Induction of amyloid-β<sub>1-42</sub> in the retina and optic nerve head of chronic ocular hypertensive monkeys

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Purpose: Recent studies have indicated that accumulation of amyloid β<sub>1-42</sub> (Aβ<sub>1-42</sub>), which is associated with the progression of Alzheimer disease, may also be responsible for retinal ganglion cell death in glaucoma. The purpose of this study was to investigate the expression and localization of Aβ<sub>1-42</sub> in the retina and the optic nerve head (ONH) of monkeys with experimental glaucoma.

Methods: Five cynomolgus monkeys with a glaucomatous left eye at 4, 9, 11, 15, and 24 weeks after laser photoocoagulation treatment were studied by immunohistochemical methods. Another two cynomolgus monkeys with a glaucomatous left eye at 133 weeks after laser photoocoagulation treatment were used to measure Aβ<sub>1-42</sub> concentrations in the retina by enzyme-linked immunosorbent assay.

Results: At 11 to 24 weeks after the laser photoocoagulation treatment, Aβ<sub>1-42</sub> was upregulated in the nerve fiber layer (NFL) and the ganglion cell layer (GCL) of the retina and the ONH, but the expression of amyloid precursor protein decreased in the NFL and ONH from levels at 9 weeks. The localizations of Aβ<sub>1-42</sub> were merged in glial fibrillary acidic protein-positive astroglial cells but not phosphorylated neurofilament heavy- or nonphosphorylated neurofilament heavy-positive axons in the retina and the ONH. Likewise, Aβ<sub>1-42</sub> concentrations in the retina of monkeys increased in the chronic stage of glaucoma.

Conclusions: These findings indicate that the upregulation of Aβ<sub>1-42</sub> after an intraocular pressure elevation could apply to monkeys since the structure of the ONH is more similar to humans than that of rodents.

Glaucoma is a multifactorial optic neuropathy characterized by retinal ganglion cell (RGC) death [1]. This irreversible RGC death results in progressive visual field loss along with decreased color sensitivity and contrast [2]. Although RGC death can be observed in patients with normal ocular tension [3], if genetic, environmental, and other factors are involved [4], elevated intraocular pressure (IOP) is a recognized risk factor for RGC degeneration in glaucoma. At present, the only well established treatment of glaucoma involves lowering the IOP; however visual field loss continues to progress in a subset of glaucoma patients even if medical and surgical treatments successfully lower the IOP [5]. Thus, new approaches to treating glaucoma, such as directly preventing RGC death, have been required in addition to regulating IOP.

Recent studies suggest that there is a significantly higher rate of glaucoma occurrence among patients with Alzheimer disease (AD), the most common form of dementia, than control subjects, suggesting a possible relationship between these two diseases [6,7]. AD is pathologically characterized by the extracellular accumulation of the amyloid β (Aβ) peptide in senile plaques within the brain [8]. Although the causal relationship between AD and Aβ still remains to be established, growing genetic and biochemical evidence strongly suggests that Aβ, especially the longer Aβ<sub>1-42</sub> isoform, plays a pivotal and early role in AD pathogenesis [9,10]. Interestingly, it has been reported that AD and glaucoma have many common features [4]. Sunderland et al. noted that levels of Aβ<sub>1-42</sub> significantly decreased in cerebrospinal fluid from AD patients in comparison with control subjects [11]. Subsequently, we reported that levels of Aβ<sub>1-42</sub> significantly decreased in the vitreous fluid from glaucoma patients in comparison with control subjects with macular hole [12]. On the other hand, a chronic elevation of IOP induces Aβ in RGCs in experimental rat glaucoma [4]. This result is consistent with some previous reports on experimental mouse...
METHODS

Animals: We used seven adult cynomolgus monkeys (Macaca fascicularis). Five monkeys aged 4–6 years (Nippon SLC, Hamamatsu, Japan) were used for immunohistochemistry. Another two monkeys was purchased from Shin Nippon Biomedical Laboratories, Ltd., (Tokyo, Japan). Two cynomolgus monkeys aged 4–5 years were used for enzyme-linked immunosorbent assay (ELISA). Each monkey was housed in an individual cage within a monkey colony. Ophthalmoscopic examinations conducted before the experiment revealed no ocular abnormalities in any of the monkeys. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the Institutional Animal Care and Use Committee of the RIKEN Center for Molecular Imaging Science and the Institutional Animal Care and Use Committee of Tokyo University.

