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Distribution of Amyloid Precursor Protein and Amyloid- β in Ocular Hypertensive C57BL/6 Mouse Eyes

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Abstract

Purpose—Amyloid precursor protein (APP) and amyloid-beta ($A\beta$) appear to participate in the pathophysiology of retinal ganglion cell (RGC) death in glaucoma. We, therefore, determined the distribution of APP and $A\beta$ in the retinas of C57BL/6 mice after induction of chronic ocular hypertension.

Methods—Ocular hypertension was induced in one eye of three-month-old C57BL/6 mice by injection of hypertonic saline into episcleral veins. After 6 weeks of documented elevated intraocular pressure (IOP), retinas were fixed with 4% paraformaldehyde and processed for immunohistochemistry with antibodies including a polyclonal antibody to the C-terminus of $A\beta$ 40 (Novartis 17-40/23) and a polyclonal antibody to the APP ectodomain (Novartis 474). Distribution and semiquantitative expression of APP and $A\beta$ immunolabeling in ocular hypertensive and control retinas were graded in a masked fashion and compared.

Results—APP and $A\beta$ immunoreactivity was found in the pia/dura, optic nerve (ON), and RGC layer of ocular hypertensive retinas, whereas APP and $A\beta$ immunoreactivity in the contralateral control eyes was detected only in the pia/dura. Comparison of ocular hypertensive and control eyes for $A\beta$ immunolabeling was significant in the ON and RGC layer ($p < 0.05$) whereas no significant difference was found when compared for APP staining.

Conclusions—High $A\beta$ and APP levels were seen in ocular hypertensive retinas, probably due to abnormal APP-splicing in the presence of elevated IOP.

Keywords

Alzheimer; Amyloid; Amyloid precursor protein; Glaucoma; Optic nerve

Introduction

Elevated IOP appears to play an important role in the pathogenesis of glaucoma, although the exact pathologic pathways leading to retinal ganglion cell (RGC) death are not fully

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understood. Evidence suggests that altered metabolism of APP and A β contributes in part to apoptosis of RGC in glaucoma.¹

Amyloid- β A4 precursor protein (APP) is a trans-membrane neuronal protein being present in several isoforms generated by alternative splicing. It is expressed throughout neuronal tissue including brain and RGCs, where it regulates neurite outgrowth, synaptogenesis, and cell survival, also playing a central role in the neuronal homeostasis.^{2,3}

APP is synthesized in RGCs, then rapidly transported to the optic nerve (ON) in small vesicles, and finally transferred to the axonal plasma membrane and synapses.⁴

The role of amyloid- β (A β) has been well documented in Alzheimer's disease (AD) and, therefore, it is interesting that recent studies imply that a significantly higher incidence of glaucoma is detected among patients with AD than in age-matched control patients, suggesting a possible relationship between these two diseases.^{5,6}

In a previous study, we demonstrated an increased accumulation of APP and A β in eyes of DBA/2J mice, which exhibit spontaneous developing, long-lasting chronic glaucoma.⁷

In the present study we aimed to determine that these changes found in DBA/2J mice were pressure related and not due to other 'intrinsic' factors of this specific mouse strain. The goal was on one hand to provide immunohistochemical evidence of the ocular distribution of APP and A β , on the other hand to quantify the amount of these peptides in C57BL/6 mice using an induced ocular hypertensive glaucoma model.⁸

Material and Methods

Animal Tissue and Iop Measurements

Ocular hypertension was induced in one eye of three-month old inbred wild type C57BL/6 mice ($n = 10$) by the Morrison method,⁹ consisting of limbal injection of hypertonic saline (1.5 M) into the episcleral veins after placement of a modified plastic occlusion ring. IOP was measured for 6 weeks in both eyes under topical anesthesia pre-operatively and on a weekly basis post-operatively using a Tonopen XL (Medtronic Solan, Jacksonville, Florida, USA). Power analysis of earlier studies⁸ indicated that in order to detect a difference in IOP of 2 mmHg, sample size of readings has to be at least 20. The mean of the recorded measures and the differences between the hypertension induced eyes and the contralateral eyes over time are illustrated in Figure 1. After 6 weeks, animals were sacrificed and perfused with 4% paraformaldehyde in phosphate-buffered saline. Treated and contralateral control eyes were obtained, coded for immunohistochemistry, and processed for semiquantitative analysis of the expression of APP and A β in a masked fashion.

