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ABSTRACT

Glaucoma is one of the leading causes of blindness, affecting 70 million people worldwide. Glaucoma is characterized by a progressive loss of retinal ganglion cells and is often associated with elevated intraocular pressure (IOP). However, patients with normal tension glaucoma (NTG), a subtype of primary open angle glaucoma (POAG), are affected without IOP elevation. Molecular pathways leading to the pathology of the disease are still unclear mainly due to the lack of animal models. Here, we describe the first animal model of NTG based on the same gene mutation found in NTG patients. The transgenic mice over expressing E50K mutation of optineurin (*OPTN*) developed phenotype which mimics the clinical features of NTG patients including degeneration of the retinal ganglion cells at normal IOP. We demonstrate that the E50K mutation in OPTN disrupts the interaction between OPTN and Rab8, a protein known to regulate vesicle transport from Golgi to plasma membrane. Wild-type OPTN and active GTP-bound form of Rab8 complex were localized adjacent to the Golgi complex. These data provide new information about the initial steps in the development of NTG at the molecular level and a new animal model of NTG suitable for therapeutic development.

INTRODUCTION

Glaucoma is characterized by progressive loss of retinal ganglion cells (RGCs), degeneration of axons in the optic nerve, and visual field defects. POAG is one of the major causes of irreversible blindness leading to vision loss in about 4.5 million people and accounting for 12% of all global blindness (Quigley et al., 1996; 2006). POAG is often associated with elevated IOP, which is one of main risk factors in glaucoma. However, characteristic degenerative changes in the retina and optic nerve, as well as visual filed loss, may occur even in the absence of elevated IOP in a subtype of POAG which is called NTG. Recent epidemiological study in Tajimi city, have resulted with more than 90% of POAG cases diagnosed as NTG (Iwase et al., 2004).

At present, at least 24 different genetic loci have been linked to various forms of glaucoma and over the last decade, four genes, *myocilin, cytochrome P4501B1*, *OPTN*, and *WDR36* have been identified as glaucoma-associated genes (Stone et al., 1997; Stoilov et al., 1997; Rezaie et al., 2002; Monemi et al., 2005). *OPTN* was the first identified gene mutation in which can lead to NTG. The *OPTN* gene contains an initial three 3 non-coding exons followed by 13 exons encoding a protein with a length of 577 amino acids. The *OPTN* mutation at nucleotide 458 (G>A) leading to substitution of glutamic acid by lysine at amino acid 50 (E50K) is tightly linked to patients with a severe NTG phenotype. While this mutation has never been reported

in the normal population (Rezaie et al., 2002; Aung et al., 2005; Alward et al., 2003), mutations in the *OPTN* gene have been observed in 13.5% of NTG families. Chalasani et al. (2007) demonstrated that the *OPTN* E50K mutation selectively induces RGC death. OPTN has been shown to interact with number of proteins including huntingtin (Faber et al., 1998), transcription factor IIIA (Moreland et al., 2000), RAB8 (Hattula and Peranen, 2000; Park et al., 2006), myosin VI (Sahlender et al., 2005), FOS (Miyamoto-Sato et al., 2005), ring finger protein 11 (Colland et al., 2004), and metabotrophic glutamate receptor 1-a (Anborgh et al., 2005).

The molecular pathways leading to NTG from a single gene mutation still remain unclear mainly due to the difficulty of working with patient's eyes at the molecular level and lack of animal models with this particular mutation. Recently, it has been reported that the glutamate transporter-deficient mice exhibit an NTG-like phenotype (Harada et al., 2007). However to date, no NTG animal models have been developed based on the gene mutation found in patients with NTG.

In this paper we show that interaction of OPTN with the GTP-form of Rab8, which occurs adjacent to the Golgi, is significantly reduced by the OPTN E50K mutation. Transgenic mice over expressing this mutant show progressive RGC loss and excavation of the optic nerve head without detectable changes in IOP similar to the phenotype observed in NTG patients. These mice represent the first OPTN-based

animal model of NTG.