Induction of experimental glaucoma: Elevated IOP was induced by applying argon blue/green laser photo-coagulation burns (Ultima 2000 SE®; Coherent Inc., Santa Clara, CA) attached to a standard slit-lamp microscope (BQ 900; Haag-Streit, Kôniz, Switzerland). IOP was measured in both eyes in each animal, using a calibrated pneumotonometer. Five monkeys aged 4–6 years (Nippon SLC, Osaka, Japan) was then placed on the eye to treat. An argon blue/green laser was focused on the mid-head (ONH) is more similar to humans than that of rodents. Hence, this is the first report that, by using experimental glaucoma monkeys, has shown the time-dependent expressions and localization of Aβ1–42 in the retina as well as in the ONH after chronic IOP elevation.

Histological analysis of the retina: After the final IOP measurement at 4 to 24 weeks after the first laser treatment, the monkeys were perfused via the common carotid artery with 0.9% saline containing 10 U/ml heparin at room temperature, followed by 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4). This was done under deep general anesthesia (sodium pentobarbital 30 mg/kg, i.v). The eyes were removed after the perfusion. The eyes were immersed in the same fixative solution for at least 24 h, soaked in 10 to 30% (w/v) sucrose, and then frozen in embedding compound (Tissue-Tek; Sakura Finetechnical Co. Ltd., Tokyo, Japan). Next, 20-µm thick coronal sections of the retina were serially cut. Frozen sections (thickness, 20 µm) cut through the optic disc of each eye were used cryostat (Leica CM1850, Leica Microsystems Inc., Buffalo Grove, IL) and stained with hematoxylin and eosin.

During the immunofluorescent staining procedures for amyloid-β1–42 (Aβ1–42), coronal sections of the retina were treated with 90% formic acid for 5 min for antigen retrieval. Coronal sections were then incubated with 10% normal goat serum (Vector Laboratories, Burlingame, CA) in 0.01 M PBS for 30 min, then incubated for 1 day at 4 °C with specific rabbit anti-Aβ1–42 polyclonal antibody (1:1,000 dilution; 171,609; Calbiochem, San Diego, CA) or anti-mouse anti-Alzheimer precursor protein monoclonal antibody (1:1,000 dilution; MAB348; Millipore, Temecula, CA), which recognizes all three isoforms of amyloid precursor protein (APP; immature, mature, and soluble APP [sAPP]), in a solution of 10% normal rabbit serum in 0.01 M PBS containing 0.3% (v/v) Triton X-100. Next, coronal sections incubated with each antibody were washed with 0.01 M PBS and then incubated for 3 h at room temperature with a mixture of an Alexa Fluor 488 F(ab')2 fragment of goat anti-rabbit immunoglobulin G recognizing both heavy and light chains (IgG; H+L; 1:1,000 dilution; A11070; Invitrogen, Carlsbad, CA) or an Alexa Fluor 488 F(ab')2 fragment of rabbit anti-mouse IgG (H+L; 1:1,000 dilution; A21204; Molecular Probes). At the end of immunostaining, Hoechst 33,342 (1:5,000 dilution) was added to the samples for 30 min to visualize the nuclei.