Immunohistochemistry and Analysis

Central retinal sections through the optic nerve were mounted on coated glass slides and deparaffinized. For all sections, a peroxidase-mediated amplification system (TSA Biotin Kit; Perkin Elmer Life Sciences, Boston, Massachusetts, USA), based on the deposition of biotinylated tyramide molecules, was used to amplify the staining signals. After rehydration, endogenous peroxidase activity was quenched in methanol containing 0.3% H₂O₂. After buffering in 0.1 M Tris-HCl, 0.15 M NaCl, and 0.05% Tween 20 and preincubation in 0.10 M Tris-HCl, 0.15 M NaCl, and 0.5% blocking reagent, the sections were incubated with primary antibody for 1 hr at room temperature. Affinity-purified polyclonal antibodies detecting the C terminus of A β 40 (A β 17–40/23) and the APP ectodomain (APP 474) were used.^{10–12} These antibodies were kindly provided by Paolo Paganetti (Novartis, Basel,

Switzerland). The optimal concentrations of the primary antibodies were experimentally determined to be 1:500 (A β 17–40/23) and 1:200 (APP 474).

After several washes, streptavidin-horseradish peroxidase (SA-HRP) was added for 30 min. The slides were rinsed before amplification with biotinyl-tyramide-reagent, which was added and incubated for 5 min. This was followed by several washing steps and further incubation with SA-HRP. Chromogenic visualization was achieved with diaminobenzidine tetrahydrochloride (DAB) as substrate (DAKO, Baar, Switzerland).

After a last washing step, the slides were counter-stained with hematoxylin and were then dehydrated through ascending alcohol washes and xylene. The slides were mounted and coverslipped with mounting medium (Eukitt; Inselspital-Apotheke, Bern, Switzerland).

To test the specificity of the primary antibody, control sections were stained simultaneously according to the same procedure, with the exception that the primary antibody was omitted. Paraffin-embedded brain sections of 24-month-old APP 23 transgenic (AD) mice served as positive controls for A β 40 and APP immunohistochemistry (Institute for Pathology, University of Basel, Basel, Switzerland; Novartis, Basel, Switzerland).¹⁰

Two masked observers assessed all amplified sections for localization and intensity of specific immunoreactivity on a semiquantitative scale, with linear grades 0 (no visible staining) to 4+ (intensity and color equaling that of the positive control, Figures 2 (A β) and 3 (APP)). Different ocular structures were graded separately, including the RGC layer, optic nerve (ON), and the pial/dural tissue around the ON. Magnifications of 100 $\times$, 200 $\times$, 400 $\times$, and 1000 $\times$ were examined using light microscopy.

Results

Measurements of IOP were performed for 6 weeks with Tonopen under topical anesthesia with a sample size of 20 readings as described above, calculating the mean value (Fig. 1). If induced hypertension was unsatisfactory, two further saline injections at weeks 2 and 4 were carried out (in 8 out of 10 eyes).

The ocular hypertension group revealed a mean IOP of 9.99 mmHg ($\pm$ 3.3 mmHg) versus 7.42 ($\pm$ 2.2 mmHg) in the contralateral control eye. Peak IOP in the ocular hypertension group was 15.6 vs. 11.6 in the control group.

