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Optic nerve alterations in apolipoprotein E deficient mice

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PURPOSE. *To study the morphologic characteristics of the optic nerve (ON) by using an experimental model of knockout mice for expression of the ApoE gene*
METHODS. *Eyeballs with the retrobulbar ON attached were obtained from 24-week-old mice. Using morphologic and morphometric techniques and light and transmission electron microscopy, the ON characteristics were determined in three groups of mice: 1) wild type mice as the controls (CG; n=15), 2) knockout mice for the ApoE gene (ApoE-G; n=15), and 3) knockout mice for the ApoE gene that were fed a cholesterol-supplemented diet (ApoED-G; n=15).*
RESULTS. *The ON cross-sectional area was significantly higher in the ApoE-G than in the CG ($p<0.001$) mice, whereas no significant changes were noticed between the ApoE-G and ApoED-G mice. Significant differences were noticed between those groups regarding the myelination index. Higher density of intra-axonal degeneration and myelin sheath alterations were found in both ApoE groups in respect to the CG.*
CONCLUSIONS. *These results suggest that ApoE knockout mice have changes in ON morphology and myelination. (Eur J Ophthalmol 2003; 13: 560-5)*

KEY WORDS. *Apolipoprotein E, Knockout, Optic nerve, Morphology, Morphometry.*

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INTRODUCTION

Apolipoprotein E (ApoE) is a 34-kDa plasma protein for the metabolism of plasma lipoproteins (1). It has an important function in maintenance of the peripheral and central nervous system (CNS) and in axonal regeneration. Endoneurial apoE is thought to play an important role in lipids distribution from the degenerating axonal and myelin membranes to the regenerating axons and myelin sheaths (2-4). The precise role of apoE in the eyes is just beginning to be investigated. It has been described that apoE is synthesized in the retina by Müller glial cells. Then, apoE is efficiently assembled into protein particles, secreted into the vitreous, and rapidly transported into the optic nerve by the retinal ganglion cells and its terminals in the lateral geniculate and superior colliculus (5, 6). There is increasing evidence of both retinal func-

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tion and cellular morphology abnormalities in apoE-deficient (apoE-/-) mice (7), which also exhibit accumulation of membrane-bounded material with ultrastructural similarities to basal linear deposits, such as those in age-related macular degeneration (ARMD) (8). ApoE protein is strategically located at the same anatomic locus where drusen are situated in human retinas, and the retinal pigment epithelium is the most likely local biosynthetic source of apoE at that location. Age-related alterations of lipoprotein biosynthesis and processing at the level of retinal pigmented epithelium and Bruch membrane may be a significant contributing factor in drusen formation and ARMD (9). The ApoE epsilon 4 allele was associated with a decreased risk and epsilon 2 allele was associated with a slightly increased risk of ARMD (10-12).

Regarding the suggested apoE pathogenic mechanisms, it is known that two apoE-promoter single nucleotide polymorphisms (SNP) previously associated with Alzheimer disease also modify the primary open-angle glaucoma phenotype (13). On the other hand, increased ApoE immunoreactivity was found in Müller cells from degenerative glaucomatous human retinas (14). In the optic nerve (ON), apoE may be involved in the mobilization and reutilization of lipids in order to repair and maintain myelin and axonal membranes, both during development and after injury (15, 16). Knowledge of the function of apoE in the retina and ON is far from complete. ApoE experimental models may assist ophthalmologists in studying the molecular processes involved in glaucoma or other optic atrophies.

In the current study, ON from knockout ApoE mice were processed using light microscopy (LM) and transmission electron microscopy (TEM), and morphologic and morphometric analyses were performed.

METHODS

Animals and experimental design

All experiments were performed in accordance with the CE statement (November 1986). Normal and homozygous (C57BL/6) ApoE mutant mice (ApoE-/-) were used. Mice were maintained in a room illuminated from 7 AM to 7 PM in standard conditions of temperature and humidity. The following three study groups of 15 mice each were included: 1) 26-week-old wild type mice (control group) (CG) (7 male, 8 female); 2) 26-week-old mutant homozygotes (ApoE-/-) (ApoE-G) (7 male, 8 female); and 3) 26-week-old mutant homozygotes (ApoE-/-) that were fed an atherogenic diet containing 15.8% fat and 1.25% cholesterol (TD 88051; Harlan/Teklad, Madison, WI) (ApoED-G) (7 male, 8 female). Food and water were provided with free access. CG and ApoE-G mice were fed a standard diet (Purina) that is low in fat and contains 0.022% cholesterol. Group characteristics are reflected in Table I.