To visualize co-localization of Aβ1–42 with glial fibrillary acidic protein (GFAP), phosphorylated neurofilament H (SMI-31) and nonphosphorylated neurofilament H (SMI-32)
double immunofluorescence was performed on sections of retina. Coronal sections of retina were washed with 0.01 M PBS and then treated with 90% formic acid for 5 min. Next, coronal sections were preincubated with 10% normal goat serum in 0.01 M PBS for 30 min, then incubated overnight at 4 °C with rabbit anti-\( \alpha \)-synuclein polyclonal antibody (1:1,000 dilution), mouse anti-GFAP monoclonal antibody (1:800 dilution; MAB360; Chemicon, Temecula, CA), mouse anti-SMI-31 monoclonal antibody (1:1,000 dilution; NE1022; Calbiochem), and mouse anti-SMI-32 monoclonal antibody (1:1,000 dilution; NE1023; Calbiochem) in a solution of 10% normal goat serum in 0.01 M PBS with 0.3% (v/v) Triton X-100. Next, coronal sections incubated with each antibody were washed with 0.01 M PBS and then incubated for 3 h at room temperature with a mixture of an Alexa Fluor 488 F(ab')2 fragment of goat anti-rabbit IgG (H+L; 1:1,000 dilution; A11070; Molecular Probes) and an Alexa Fluor 546 F(ab')2 fragment of goat anti-mouse IgG (H+L; 1:1,000 dilution; A-11018; Molecular Probes). At the end of immunostaining, Hoechst 33,342 (1:5,000 dilution) was added to the samples for 30 min to visualize the nuclei.

Images of the retina and ONH were taken using a microscope (BX50; Olympus, Tokyo, Japan) fitted with 4×, 20×, and 40× microscope objective lenses. The images visualized by hematoxylin and eosin stain (Figure 1) were taken using a charge-coupled device camera (MicroPublisher 5.0RTV; QIMAGING, Burnaby, Canada), and the cell counts in the ganglion cell layer (GCL) and inner nuclear layer (INL) at a distance between 1,750 and 2,200 μm from the optic disc toward the macula were measured on the images in a masked fashion by a single observer (Y.I.). Data from six sections were averaged for each eye, and the values obtained were used to evaluate the GCL cell counts. Immunofluorescence images (Figure 2 and Figure 3) were taken using a cooled charge-coupled device camera (DP30BP; Olympus) via Metamorph (Universal Imaging Corp., Downingtown, PA). The double immunofluorescence images (Figure 4 and Figure 5) were taken using a confocal microscope (FV10i; Olympus). The intensities of immunoreactivity in the retina and ONH were scored by a single observer who was blinded to the animals’ data. Background immunoreactivity was represented with a minus sign (−). One plus (+) represented weak immunostaining intensity, two pluses (++) represented moderate immunostaining intensity, and three pluses (+++) represented robust immunostaining intensity (see Table 1).

**Enzyme-linked immunosorbent assay of the retina:** To measure soluble or insoluble A\( \beta \)1-42, monkey retinas were homogenized in tris-buffered saline (TBS) solution consisting of 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and protease inhibitors (P8340; Sigma-Aldrich, St. Louis, MO) and then centrifuged at 500,000 × g for 20 min at 4 °C. The pellets were dissolved in 200 μl of 1% Triton X-100 and then centrifuged at 500,000 × g for 20 min. The supernatants were used as the Triton X-100 soluble fraction, while the pellets were dissolved in 200 μl of 2% sodium dodecyl sulfate (SDS)/TBS/protease inhibitors and then homogenized. The homogenates were incubated for 15 min at 37 °C, and then centrifuged at 500,000 × g for 20 min. The supernatants were collected and then dried by speed vacuum. Dried supernatants were dissolved in dimethyl sulfoxide and used as the Triton X-100 insoluble fraction. The concentration of A\( \beta \)1-42 in the Triton X-100 soluble and insoluble fractions of the monkey retina was measured using the A\( \beta \)1-42 ELISA Kit (292–64501; Wako, Osaka, Japan). ELISA for A\( \beta \)1-42 was performed according to the manufacturer’s protocol, and the monoclonal antibodies, BC05, contained in this kit specifically detects the C-terminal protein of A\( \beta \)1-42. In this assay of standard solution and the sample solutions dispersed 100 μl into wells for 1 day at 4 °C. The monoclonal antibody, BC05, contained in this kit specifically detect the C-terminal protein of A\( \beta \)1-42. Next, the wells were washed 3 times with wash solution, and then, dispensed 100 μl HRP-conjugated antibody solution into the wells for 1 h at 4 °C. Next, the wells were washed 3 times with wash solution, and then incubated for 30 min at room temperature with 100 μl TMB solution and then added 100 μl stop solution. The absorbance was measured at 450 nm using VARIOSKAN FLASH (Thermo Fisher Scientific, Waltham, MA). The absorbance was measured at 450 nm using VARIOSKAN FLASH (Thermo Fisher Scientific, Waltham, MA).