Histopathochemically, different structures within the back of the hypertonic eyes of C57BL/6 mice displayed specific APP (Figure 4) and A β (Figure 5) immunoreactivity, above all the optic nerve, the pial/dural tissue and the RGCs compared to the contralateral control eye (Figure 6 (APP) and Figure 7 (A β)). The vitreous also revealed immunohistochemical staining, as described elsewhere.^{13,14} In adjacent control sections where the primary antibody was omitted, no immunolabeling was noted. Tables 1 and 2 represent the average staining intensities of the two antibodies (APP 474 and A β 17–40) in ocular hypertensive and control eyes. Figures 8 (APP) and 9 (A β) illustrate their respective frequency distributions. Immunolabeling in each group was compared statistically using the student's *t*-test.

Highest staining intensities of APP and A β after 6 weeks of hypertension were found predominantly in the pial/dural tissue around and in the ON and in the RGC layer. Figure 5 shows an example of an A β —stained ocular hypertensive mouse retina in contrast to the control (Figure 7). There was no correlation between amount of raised IOP and intensity of immunolabeling, but staining intensities in the ocular hypertension group were elevated compared to the control eyes, as described below. The association between IOP in individual

eyes and their respective staining intensity is illustrated in Figures 10 (APP) and 11 (A β). There were no differences in comparing the re-injected to the other mice.

In sections stained for APP, eyes with ocular hypertension showed greater staining intensities in every anatomical structure. The same eyes stained for A β also revealed greater staining intensities compared to the contralateral control eyes. Statistical significance (p 0.05) was found for A β when comparing the RGC layer and ON.

Discussion

The present study confirmed our prior findings of elevated APP and A β accumulation in hypertensive eyes, showing that these changes already occur after 6 weeks of surgically induced hypertension in young, otherwise healthy mice (C57BL/6). In earlier studies, McKinnon et al. confirmed elevated amyloid levels in an induced glaucoma rat model by western blotting and ELISA, where protein levels found were higher for A β 40 than A β 42 in ocular hypertensive retinas compared to control retinas.^{1,15} Therefore, we used an antibody targeting A β 40, not being prone to aggregation and plaque formation, such as A β 42, which could explain why no plaque formation typically seen in AD was found in these C57BL/6 mouse eyes.

Our findings demonstrate elevated APP and A β accumulation in distinct ocular tissues, mainly in the ON, the RGC layer, and the pial/dural complex. These changes might be due to an altered metabolism of APP elimination in these hypertensive eyes. Martin et al.¹⁶ demonstrated that a disruption of dynein transport contributes to disturbed retrograde axonal transport in glaucoma. APP is known to interact with cytoplasmic kinesin transport cargo adaptors and alteration in axonal transport is directly involved in disease progression,¹⁷ which may explain why similar distribution patterns of dynein accumulation in glaucomatous optic nerves are seen when compared to the distribution of accumulated APP found in our study. Our results suggest that APP is naturally occurring in pial/dural structures, being metabolized and transported in optic nerve axons, where the disruption of this equilibrium due to elevated intraocular pressure might lead to increased accumulation of A β . This and the relatively short time of hypertension might explain why the differences in staining were only significant regarding A β -immunohistochemistry.

Elevated concentrations of A β in mature neurons, as seen in AD, are responsible for neuronal degeneration¹⁸ and, furthermore, induce apoptosis in RGCs in a dose- and time-dependent manner.¹⁹

Explanations for the highest A β staining in the pial/dural tissue in ocular hypertensive eyes might be due to an amyloid-like microangiopathy, well known in AD. Additionally, microglial cells that are located around vessels express a scavenger-receptor that is supposed to act as a ligand for A β .²⁰