MATERIALS AND METHODS

Development of transgenic mice over expressing mutant OPTN

Total RNA was extracted from a fresh C57BL/6N mouse brain tissue using TRIzol (Invitrogen, Carlsbad, CA) and reverse-transcribed into first-strand cDNA using oligo-dT adaptor primer and SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). *OPTN* cDNA was amplified by PCR using oligonucleotides 5'-cggaattccgatgtcccatcaacctctgag-3' and 5'- cggaattccgtcaaatgatgcagtccatca-3' as primers. The amplified DNA fragment was purified using a MinElute gel extraction kit (Qiagen, Hilden, Germany), ligated into pBluescriptII (KS-) (Agilent Technologies, Santa Clara, CA) and sequenced using the M13 primers and ABI PRISM 3130 (Applied Biosystems, Foster City, CA). Site-directed mutagenesis was carried out to produce cDNA corresponding to the deletion of the E50K mutation, the first leucine zipper (1st LZ del), deletion of the second leucine zipper (2nd LZ del) and the H486R mutation. The following primers were used: 5'-cagctcaaactcaactccgg -3' and 5'-atgctccacttcctgctcca -3' for1st LZ del , 5'-aaatgaaggaactcctggttaagaaccaccagctgaaagaa-3' and 5'-ttctttcagctggtggttcttaaccaggagttccttcattt-3' for E50K, 5'-gagaccatggccgtcctc-3' and

5'-caacatcttgtccacctttctg-3' for 2nd LZ del, and

5'-gcagcaagagagaagattcgtgaagaaaggagcagc-3' and

5'-gctgctcctttcttcacgaatcttctctcttgctgc-3' for H486R. The identities of all clones were confirmed by sequencing. Plasmids were digested with EcoRI, purified by agarose gel electrophoresis, and recovered using the MinElute gel extraction kit according to the manufacturer's protocol. The cDNA inserts were ligated into EcoRI digested pCMVHA vector (Takara Bio USA, Madison, WI). HA-tagged *OPTN*s were amplified by PCR using oligonucleotides 5'- ccgctcgagcgccaccatgatgtacccatacgatgttcc-3' and 5'-ccgctcgagcggtcaaatgatgcagtccatca-3' as primers. The amplified DNA fragments were purified using a MinElute gel extraction kit (Qiagen), ligated into the pCAGGS vector and sequenced as above. cDNA inserts were released from the pCAGGS vector using SalI and BamHI. These restriction fragments were injected into pronuclear stage BDF1/C57BL6N embryos and transgenic mice were generated at PhoenixBio Co., Ltd (Tochigi, Japan). Offspring were screened for the transgene by isolating genomic DNA from tail biopsies followed by PCR. Primers used for PCR were

5'-ctctagagcctctgctaaccatgt-3' and 5'- ccatggccataagagcgtaa -3'. All experiments with mice were performed in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Vision Research.

Fundus photography

Mice were anesthetized by aspiration of isoflurane (Mylan, Canonsburg PA). Optic disk imaging was performed as previously described (Coban et al., 2003). Fundus images were obtained using 2 mm gonio lens (Ocular Instrument, Bellevue, WA) and slit lamp (SR-D7, Topcon, Tokyo, Japan) with digital camera (D100, Nikon, Tokyo, Japan).

Light microscopic histopathology of the optic nerve

After deep anesthesia, mouse eyes were dissected and immersed in Davidson solution fixative overnight at 4°C. The eyes were embedded in paraffin and sectioned at 5 μm thickness along the vertical meridian through the optic nerve head. After deparaffinization, and rehydration, sections were stained with hematoxylin and eosin (HE staining).

Electroretinogram (ERG)

Mice (15 month) were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (25 mg/kg). Pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. ERGs were recorded by Mayo Co. (Nagoya, Japan). Standard Flash ERG was obtained using flash intensity 2.50cd・

s/m^2 with a single flash. To isolate cone response, eyes was light adapted for 10 minutes with a 25cd/m^2 adapting field. Cone ERGs were obtained using flash intensity 2.50cd・s/m^2 presented at 0.5Hz with 50 flashes averaged for this intensity. Electrical signals were amplified with 8,300 gain and a bandwidth of 0.5 to 500Hz, electrical signals were digitized at a 10kHz rate using Evoked Potential/EMG Measuring System (Neuropack μ MEB-9102, Nihon Kohden Corporation, Tokyo, Japan).