**RESULTS**

**Retinal damage after intraocular pressure elevation:** Although the nontreated right eyes did not display morphological changes in the retina and ONH, the glaucomatous eyes exhibited time-dependent changes (decreased cell number of the GCL and increased glaucomatous cup depth of the ONH) after IOP elevation (Figure 1 and Table 1). The mean cell number of the GCL was 839.3, 971.1, 360.7, 253.7, and 116.7 cells/mm in the fellow eyes of monkeys 1 to 5, respectively.

**Expression and localization of amyloid β\( \beta \)1-42 in the glaucomatous monkey eye:** The IOP data and A\( \beta \)1-42 immunoreactivities
in the retina and ONH are summarized in Table 1. Aβ\textsubscript{1–42} immunoreactivity was present in the nerve fiber layer (NFL), GCL, and ONH of the monkey retina at 11, 15, and 24 weeks but not 4 or 9 weeks after the first laser treatment in this study (Figure 2). The Aβ\textsubscript{1–42} deposits (green) were detected overlying the NFL and/or surrounding cells in the GCL (Figure 2). On the other hand, there was a decrease in the APP expressions in the NFL and ONH at 9, 11, and 15 weeks after the first laser treatment (Figure 3).

Next, to identify the localization of Aβ\textsubscript{1–42}-positive amyloid deposits, double immunofluorescence was performed for Aβ\textsubscript{1–42} and GFAP, SMI-31, or SMI-32 in the retina after IOP elevation. In the present study, Aβ\textsubscript{1–42} immunoreactivity was markedly increased in the monkey at 11 weeks after the first laser treatment (Figure 2, Table 1). We therefore used

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Figure 1. Morphological changes in retina and optic nerve head after chronic elevation of intraocular pressure. Representative photographs A: shows hematoxylin and eosin staining sections of retina and optic nerve head obtained from the eyes that had the pressure in their left eye elevated for 4 and 15 weeks and nontreated eye. Each scale bar indicates 100 μm for retina and 250 μm for ONH. Abbreviations are as follows: IOP represents intraocular pressure; GCL represents ganglion cell layer; IPL represents inner plexiform layer; INL represents inner nuclear layer; OPL represents outer plexiform layer. Fundus photographs in monkey. Representative fundus photographs showing nontreated eye, and treated eye at 133 weeks after IOP elevation B.
the retinal section obtained from the monkey at 11 weeks after the first laser treatment. Figure 4 shows representative photographs of the retina double immunostained with Aβ1-42 and GFAP, SMI-31, or SMI-32 at 11 weeks after IOP elevation. GFAP-positive astroglial cells in the NFL and the GCL of the retina but not SMI-31- or SMI-32-positive axons.
were found to express Aβ\textsubscript{1–42} at 11 weeks after the laser treatment. Concerning the localization of Aβ\textsubscript{1–42}-positive amyloid deposits in the ONH, Aβ\textsubscript{1–42} was co-localized with GFAP-positive astroglial cells as well as Aβ\textsubscript{1–42} localization in the retina (Figure 5).

Concentrations of amyloid β\textsubscript{1–42} in Triton X-100 insoluble and soluble fractions of retinal extracts: The concentrations of retinal tissue Aβ\textsubscript{1–42} as determined by ELISA are shown in Table 2. The Triton X-100 insoluble Aβ\textsubscript{1–42} concentrations in retinas were 1.41 and 0.76 pmol/mg protein in the glaucomatous left eye and nontreated right eye, respectively, of monkey 6, while those of monkey 7 were 4.56 and 0.81 pmol/mg protein, respectively. The Triton X-100 soluble Aβ\textsubscript{1–42} concentrations of the retina were 0.27 and 0.12 pmol/mg protein in the glaucomatous left eye and nontreated right eye, respectively, of monkey 6, while those of monkey 7 were 0.76 and 0.43 pmol/mg protein, respectively. Compared with the nontreated right eye, the Triton X-100 insoluble and soluble Aβ\textsubscript{1–42} concentrations of the retina increased in the glaucomatous left eye in both monkeys 6 and 7.

