OPA1 Deficiency Associated with Increased Autophagy in Retinal Ganglion Cells in a Murine Model of Dominant Optic Atrophy

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PURPOSE. To examine retinal ganglion cell (RGC) and axonal abnormalities in an ENU-induced mutant mouse carrying a protein-truncating nonsense mutation in OPA1. Mutations in the OPA1 gene cause autosomal dominant optic atrophy (ADOA) in which loss of RGCs followed by myelin degeneration in the optic nerve leads to progressive decrease in visual acuity.

METHODS. Ultrastructure of the optic nerve was examined in heterozygous mutants and wild-type littermate controls at 6, 9, and 24 months using electron microscopy. The RGC layer was examined at 6 and 24 months.

RESULTS. There was an increase in the number of autophagosomes in the RGC layer in heterozygous mutants compared with wild type at 24 months. Signs of optic nerve degeneration were seen as early as 9 months in Opa1+/− mice, with more severe degeneration by 24 months. By 24 months, degeneration of axons was also seen in control mice. Numbers of opaque mitochondria in the Opa1+/− mice increased at 6 and 24 months, possibly representing an increase in the density of cristae to fulfill the energy requirements of the axon. In addition, mitochondria with vesiculation of the inner membranes, similar to the mutant mitochondria described in a mouse model of Charcot-Marie-Tooth type 2A, were observed.

CONCLUSIONS. Mutations in OPA1 cause pathologic changes to optic nerve axons that are similar to, but occur earlier than, age-related degeneration. Increased autophagy is likely to result from an increase in abnormal mitochondria and could be one mechanism contributing to RGC loss and subsequent optic atrophy seen in ADOA. (Invest Ophtalmol Vis Sci. 2009;50:2567–2571) DOI:10.1167/iovs.08-2913

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Supported in part by Medical Research Council Grant G108/523. Submitted for publication September 19, 2008; revised November 7, 2008, and January 3, 2009; accepted April 16, 2009.

Disclosure: K.E. White, None; V.J. Davies, None; V.E. Hogan, None; M.J. Piechota, None; P.P. Nicholls, None; D.M. Turnbull, None; M. Votruba, None

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heterozygous mice. All tested Opa1+/−/ and Opa1+/+ mice were homozygous wild-type for the rdl allele.

All experiments were approved by the animal ethics committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Home Office Animals in Scientific Procedures Act of 1985.

Electron Microscopy
Three Opa1+/− mice and three littermate wild-type controls were examined at 6 months, and five Opa1+/− mice and six littermate controls were examined at 24 months. The mice were killed by lethal intraperitoneal injection of phenobarbitone and were perfusion fixed with a mixture of 4% paraformaldehyde and 5% glutaraldehyde in cacodylate buffer. The eye was dissected out, and the optic nerve and small pieces of the retina were removed. Optic nerves were also available from three Opa1+/− and three littermate controls at 9 months, though these samples were immersion rather than perfusion fixed. All samples were postfixed in osmium tetroxide, dehydrated in acetone, and embedded in epoxy resin.

Optic Nerve
Transverse ultrathin sections (approximately 90 nm) were taken from the retinal end of the optic nerve, with care taken to avoid the lamina cribosa. Sections were placed on copper grids, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (CM100; Philips, Eindhoven, Netherlands).

Fifteen systematically sampled random images of each optic nerve were taken at a magnification of 7900× for the analysis of axonal degeneration. An unbiased counting frame was superimposed on each image with image analysis software (Image Pro 6.2; Media Cybernetics, Bethesda, MD), and the number of axons within the frame was counted. The number of abnormal axons was also counted, with abnormalities classified as “watery” degeneration, “dark” degeneration, myelin clumping with redundant loops of myelin, or demyelination (Fig. 1). The number of abnormal axons in each classification was expressed as a percentage of the total number of axons. All axons were classified by a single masked observer (VH). The images were also used to estimate the number of axons per area of optic nerve to determine whether there was any axonal loss.

Another 15 images were taken at a higher magnification (×13,500) for analysis of mitochondrial morphology. Mitochondria were classified as either clear or opaque, depending on the electron density of the mitochondrial matrix (Fig. 2), and were expressed as a percentage of the total number of mitochondria analyzed. Any abnormal-appearing mitochondria were noted. All mitochondria were classified by a single masked observer (KW).

Retina
Ultrathin sections were taken through the tissue such that all the layers of the retina could be distinguished. Three blocks of retina were sectioned from each animal. On average, 45 systematically sampled random images at a magnification of ×4600 were taken of the retinal
ganglion cell layer in each mouse. Autophagosomes were observed in the ganglion cell layer and the surrounding axons. These were counted and expressed as number per unit area. An autophagosome was defined as a double membrane-bound structure that might or might not be fused with a lysosome (Fig. 3A).

**Statistical Analysis**

Analysis was conducted with statistical analysis software (SPSS 11.0; SPSS Inc., Chicago, IL). Comparisons between groups were performed with Student’s t test or ANOVA. Two-tailed P < 0.05 was considered statistically significant.