Immunohistochemistry

The eyes were sectioned at 5 μm thickness along the vertical meridian through the optic nerve head. After deparaffinization and rehydration, the tissue sections were treated with Target Retrieval Solution (DakoCytomation, Denmark). The sections were incubated with blocking solution for 1 h followed by overnight incubation with primary antibody against HA tag (1:500 dilution; Sigma-Aldrich, St. Louis, MO), tublin β III isoform (1:100 dilution; Millipore, Billerica, MA), OPTN (1:500 dilution; kind gift from Mansoor Sarfarazi), or Calretinin (1:500 dilution; Sigma) in PBS containing 1% BSA at 4°C. Slides were washed in PBS and then incubated with Alexa 488 or Alexa 568 (1:500 dilution; Invitrogen) conjugated secondary antibody and with 4', 6' –diamidino-2-phenylindole (DAPI) for nuclear staining for 1 h at room temperature.

The stained tissues were examined using confocal fluorescence laser microscope (Radiance 2000, Bio-Rad Laboratories, Hercules, CA). For negative control of the immunohistochemical staining, the sections were incubated with blocking solution without primary antibody (data not shown).

Whole-mount immunostaining

The whole-mount immunostaining was performed essentially as described (Jakobs et al., 2005). Anterior parts were dissected from enucleated eyes. The posterior parts were fixed in 4% PFA/PBS for 2 hours on ice and then incubated with the anti-SMI32 (1:200 dilution; Sternberger Monoclonals, Baltimore, MD), anti-GFAP (1:200 dilution; Millipore) or anti-active caspase-3 (1:200 dilution; Imgenex, San Diego, CA) antibody for 7 days at 4°C. Slides were washed in PBS containing 0.1% Triton X-100 and then incubated with Alexa 488 or Alexa 568 (1:500 dilution; Invitrogen) conjugated secondary antibody and with 4', 6' –diamidino-2-phenylindole (DAPI) for nuclear staining for 2 days at 4°C. The retinas were then mounted with Vectashield (Vector Laboratories, Burlingame, CA) and evaluated on a confocal microscope.

Measurement of intraocular pressure (IOP)

The average IOP for each genotype was recorded. IOP was measured using an

impact-rebound tonometer (Colonal Medical Supply, Franconia, NH) and optical interferometry tonometer (FISO Technologies, Quebec, Canada) for mice of each genotype as described (Senatorov et al., 2006). Using the rebound tonometer, we were able to measure IOP in awake and non-sedated mice of various ages, while optical interferometry tonometry was performed on anesthesized animals. Measurement of IOP was always performed in the morning between 10 and 12 a. m. The numbers of mice successfully assessed for each genotype and age were 18 weeks and 12 month.

Measurement of OPTN-Rab8 interaction

OPTN-Rab8 interaction analysis was performed using *CoralHue*® Fluo-chase Kit (MBL, Tokyo, Japan). Based on the instruction manual, we constructed *OPTN* wild type, *OPTN* E50K, and Rab8 wild type, Rab8 GDP-bound (T22N) inactive form, and Rab8 GTP-bound (Q67L) active form recombinant fusion protein plasmids with both N-terminal and C-terminal fluorescence protein (Fig. 7A). RGC-5 and COS1 cells were transfected by each pairs of the plasmid mixtures using Fugene HD (Roche). 48 h after transfection, the medium was replaced to phosphate buffered saline (PBS) and the cells were observed with inverted microscope (Eclipse TE300, Nikon). To observe OPTN-Rab8 complex, cells were fixed 48 h after transfection with 4%

paraformaldehyde in PBS on ice for 20 minutes. Cells were incubated in blocking buffer (3% bovine serum albumin, 0.1% Triton X-100 in PBS) and then with anti-GM130 antibody (BD bioscience, San Jose, CA) at room temperature for 1 h each. Cells were washed three times with PBS-T (0.1% Triton X-100 in PBS) and then incubated with Alexa-568 conjugated secondary antibody for 1 h at room temperature. Slides were washed, mounted, and analyzed by confocal microscopy.