**DISCUSSION**

In the present study we observed the expression and localization of Aβ\textsubscript{1–42} in the retina and the ONH of monkeys with experimental glaucoma by using immunohistochemistry and ELISA. Advanced stage of RGC death and glaucomatous cupping in the ONH were observed to be time dependent at 11, 15, and 24 weeks after chronic IOP elevation.

APP is processed through non-amyloidogenic or amyloidogenic pathways. In the non-amyloidogenic pathway, α-secretase cleaves APP within the Aβ domain, thus preventing Aβ generation [17-20]. In contrast, APP cleaved
by β- and γ-secretases produces Aβ, which may aggregate, deposit, and form ion channels in the cell plasma membrane, leading to neuronal death \([17-21]\). In the present study, APP expressions decreased in the NFL and ONH from 9 weeks after IOP elevation. APP might therefore be expected to decrease at a rate equal to the increased accumulation of Aβ.
In addition, RGC loss after chronic IOP elevation was consistent with the increased expression of Aβ\textsubscript{1–42} in the retina and ONH. These findings indicate that an increase in expressions of Aβ\textsubscript{1–42} may play a role in the progressive degeneration of RGCs and their axons that is usually associated with elevated IOP in primates and rodents [3,13,14]. Likewise, concentrations of Aβ\textsubscript{1–42} in the retina increased in the chronic stage (at 133 weeks after chronic IOP elevation) of glaucoma.

Figure 5. Immunolocalizations of amyloid β\textsubscript{1–4} (Aβ\textsubscript{1–42}) in the optic nerve head of cynomolgus monkeys. Representative photographs showing of amyloid β\textsubscript{1–4} (Aβ\textsubscript{1–42})/glial fibrillary acidic protein (GFAP: A). Aβ\textsubscript{1–42}/phosphorylated neurofilament H (SMI-31: B) and Aβ\textsubscript{1–42}/non-phosphorylated neurofilament H (SMI-32: C) immunofluorescence stainings from monkey optic nerve head (ONH) at 11 weeks after laser photocoagulation treatment. Aβ\textsubscript{1–42} was co-localized with GFAP but not SMI-31 or SMI-32 in the ONH at 11 weeks after laser photocoagulation treatment. Horizontal scale bars indicate 30 µm.
in monkeys (Table 2). However, increased expressions of Aβ\textsubscript{1–42} were not found in the retina and ONH of the monkeys at the early phase (4 and 9 weeks) after IOP elevation (Figure 2). Thus, we were able to observe increased expressions of Aβ\textsubscript{1–42}, consistent with RGC loss and glaucomatous cupping in monkeys, even with the small sample sizes of our study.

The expression of Aβ\textsubscript{1–42} in GFAP-positive astroglial cells was upregulated in the NFL and the GCL of the retina as well as in the ONH in experimental glaucoma in monkey eyes. The presence of large numbers of astroglial cells associated with Aβ\textsubscript{1–42} in the retina and the ONH suggests that these lesions may generate chemotactic molecules that mediate astroglial cell recruitment after chronic IOP elevation [22-24]. In fact, recruited astroglial cells assemble at the Aβ, most likely prolonging neuroinflammation [24]. Furthermore, retinal abnormalities, such as an increase in astrocyte glial cells, atrophy of the NFL, and loss of cells in the GCL, have been observed in AD patients [25-27], and accumulation of Aβ in the retina of the 27-month AD mice model promoted the overexpression of monocyte chemotactic protein-1 by cells in the GCL, leading to loss of cells in the GCL [28]. Therefore, the role of Aβ\textsubscript{1–42} co-localized with astroglial cells may not be beneficial under certain conditions related to chronic stress.