Collection of blood and plasma lipid assay

Blood was withdrawn from tails to measure plasma cholesterol and triglyceride levels using enzymatic procedures (Sigma, St. Louis, MO).

ON morphologic and morphometric analysis

Light microscopy - Mice previously anesthetized with ether were killed with a guillotine. The eyes and ON were then enucleated and the ON sectioned 1-3 mm behind the eyeball. Samples were fixed in 2% glutaraldehyde and 3% glutaraldehyde in 0.1 M pH 7.4 cacodylate buffer. Standard dehydration of the specimens was performed by embedding in epoxy resin (EPON). Semithin sections (1 mm) were cut in an ultramicrotome, stained with 2% toluidine blue, and examined using a LM (Olympus). To assess the morphometric analysis, micrographs were taken at 10x and 100x, scanned, and digitalized (Adobe Photo Deluxe, PC Windows Millenium). Major and minor diameters of the ON cross section were measured (Scion image, Scion Corporation, PC Windows Millenium), as shown in Figure 1. The ON cross-sectional area was calculated from the shortest diameter (a) and the longest perpendicular diameter (b), excluding meningeal covering, according to the following equation:

$$\text{Area l} = \pi \cdot a \cdot b/(A \cdot Fc)^2$$

where A = magnification constant and Fc = Olympus l m constant.

Transmission electron microscopy - Eyes were first examined under LM to determine areas of interest. Ultrathin sections of approximately 0.5 mm were cut and collected on copper grids. Sections were stained

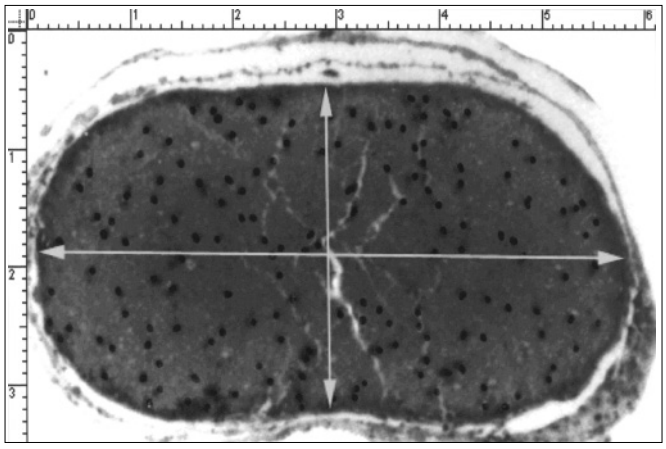


Fig. 1 - *Mouse optic nerve cross-section. Digitalized photograph measures the major and minor diameter. (Toluidine blue 10x)*

TABLE I - CHARACTERISTICS OF MICE

Characteristics	ApoE-G	ApoED-G	CG
Weight, g	23.5 ± 1.5	22.9 ± 1.7	23.2 ± 1.4
Sex, M/F	7/8	7/8	7/8

There were 15 26-week-old mice in each group

with 4% uranyl acetate and lead citrate. Ultrathin sections were evaluated by TEM and photographed at 10,400x and 25,000x magnification. All micrographs were digitalized, as previously described.

The total numbers of normal/altered axons in the electron micrographs of similar magnification and study area from the three groups were counted. The number of altered axons and myelin was expressed as a percentage of the total number of counted axons.

The myelination index is expressed as the difference between the myelin thickness and the mean of the major and minor axon diameter. The results were assembled in four different subgroups depending on the axon diameter. The first subgroup was formed by axons with a mean diameter <1 mm (SG1); second subgroup, axon diameter between 1 and 1.5 mm (SG2); third subgroup, between 1.5 and 2 mm (SG3); fourth subgroup, axons >2 mm (SG4).

Statistical analysis

Data were subjected to statistical analysis by Primer of biostatistics (McGraw-Hill), version 3.01. Student t-test and comparison of proportions was performed.

RESULTS

Serum cholesterol

Serum cholesterol levels were measured in the 26-week-old mice in all groups. Whereas the CG had cholesterol levels of approximately 118 ± 6 mg/dl and triglycerides 41 ± 3 mg/dl, the experimental groups had elevated levels of both parameters. ApoE-deficient mice (ApoE-G) fed regular mouse chow had average cholesterol levels of 425 ± 16 mg/dl and triglycerides 65 ± 23 mg/dl, whereas apoE-deficient mice that were fed a cholesterol-containing diet (ApoED-G) had the highest cholesterol and triglyceride levels (1561 ± 12 mg/dl and 521 ± 23 mg/dl).