Measurement of OPTN-Rab8 interaction by quartz crystal microbalance (QCM)

The OPTN-Rab8 interaction was examined by highly sensitive 27MHz quartz crystal microbalance (QCM) (Hama et al., 2004). Rab8, OPTN, and OPTN E50K were cloned into expression vector pTrcHisB and transformed into E. coli strain BL21. Protein extracts were applied to equilibrated 1 ml Ni-chelate affinity column (Qiagen, Hilden, Germany) for purification. The gold plate on the QCM sensor chip (Initium, Tokyo, Japan) was cleaned with a mixture of sulfuric acid and hydrogen peroxide (1:3) for 5 min followed by immediate washing with water. One hundred microliters of 0.1 M glycine-HCl (pH 2.4) was then applied to the gold plate and incubated for 10 s. The sensor chip was washed again and covered with water until 150 μg of Rab8 was bound to the gold plate. Rab8 bound sensor chip was dipped into 8 ml reaction chamber containing 25% blocking buffer in PBS. Sensor chip vibration was monitored

until stabilized, then less than 8 μl of purified wild type or mutant OPTN were injected into the reaction chamber. A new sensor chip bound with Rab8 was used for each OPTN variant.

GST pull-down assay

Rab8 cDNA was cloned into expression vector pET42b and expressed in E. coli strain BL21 (DE3) to produce glutathione-S-transferase (GST) fused Rab8 protein. Supernatant fraction containing His-OPTN and His-OPTN E50K were incubated with GST and glutathione-Sepharose beads at 4°C for 30 min with continuous agitation. Each supernatant was then incubated with GST alone or GST-Rab8 with glutathione-Sepharose beads. The beads were then washed 3 times with binding buffer before eluting the bound proteins with buffer containing 20 mM glutathione. Eluted samples were analyzed by SDS-PAGE followed by western blotting using anti-Xpress tag antibody (Invitrogen, Carlsbad, CA).

The ocular coherence tomography analysis of patients with OPTN E50K mutation

Four NTG patients with severe visual field loss were identified in a three generation family by the Department of Ophthalmology of Gifu University Graduate School of Medicine. Affected 48 years old female (patient 2) was compared with

unrelated unaffected male (45 yrs) for ocular coherence tomography analysis (OCT2000, Carl Zeiss Meditec, Dublin, CA).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Scheffe's procedure after the ANOVA test for multiple comparisons of mean values. $P < 0.05$ was considered statistically significant.

RESULTS

Development of NTG transgenic (TG) mice

The chicken beta-actin promoter (pCAGGS) with CMV enhancer was used for transgene expression and was kindly provided by Dr. Junichi Miyazaki of Osaka University. HA-tag was inserted at the N-terminus of OPTN constructs for detection of proteins expressed by the transgene. Five independent Tg mice lines overexpressing *OPTN* wild type (Wt Tg), mutant E50K (E50K Tg), mutant 1st leucine zipper deletion (1st LZ del), mutant 2nd leucine zipper deletion (2nd LZ del), and mutant H486R (H486R Tg) were developed using the BDF1 and C57BL/6J strain (Fig. 1A). In the *OPTN* E50K mice, early symptoms were observed after 16 month leading to abnormal curvature around the optic disc (Fig. 1B). The distribution and protein expression level

of endogenous and mutant OPTN in the retina are shown as immunostaining with OPTN antibody kindly given by Dr. Mansoor Sarfarazi of University of Connecticut and anti-HA tag antibody respectively (Fig. 1C). Both endogenous and mutant OPTN was detected ubiquitously in all retinal layers at approximately 2-fold for all transgene constructs (data not show).