**RESULTS**

**Optic Nerve**

The quantification of structural changes seen in the optic nerve axons is illustrated in Figure 4. At 6 months, there were no differences in the structure of the axons between *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>* mice compared with wild-type controls (*P* = 0.016). There was a significant reduction in the number of autophagosomes with age in the wild-type controls (*P* = 0.001). *Significant differences between *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>* mice. #Significant differences between 6 and 24 months.

**Figure 3.** (A) Electron micrograph of early- and late-stage autophagy in retinal ganglion cell layer from a 24-month-old mouse. In the earlier stage, the double-membrane bound structure (arrow) is fusing with a lysosome (arrowhead). At the later stage (asterisk), fusion with the lysosome has taken place, and the structure is undergoing proteolysis. (B) Graph showing the numbers of autophagosomes per unit area of the retinal ganglion cell layer in *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>* mice. At 6 months, there was no difference. At 24 months, there was a significant increase in autophagosomes in the *Opa1<sup>+/−</sup>* mice compared with wild-type controls (*P* = 0.016). There was a significant reduction in the number of autophagosomes with age in the wild-type controls (*P* = 0.001). *Significant differences between *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>* mice.

**Figure 4.** Graphs showing quantification of structural changes in the optic nerve axons at 6, 9 and 24 months in *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>* mice. There were no differences in axonal structure between *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>* mice at 6 months. At 9 months, there was evidence of watery and dark degeneration in the *Opa1<sup>+/−</sup>* mice, but this did not reach statistical significance. At 24 months, there was a significant increase in the percentage of axons with disorganized myelin (clumping) in the *Opa1<sup>+/−</sup>* compared with wild-type controls (*P* = 0.037). All types of degeneration were significantly increased with age in *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>* mice, and there was a significant reduction in the number of axons per area optic nerve, indicating axonal loss. There were increased numbers of opaque mitochondria in *Opa1<sup>+/−</sup>* compared with wild-type controls at 6 (*P* = 0.027) and 24 (*P* = 0.004) months. *Significant differences between *Opa1<sup>+/−</sup>* and *Opa1<sup>+/−</sup>*. #Significant differences between 6 and 24 months.
mice and littersmate controls (Figs. 4A-D). At 9 months, the Opal+/− mice were beginning to show evidence of watery and dark degeneration compared with littersmate controls (Figs. 4A, B), though these differences did not reach statistical significance (P = 0.06, P = 0.08, respectively). At 24 months, Opal+/− mice had a significantly higher percentage of axons with disorganized myelin (clumping) than did littersmate controls (3.28% ± 1.60% vs. 1.62% ± 0.45%; P = 0.057; Fig. 4C). There was no difference in the number of axons per area of optic nerve between Opal+/− and Opal+/+ mice at 6, 9, and 24 months (Fig. 4E).

Mitochondria with clear matrix and opaque matrix were found in Opal+/− and Opal+/+ mice. However, the percentage of opaque mitochondria was significantly increased in the Opal+/− mice at 6 and 24 months (45.58% ± 5.60% vs. 28.70% ± 0.66%; P = 0.027 and 47.43% ± 18.14% vs. 15.25% ± 9.24%; P = 0.004), respectively; Fig. 4F). Some mitochondria in the optic nerves of 24-month-old Opal+/− mice had structural abnormalities and vesiculation of the inner membranes (Fig. 2C).

The structural abnormalities of the optic nerve axons were not restricted to the Opal+/− mice. Opal+/− mice also underwent age-related degeneration of the axons. With increasing age, there was a significant increase in the percentage of axons with watery degeneration (Fig. 4A; P = 0.008) and myelin clumping (Fig. 4C; P = 0.015). At 24 months, two Opal+/− mice and one Opal+/− mouse had extensive demyelination of the optic nerve. Opal+/− and Opal+/− mice experienced significant reductions in the number of axons per area of optic nerve with increasing age (Fig. 4E; P = 0.009 and P = 0.002, respectively) indicating age-related axonal loss.

Retina

Almost all the mitochondria in the RGCs had clear morphology, though there was an occasional mutant mitochondrion in the 24-month-old Opal+/− mice, as seen in the optic nerve axons.

At 6 months, there was no difference in the number of autophagosomes in the RGCs and surrounding axons between Opal+/− and Opal+/+. At 24 months, however, there were significantly more autophagosomes in the Opal+/− mice than in the Opal+/+ mice (4.25 ± 1.38 vs. 2.57 ± 0.17 μm; P = 0.016; Fig. 3B). The number of autophagosomes in the 24-month-old Opal+/− mice was significantly lower than in the 6-month-old control mice (P = 0.001). There were no statistically significant age-related differences in the Opal+/− mice.

Discussion

In this study, we describe structural abnormalities in the optic nerve and the RGC layer of an Opal+/− mouse model of ADOA. We previously published data showing abnormalities in the myelination of the optic nerve in 9- and 18-month-old Opal+/− mice.12 and our data here confirm that this is the case up to 24 months of age.