ON morphometric study

The results of the measures of the transversal areas are shown in Table II (results are expressed in mean ± SE). The cross-sectional areas were higher in the ApoE-G and ApoED-G than in the CG (21,500 mm^2 ± 2,300 mm^2 and 20,400 mm^2 ± 2,500 mm^2 versus 15,000 mm^2 ± 1,600 mm^2; $p<0.01$). There were no significant differences between ApoE-G and ApoED-G.

Morphologic study

Myelin sheath degeneration (Fig. 2)-The total number of axons in the CG was 941; there were 850 in the ApoE-G and 1062 in the ApoED-G. The percentages of axons with profiles of myelin sheath degeneration were 8% in the CG, 16% in the ApoE-G, and 15% in the ApoED-G (Tab. III). Statistical analysis using the comparison of proportions test showed a difference of 8% ± 1.5% (standard error of the difference), $p<0.005$, when we compared CG with ApoE-G, and 7% ±1.4%, $p<0.005$, when comparing CG with ApoED-G.

Using TEM, we examined axonal degeneration signs such as axolemma swelling and axoplasm vacuolation (Fig. 2). The percentages of axons with myelin sheath degeneration were 7% in the CG, 15% in the ApoE-G, and 16% in the ApoED-G (Tab. III). Statisti-

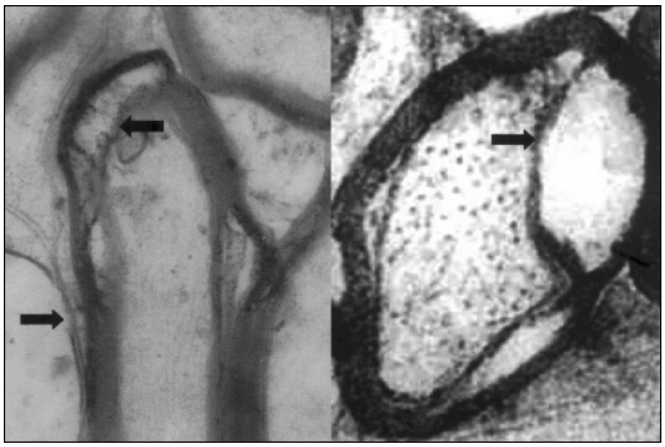
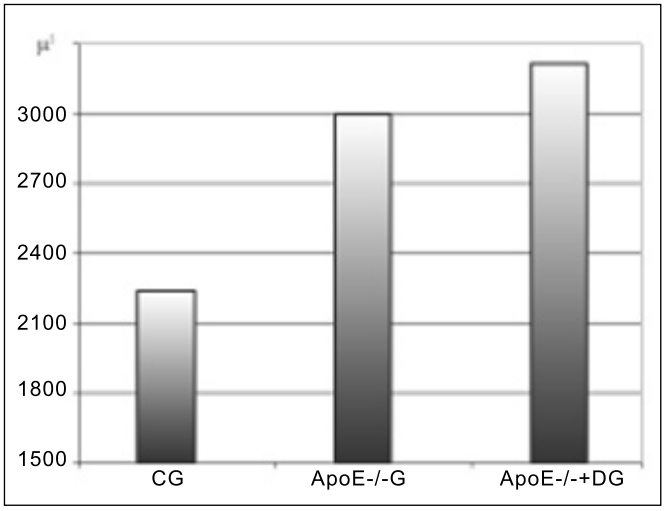


Fig. 2 - Left. *Axons with myelin sheath alterations (arrows). Ultrathin 0.5 mm, 10,400x.* **Right.** *Image suggesting an intra-axonal degeneration. Axolema swelling. Axoplasm vacuolation (arrow). Ultrathin 0.5 mm, 10,400x.*

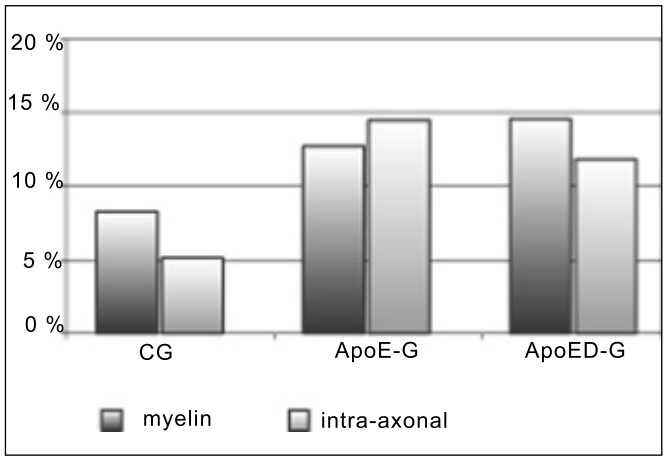
TABLE II - MORPHOMETRIC STUDY OF OPTIC NERVE AREAS: AREAS OF ApoE AND ApoE DIET ON WERE HIGHER THAN CONTROL AREAS (M^2) (mean± SE)



cal analysis using the comparison of proportions test showed a significant difference of 8% ± 1.5% (standard error of the difference), $p<0.005$, when we compared CG with ApoE-G, and 9% ± 1.4%, $p<0.005$, when comparing CG with ApoED-G.