Loss of RGCs in E50K Tg mice

Paraffin eye sections were stained with hematoxylin-eosin to analyze morphological changes in the retina of Tg mice (Figure 2A). *OPTN* wild type (Wt Tg), 1st leucine zipper deletion (1st LZ del), 2nd leucine zipper deletion (2nd LZ del), and mutant H486R (H486R Tg) mice did not show any abnormalities after 16 months. However, expression of E50K mutant (458 G>A) produced significant changes in the eye. In 16 month old mice, retinal thickness and RGC number were reduced by approximately 43% and 20%, respectively in OPTN E50K Tg as compared with normal animals (Fig. 2B). Kinetics of RGC loss and retinal thinning for E50K Tg mice are shown in Fig. 2C. Statistically significant RGC loss and retinal thinning were detected 12 months after birth (Fig. 2C). To determine whether retinal function is affected by the thinning of retinal layers, electoretinograms (ERG) were obtained. E50K Tg mice exhibited abolition of the negative wave that normally follows the

transient b-wave, which originates from RGCs and is termed the photopic negative response (PhNR) (Fig. 2D arrow). The a-wave and b-wave were normal in E50K Tg mice. We concluded that E50K was the only mutation among the mutations made expression of which could lead to significant abnormality of retinal histology and function.

Synapse disruption and RGC cell death in OPTN E50K mice

Immunostaining for calretinin, a specific marker for RGCs and amacrine cells, showed loss of the synapses of these cells in the inner plexiform layer (IPL) of 16 month OPTN E50K Tg mice (Fig. 3A arrow). We confirmed excavation of the optic nerve head and immunostaining with anti-tubulin ßIII antibodies confirmed significant thinning of the nerve fiber layer in E50K Tg mice (Fig.3B arrow). Considering the abundant expression of mutant *OPTN* E50K in all retinal layers, these data indicate that RGC and nerve fiber layer are more sensitive to the expression of E50K *OPTN* and this may be the cause for NTG phenotype.

RGC and glia cell loss in OPTN E50K retina

The whole mount retina was immunostained for SMI32, a neurofilament marker for alpha-type RGCs. The number of RGCs and length of extending axons in *OPTN*

E50K Tg mice were significantly reduced compared to normal or *OPTN* Wt Tg mice (Fig. 4). Double staining for SMI32 and activated caspase-3 revealed apoptotic cells above the RGC layer in *OPTN* E50K Tg mice (Fig. 5A, B). These caspase-3 positive cells were also positively stained for glial fibrillary acidic protein (GFAP) (Fig. 5C-E), a marker for astrocytes. Interestingly, the apoptotic astrocytes were densely observed in the peripheral (Fig. 5F) compared to the central region of retina (Fig. 5G). The location and the density of apoptotic astrocytes matched the apoptotic localization of retinal neurons resulting as thinning of the peripheral retina (Fig. 2A).

IOP measurement of OPTN E50K Tg mice

IOP was measured using two different non-invasive techniques: an impact-rebound tonometer at wake condition and an optical interferometry tonometer utilizing a fiber optic pressure sensor at anesthetized condition (Fig. 6). All measurements were done between 10:00 AM and noon to minimize the influence of diurnal fluctuation of IOP. The average IOP reading from both devices gave similar IOP for mutant and wild-type mice in the normal range of 15 ± 1 mm Hg for all examined ages. These results demonstrated that glaucoma-like pathological features of mutant E50K Tg mice are not due to changes in IOP.

Disruption of OPTN-Rab8 interaction by E50K mutation

Previous studies have demonstrated that OPTN is involved in a number of cellular processes through interaction with multiple proteins. E50K mutation is located near the N-terminus of the protein where the predicted binding site of Rab8 is located (Hattula et al., 2000) suggesting a possible alteration of OPTN-Rab8 interaction in *OPTN* E50K mutants. To test this hypothesis, we compared the protein-protein interaction of OPTN (Wt, E50K) with Rab8 (Wt, T22N inactive GDP-form, and Q67L active GTP-form) in RGC5 and COS1 cells using *CoralHue*® Fluo-chase Kit. We observed over 4-fold greater interaction between wild-type OPTN and the active form of Rab8 compared with the inactive form of Rab8. No interactions between OPTN E50K and all forms of Rab8 were observed (Fig. 7B). This observation was further confirmed by QCM interaction assay and co-immunoprecipitaion assay (Fig. 7C). Interaction between host (Rab8) and guest (OPTN) protein was measured by the decrease in frequency of quartz crystal by increase of total mass (OPTN + Rab8). Purified Rab8 was fixed on the QCM sensor chip for measurements of interaction with OPTN Wt and mutant OPTN E50K. As shown in Figure 7C, interaction with Rab8 was completely disrupted by E50K mutation. These results were further supported by the glutathione S-transferase (GST)-pull down experiment using Rab8-GST fusion protein and OPTN Wt and OPTN E50K (Fig. 7D). Increased band intensity was observed for

OPTN wild-type compared to OPTN E50K, showing that the wild type form engaged in a stronger interaction with GST-Rab8 protein.

