDNF, the overall BDNF concentration was 0.158 pg/mL (n=21): 0.196 pg/mL (n=16) in control cases and 0.034 pg/mL (n=5: **Fig. 2B**) in glaucoma cases.

### Discussion

BDNF is transported anteriorly and retrogradely through optic nerve fibers<sup>21-23</sup>, and the BDNF receptor TrkB is expressed in RGCs, which degenerate in glaucoma patients<sup>24-26</sup>. BDNF dysfunction is a putative cause of RGC death in glaucoma. Experimental studies have shown that reduced axonal transport induced by elevated IOP results in reduced BDNF and subsequent RGC death<sup>7,27</sup>.

BDNF in aqueous humor is considered an important biomarker for glaucoma. Therefore, in this experiment, BDNF concentrations were also measured in the aqueous humor of glaucoma patients. The most commonly used ELISA kit (human BDNF Quantikine ELISA kit; R&D Systems), the most sensitive commercially available ELISA kit (ultrasensitive human BDNF ELISA kit; Aviscera Bioscience), and Simoa, an ultrasensitive digital ELISA contract analysis service, were used. However, BDNF concentrations were still below the measurement limit. Conventional ELISA measurements are limited to pg/mL levels of detection. Simoa (SIngle-Molecule Array) is a single-molecule digital detection technology with a femtomolar (fg/mL) detection sensitivity, ie, 1,000 times higher than that of conventional ELISA<sup>20</sup>. Even with this technique, the detection rate was below 50%, and BDNF concentration in measurable samples was 0.2 pg/mL or less. The protein-stabilizing cocktail is a versatile stabilizing solution that increases the shelf-life of proteins during storage. However, the protein-stabilizing cocktail did not result in dramatic improvement. A potential reason why we were unable to measure BDNF in aqueous humor is that it is present in very small amounts and degraded before measurement. Sakane et al. reported that the therapeutic protein of BDNF has a short half-life in vivo (<2 minutes)<sup>28</sup>. Vitreous BDNF has not been measured because of its short half-life but might be more abundant than in the aqueous humor, as the vitreous contacts the retina. In the future, measurement of BDNF in the vitreous may be useful.

Zhang et al. used multiplex bead-based immunoassays (Luminex, Austin, TX, USA) with Human Neurodegenerative Disease Panel 3, which includes BDNF with a detection limit of 2.4 pg/mL. Their average measured BDNF concentration was 0.89 pg/mL, which was below

the detection limit<sup>19</sup>. As was the case for our experiment 3, these data may be unreliable. Shpak et al. used the BDNF Emax ImmunoAssay System kit (Promega Corporation, Madison, WI, USA), which has a detection limit of 7.8 pg/mL. Their average measured BDNF concentration in glaucoma patients was 35.2 pg/mL, which is within the measurement range<sup>17</sup>. Uzel et al. used the RayBio human BDNF ELISA kit (Raybiotech Inc, Peachtree Corners, GA, USA), which has a detection limit of 66.6 pg/mL. The average BDNF concentration in glaucoma patients was 9.36 ng/mL<sup>18</sup>. This value is extremely high and thus interesting.

BDNF has great potential as a neuroprotective factor. In recent years, ocular gene therapy has been performed frequently. Use of an adeno-associated virus vector for gene transfer into the inner retina is a new strategy for BDNF replacement<sup>29-32</sup>. An effective gene transfer method of intravitreal injection has also been developed for non-human primates<sup>33,34</sup>. The possibility of ocular gene therapy with BDNF for glaucoma requires monitoring of BDNF in the retina. Because it is impossible to measure BDNF in the retina, measurement of BDNF in aqueous humor is important. Past and present evidence indicates that BDNF levels in aqueous humor vary widely. The present experiments used Simoa, an ultrasensitive ELISA, but 50% or more of the samples were below the detection limit. In the future, this will be important basic data, because of the role of BDNF gene expression in ocular gene therapy.

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**Conflict of Interest:** The authors declare no conflict of interest.

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