Accumulation of APP and A β is a recognized characteristic of the aging brain, though the mechanisms are not yet fully understood.²¹ As the retina is an integral part of the central nervous system, it is probable that these aging changes also occur in the retina. To exclude this confounding factor of amyloid accumulation, only 'young' animals (3 months of age) were used for the induction of elevated intraocular pressure. There was no correlation comparing the height of IOP-raise and the intensity of immunolabeling, suggesting that the accumulation of APP and A β is probably a consequence of a threshold excess in IOP elevation. The peak of IOP does not seem to be important, connoting that the accumulation is initiated as soon as the individual limit is exceeded even over a short period of time. Furthermore, it is suggested that RGC death after IOP elevation occurs in two phases: the fast phase, taking place in the first 3 weeks with a rate of 12% RGC loss per week, followed

months later by a second phase.²² Therefore, a pressure-related difference in RGC loss might be detectable after several months and not yet fully after 6 weeks of induction,²³ explaining why there was no visible difference in RCG-density in the two groups.

Guo et al.¹⁹ were able to show significant reduction of RGC apoptosis when targeting the amyloid pathway. New medical treatment modalities for glaucoma may, thus, be tested to treat the secondary changes including known neuroprotective anti-Alzheimer drugs, such as cholinesterase-inhibitors, or “anti-amyloid”-therapeutic strategies that decrease A β -production via secretase-inhibitors²⁴ and caspase inhibitors,²⁵ or by interfering with A β -aggregation using A β -vaccination.^{26,27}

In summary, when compared to our earlier studies where similar investigations were performed on a congenital glaucoma mouse strain (DBA/2J),⁷ the results found here were highly similar. We propose that accumulation of APP and A β in RGCs is a secondary, direct consequence of elevated IOP, and is not due to other factors intrinsic to particular spontaneous mouse glaucoma models.

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Figure 1.
Time course of the means of the IOP differences between the hypertension induced and their contralateral control eyes.

Figure 2.
A β staining in a brain section of a 24-month-old APP23 transgenic (AD) mouse (positive control, original mag. 200 $\times$) with immunolabeling (reddish-brown).

Figure 3.
APP staining in a brain section of a 24-month-old APP23 transgenic (AD) mouse (positive control, original mag. 200×) with immunolabeling (reddish-brown).

Figure 4.
APP staining in an ocular hypertensive mouse retina (original mag. 200×).

Figure 5.
Aβ staining of an ocular hypertensive mouse retina (original mag. 200β). Immunolabeled structures appear reddish-brown, e.g., in the ON.

Figure 6.
APP staining of the contralateral eye (no hypertension) of the same mouse as in Figure 4 (original mag. 200×).

Figure 7.
Aβ staining of the contralateral eye (no hypertension) of the same mouse as in Figure 5 (original mag. 200×).

Figure 8.

Frequency distribution of APP staining grades for RGC, ON, and pia/dura, in induced ocular hypertensive C57BL/6 mice and their control eyes (0 = no staining, 4 = most intense staining).

Figure 9. Frequency distribution of A β staining grades for RGC, ON, and pia/dura, in induced ocular hypertensive C57BL/6 mice and their control eyes (0 = no staining, 4 = most intense staining).

Figure 10.

Association between individual APP staining intensity and mean IOP in 10 IOP elevated and 10 control eyes (solid lines = linear regressions for elevated IOP eyes).

Figure 11.

Association between individual Aβ staining intensity and mean IOP in 10 IOP elevated and 10 control eyes (solid lines = linear regression for elevated IOP eyes).

Table 1

Distribution of APP staining for RGC, ON, and pia/dura, in induced ocular hypertensive C57BL/6 mice and their control. Mean staining intensity of each anatomical structure (0 = no staining, 4 = most intense staining)

APP	RGC	ON	Pia
Ocular hypertensive	1.35	1.8	1.9
Control	1.11	1.4	2

Table 2

Distribution of A β staining for RGC, ON, and pia/dura, in induced ocular hypertensive C57BL/6 mice and their control. Mean staining intensity of each anatomical structure (0 = no staining, 4 = most intense staining)

A β	RGC	ON	Pia
Ocular hypertensive	2.3 *	2.4 *	2.8
Control	0.9	1.25	2.1

* Student's *t*-test, *p* = 0.05.