Localization of OPTN-Rab8 complex

CoralHue® Fluo-chase Kit was also used for the localization of OPTN-Rab8 in two cell lines, RGC5 and COS1. Co-immunostaining of OPTN-Rab8 complex with Golgi marker GM130 clearly indicated that the interaction take place adjacent to the Golgi network (Fig. 7E).

Optical coherence tomography (OCT) analysis of patients with OPTN E50K mutation

A family with 4 NTG patients carrying the *OPTN* E50K mutation through three generations was identified by the Department of Ophthalmology at Gifu University School of Medicine (Fig. 8A). All patients have severe visual field defects at normal IOP between 9-19 mm Hg for both eyes (Fig. 8). OCT analysis was performed to evaluate retinal abnormality. Thinning of the retina, especially retinal nerve fiber layer (NFL) and ganglion cell layer (GCL) was observed in patients compared to controls (Fig. 8). The phenotype of the *OPTN* E50K Tg mice mimics clinical features of the patient 2.

DISCUSSION

We report the first NTG mouse model that was developed using the *OPTN* gene mutation found in NTG patients. Similar to human NTG patients, the *OPTN* E50K Tg mice show progressive RGC loss in the peripheral retina without elevation of IOP. OPTN Wt, 1st LZ deletion, 2nd LZ deletion, and H486R Tg mice did not show any sign of abnormality even after 16 month. Tg mice lines expressing OPTN E50K showed significant thinning of the NFL and INL (Fig. 2, 3, 4). Expression of transgene driven by ubiquitous chicken beta-actin promoter with CMV enhancer gave similar distribution of mutant OPTN as the endogenous OPTN (Fig. 1C). Considering the fact that mutant OPTN is expressed over the entire retina, it is noteworthy that the cell death occurred mainly in the RGCs and astrocytes. These cells may be more sensitive to deleterious effects of OPTN E50K expression than other retinal cells and this may be the reason for the NTG phenotype. The amount of RGCs was reduced by 20 % in *OPTN* E50K Tg mice as compared with Wt 16 month old animals. Slow progression of RGC death resembles that in human glaucomatous patients.

Retinal function was shown to be disrupted by *OPTN* E50K mutation. The amplitude of the photopic negative response (PhNR) by E50K Tg mice was marked as slow negative wave after the b-wave often being replaced by a prolonged positive phase above base line (Fig. 2D). The PhNR was probably primarily a reflection of the

spiking activity of RGCs although other spiking cells in the inner retina, such as amacrine cells, may also contribute to PhNR. These data agree with previous human ERG studies showing PhNR disturbances in glaucoma patients (Rangaswamy et al. 2007; Machida et al. 2008).

Surprisingly, apoptotic astrocytes were more abundant in the NFL of the peripheral retina and reduced around the optic nerve head (Fig. 5F, G). Previous studies have shown activated astrocytes in the glaucomatous retina and optic nerve lamina region (Wang et al., 2002; Howell at al., 2007). Glial cells are important structural and functional components of the nervous system, including the retinal astrocytes which originate embryologically in optic nerve and subsequently migrate to their final location in the whole retina (Watanabe and Raff, 1988). It is still unclear whether which cell type, an astrocytes or retinal ganglion cells, is affected before the other. Future study using this animal model may provide new information on why peripheral retinas are more affected in human glaucoma.

OPTN was discovered as the first gene shown to be associated with NTG (Rezaie et al., 2002). The molecular mechanism leading to the disease is still unknown. OPTN E50K mutation at the N-terminus of the protein is responsible for severe NTG while H486R mutation at the C-terminus is responsible for JOAG with elevation of IOP (Stone et al., 1997; Willoughby et al., 2004). Differences of phenotype observed for