On the other hand, astroglial cells are important for Aβ\textsubscript{1–42} degradation and clearance. In this processes, astroglial cells detect Aβ\textsubscript{1–42} deposits and then cover them. Subsequently, Aβ\textsubscript{1–42} is incorporated into the astroglial cells, which degrade the Aβ\textsubscript{1–42} [22,23]. In fact, our previous study indicated that

### Table 1. Changes in Intraocular Pressure (IOP) and Aβ\textsubscript{1–42} Immunoreactivity after Laser Photocoagulation Treatment of the Left Eye in Cynomolgus Monkeys.

| Animal number | Duration (weeks) | Mean IOP (mm Hg) | Grades of Aβ1–42 immunoreactivity | Retina | ONH |
|---------------|-----------------|------------------|----------------------------------|--------|-----|
| 1             | 4               | Left 48.7        | −                               | −      | −   |
|               |                 | Right 26.2       | −                               | −      | −   |
| 2             | 9               | Left 40          | −                               | −      | −   |
|               |                 | Right 23.2       | −                               | −      | −   |
| 3             | 11              | Left 60.2        | +++                             | +      | −   |
|               |                 | Right 23.2       | −                               | −      | −   |
| 4             | 15              | Left 62.1        | ++                              | +      | −   |
|               |                 | Right 26.8       | −                               | −      | −   |
| 5             | 24              | Left 49          | ++                              | +      | −   |
|               |                 | Right 25.5       | −                               | −      | −   |

This table identifies the duration of laser photocoagulation treatment, mean IOP, and Aβ\textsubscript{1–42} immunoreactivities in the retina and optic nerve head (ONH) after laser photocoagulation treatment. The left eye was treated with laser photocoagulation and the right eye was used as a non-treated control in each monkey. Abbreviations: tissue not stained (−), weak immunostaining intensity (+), moderate immunostaining intensity (++), and robust immunostaining intensity (+++). The grading was performed by a single observer who was blinded to the animals’ data.

### Table 2. Aβ1–42 Concentrations of Retina in the Chronic Stage of a Glaucomatous Left Eye.

| Animal number | Duration (weeks) | Mean IOP (mm Hg) | ABI–42 (pmol/mg protein) |
|---------------|-----------------|------------------|--------------------------|
|               |                 |                  | Triton X-100              |
|               |                 |                  | Insoluble fraction | Soluble fraction |
| 6             | 133             | Left 30.4        | 1.41                     | 0.27             |
|               |                 | Right 18.8       | 0.76                     | 0.12             |
| 7             | 133             | Left 29.5        | 4.56                     | 0.76             |
|               |                 | Right 19.6       | 0.81                     | 0.43             |

This table identifies duration of laser photocoagulation treatment, mean intraocular pressure (IOP), and concentrations of Aβ\textsubscript{1–42} in Triton X-100 insoluble and soluble fraction of retina after the laser photocoagulation treatment. The left eye was treated with the laser photocoagulation and the right eye was used as a non-treated control in each monkey. The concentrations of retinal tissue Aβ\textsubscript{1–42} were measured by ELISA.
the activity of neprilysin, a zinc-dependent metalloprotease involved in the physiologic degradation of Aβ\textsubscript{1-42} and Aβ concentrations in vitreous fluid displayed converse changes in patients with proliferative diabetic retinopathy (compared to patients with macular hole), with a significant inverse correlation between the two parameters [29]. We also found that concentrations of Aβ\textsubscript{1-42} significantly decrease in the vitreous fluid of glaucoma patients (compared to macular hole patients) [12]; however, we did not investigate neprilysin activity and Aβ\textsubscript{1-42} concentrations in vitreous fluid obtained from monkeys with experimental glaucoma in this study. Furthermore, Aβ\textsubscript{1-42} in the retina appears to be eliminated by the action of metalloproteases, which are localized in astroglial cells closely associated with the accumulation of Aβ\textsubscript{1-42} [30,31]. These findings suggest that astroglial cells possess the requisite elements for Aβ\textsubscript{1-42} degradation. However, we could not conclude from our data to clarify it, and therefore further experiments will be needed to clarify the precise mechanisms.

In conclusion, the present study has provided novel information concerning the time-dependent expressions and localization of Aβ\textsubscript{1-42} in the retina and ONH in monkeys after chronic IOP elevation. These findings indicate that the upregulation of Aβ\textsubscript{1-42} after an IOP elevation applies to monkeys as well as rodents. Therefore, the modulation of Aβ\textsubscript{1-42} potential target for therapeutic interventions against glaucoma.

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