Two types of axonal degeneration have been described in the literature.15,14 One is dark degeneration, in which the axons have a dense axoplasm possibly because of neurofilament aggregation,12 and the other is watery degeneration, in which the axoplasm either is empty or is filled with an amorphous granular material. Both types of degeneration were seen in the Opal+/− and Opal+/+ mice, though watery degeneration was more prominent. The age-related changes seen in the control mice made it difficult to remove structural abnormalities caused by the OPA1 mutation and those caused by the aging process. However, we hypothesized that from the time abnormalities appear in the Opal+/− mice, the OPA1 mutation results in premature aging of the optic nerve, and that control mice might eventually catch-up with Opal+/− mice in axonal degeneration.

The age-related changes seen in the optic nerves of our control mice are similar to those reported in the optic nerves of rhesus monkeys,16 in which the most prominent changes were axonal degeneration (both dark and watery) and reduced numbers of nerve fibers. However, the extensive demyelination found in two of our controls and in one Opal+/− mouse was unexpected and not paralleled by similar levels of demyelination in the optic nerves of humans17 or monkeys.16

The presence of two populations of mitochondria (clear and opaque) in optic nerve axons has been observed recently by our group in an Opa3 mutant mouse model of Costeff syndrome in humans.18 However, given that both types are present in Opal+/− and Opal+/− mice (though the opaque type is more prevalent in Opal+/− mice), they may simply represent an increase in the density of cristae to fulfill the energy requirements of the axon.

Perhaps of more relevance was the observation that some mitochondria in the older Opal+/− mice had an abnormal cristate structure similar to that of mutant mitochondria described in a mouse model of Charcot-Marie-Tooth type 2A.19 This neurodegenerative disease is caused by mutations in the mitochondrial fusion gene Mfn2.20 Mfn2 is one of the three GTPases involved in mitochondrial fusion.10 The others are Mfn1 and OPA1.27 Thus, it is highly feasible that mutations in OPA1 could cause morphologic changes to the mitochondria similar to those seen with mutations in Mfn2.

We also looked at the structure of the RGCs, and, though a few mitochondria had abnormal cristate organization, the most prominent feature of the RGCs and the surrounding axons was the presence of several double membrane-bound structures that were identified as autophagosomes.24 Although biomarkers have been used to identify autophagosomes, this method is compromised because of a lack of differential gene expression; transmission electron microscopy remains the most accurate way to evaluate autophagy in tissue.22 Even so, electron microscopy is not without its limitations, and it is possible to overestimate autophagy by misidentifying other vacuoles as autophagic. In our study, we counted only structures that were bound by a double membrane or were obviously fusing with a lysosome. We did not try to count late-stage autophagic vacuoles because these cannot be distinguished from heterophagic vacuoles. Given that large numbers of nonspecific vacuoles were present in the cells, we feel we were more likely to have underestimated rather than overestimated the level of autophagy occurring in these cells.

Autophagy is a cellular pathway for the clearance of proteins and organelles; therefore, some degree of autophagy is necessary to maintain normal cellular homeostasis.25 Different tissues require different levels of autophagy, but it is particularly important in quiescent cells such as neurons.24 Thus, it was not surprising that autophagosomes were observed in the RGCs of our mice. In addition, the reduction in the number of autophagosomes in the older control mice is consistent with the published data on autophagy decline with age.25,26

What is even more interesting is that there were more Opal+/− mice than littermate controls. In addition, there was no age-related decline in autophagy in the Opal+/− mice. Increased numbers of autophagosomes are a feature in a number of neurologic diseases, but the question is whether this is a result of the increased formation of autophagosomes or of the decreased lysis and breakdown of the autophagosomes.27 The large amounts of nonspecific vacuoles and cytoplasmic debris that were also present suggest that the formation of autophagosomes was increased in this study.

Why then would a mutation in OPA1 cause increased autophagy? Downregulation of OPA1 results in a loss of the mito-
chondrial membrane potential and, thus, the formation of depolarized mitochondria, which are dysfunctional and must be “rescued” or eliminated. It has been suggested that mitochondrial fusion is a mechanism by which depolarized mitochondria are recovered, whereas autophagy has been shown to target depolarized mitochondria for digestion and elimination. If fusion and autophagy compete with each other for mitochondrial fusion is impaired, autophagy is more likely to be the winner. This hypothesis is supported by a recent study in which overexpression of OPA1 decreased mitochondrial autophagy.

It could be argued that this increased autophagy would maintain cellular homeostasis and have no detrimental effect on the cell. However, because RGC loss is the primary pathologic characteristic of ADOA, it is possible that the autophagic process becomes overloaded, resulting in an accumulation of defective mitochondria, the release of cytochrome c, and ultimately the apoptosis of RGCs.

In conclusion, we have shown that mutations in OPA1 cause pathologic changes to optic nerve axons that are similar to, but occur earlier than, age-related degeneration. In addition, we suggest that increased autophagy could be one mechanism contributing to RGC loss and the subsequent optic atrophy seen in ADOA.

Acknowledgments
The authors thank Tracey Davey and Vivian Thompson (EM Research Services, Newcastle University, UK) for technical assistance.

References