The myelination index showed a statistical difference when we compared CG with ApoE-G and CG with ApoED-G in the SG1, SG2, and SG3 subgroups (Student t-test, $p<0.005$).

TABLE III - PERCENTAGE OF AXONS WITH MYELIN SHEATH AND INTRA-AXONAL DEGENERATION



DISCUSSION

We have attempted to characterize the ON in genetically targeted ApoE mice. This is an essential step for future research regarding the role of ApoE gene in the pathogenic mechanisms of the retinal and ON diseases. If ApoE-KO mice exhibit abnormalities in cellular morphology and function within the CNS (15, 16), therefore, morphologic alterations should be expected in the ON. With the experimental model of knockout ApoE mice used in the present study, qualitative and quantitative differences in ON size and morphology and significant differences in myelination and axonal outgrowth were found with respect to the controls.

ApoE-KO mice have elevated serum cholesterol levels and higher and earlier incidence of atherosclerosis than their counterparts (10). As a consequence of this, it seems reasonable to speculate that either the impaired nerve blood flow due to atherosclerosis and/or the high cholesterol levels reached by these animals can be the responsible ON alteration. In fact, by 14 weeks of age the animals showed atherogenesis within the aortic sinus, but not in the thoracic aorta (17). As we have used young adult mice, only the earlier stages of atherosclerotic plaque formation can be seen. However, it must be taken into account that peripheral blood vessel thickness and brain blood flow appeared normal in the experimental model used herein, as previously described (4, 17). Furthermore, it has

been shown that nerves receiving impaired blood flow displayed a characteristic pattern of degeneration that begins at the core of the nerve and progresses outward. We have not detected any sign of this or similar morphologic patterns in the ON during our research. Moreover, we have not found differences in ON size, macroglial cell and axonal characteristics, or the processes of outgrowth and myelination between the ApoE knockout group and the ApoE knockout group fed with hypercholesterolemic diet. All concepts suggest that the atherosclerotic mechanism cannot be considered when analyzing the ON anomalies in the knockout ApoE mice.

It has been suggested that ApoE protects neurons from injury and tries to help them in the processes of self-preservation and repair after cellular damage. This hypothesis is consistent with the increased apoE production *in vivo* following injury and other similar activities that has been documented by *in vitro* experiments (16). Indeed, ApoE knockout mice are particularly susceptible to nervous system trauma induced by focal ischemia (19). How, then, can we justify our findings in these uninjured models? Fullerton et al (3) suggested that highly oxidative endoneural environment should act as a self-injury factor, and that the loss of apoE might act leaving the axons especially vulnerable to injury. This mechanism, which has been described in the peripheral nervous system, should also be the origin of our present ON findings. Regarding the defense mechanisms against free radical damage, Moghadasian et al (4) measured the enzymatic defense activities of glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase in the ApoE knockout mice, and no noticeable differences in antioxidant activities were reported when the knockout and wild type mice were compared (4). These authors concluded that although apoE deficiency along with antioxidative stress are considered the major cause of numerous disorders, including neurodegenerative diseases, no significant alterations in endogenous enzymatic antioxidant activities were found.

Although no myelin alteration has been proved in the PNS (3), it is known that the apoE is involved in the trafficking of lipids in the CNS and remyelination processes. Therefore, these latter may be defective in patients with different apoE isoforms (20). Per our results, it is reasonable to believe that morphologic alterations in axons and myelin sheaths of the ON should be present in the genetically targeted ApoE mice. New morphologic and morphometric studies are needed to further clarify the presence of myelin defects in relation to neurodegenerative processes closely related to impaired vision.

Even with the presence of myelin sheath alterations, we have not found any apoptotic profiles, nor apoptotic bodies or phagocytosis phenomena, which would prove the activation of apoptotic processes in the ON of the ApoE-deficient mice (21).

Taken together, our results suggest that apoE deficit might be responsible for altered ON morphogenesis by mechanisms not fully understood. Clarifying these is our main goal for future studies.

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