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Optic nerve alterations in PTEN$^{+/-}$ mice

E. LÓPEZ-SÁNCHEZ[1], E. FRANCÉS-MUÑOZ[2], J.L. MENEZO[2,3], M.D. PINAZO-DURÁN[4]

[1]Hospital Arnau de Vilanova
[2]Hospital "La Fe"
[3]Fundación Oftalmológica del Mediterráneo (FOM)
[4]Ophthalmology Research Unit "Santiago Grisolía," Valencia - Spain

PURPOSE. *To study the morphologic characteristics of the optic nerve (ON) by using an experimental model of knockout mice for the expression of the PTEN gene, mainly involved in cell cycle arrest, apoptosis control, and cell size regulation.*
METHODS. *The eyeballs with the retrobulbar ON attached were obtained from 26-week-old mice. By using morphologic and morphometric techniques, light and electron transmission microscopy, the ON characteristics were determined in two groups of mice: 1) "wild type" mice as the control group (C-G; n=15), 2) heterozygous knockout mice (+/-) for the PTEN gene (PTEN-G; n=15). Glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) were studied using Western blot and immunoblotting approaches.*
RESULTS. *The ON cross-sectional area was significantly higher in the PTEN-G than in the C-G ($p<0.001$). The axon sizes in mutant animals were much larger than in wild-type mice ($p<0.001$). No significant differences were noticed between those groups regarding the number of axons forming the ON and the presence of intra-axonal degeneration, myelin sheath alterations, or axoplasm density. No differences were detected in developmental marker protein expression.*
CONCLUSIONS. *The morphologic and morphometric results suggest that heterozygous PTEN knockout mice had hypertrophic ON without ultrastructural alterations. (Eur J Ophthalmol 2006; 16: 440-5)*

KEY WORDS. *Knockout, Morphology, Morphometry, Optic nerve, PTEN*

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INTRODUCTION

The tumor suppressor gene PTEN is one of the most frequently mutated genes involved in the development of human cancer. PTEN mutations are found in a wide variety of cancers such as prostate cancer, endometrial carcinomas, and glioblastomas (1).

The protein/lipid phosphatase PTEN plays a significant role not only in inducing cell cycle arrest and programming apoptosis, but also in other aspects of cell physiology, including the regulation of cell adhesion, migration, differentiation, and growth (2, 3).

PTEN is an inefficient protein phosphatase. This suggests that the substrate of PTEN might be the phosphatidylinositol (3,4,5)-triphosphate (PIP-3). The PIP-3 is dephosphorylated by PTEN to phosphatidylinositol (4,5)-diphosphate (PIP-2). In the absence of PTEN, the binding of Akt to PIP-3 phosphorylates and inactivates members of the forkhead family of transcription factors, the proapoptotic proteins Bad and caspase-9, and blocks cytochrome c release and Fas-dependent apoptosis through unknown pathways.

This results in cell protection from several apoptotic stimuli. Akt also downregulates p27 levels resulting in stabilization of cycline D1, thus leading to increased cell proliferation (3-5).

Although the spectrum of disorders affecting the PTEN

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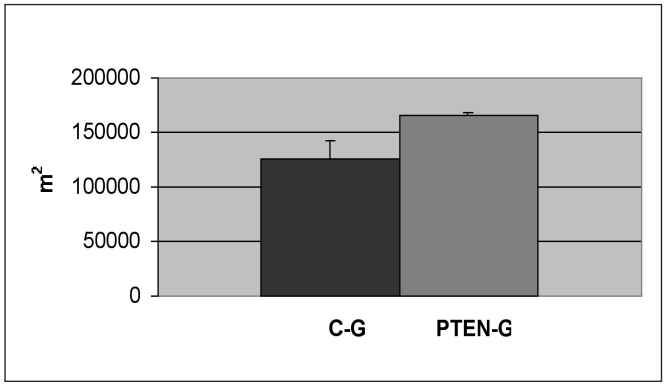

Fig. 1 - *Morphometric study. Optic nerve areas: PTEN-G areas were higher than control areas (μm²) (p<0.01).*

mutant mice varies between the different strains, all homozygous PTEN mutants exhibit early embryonic lethality (6).

Surprisingly, even the inactivation of just one PTEN allele has a dramatic impact on the survival and proliferation of selected cell populations such as lymphocytes. PTEN-/+ mutants developed a lethal autoimmune syndrome with features reminiscent of those observed in Fas-deficient mutants (7). Those mutant mice also developed tumors in multiple tissues like breast or endometrial cancer (8, 9), and offer an excellent model system for the investigation of PTEN-related hamartoma syndromes (10).

Overexpression of PTEN in different mammalian tissue culture cells affects several processes including cell proliferation, cell death, and cell migration (11, 12).

Huang et al have characterized the Drosophila PTEN gene and presented evidence that both inactivation and overexpression of PTEN affects cell size. While loss of PTEN affects the sizes of individual mutant cells, which are then much larger than their neighboring wild-type cells, this condition seems to have no effect on the pattern of the impaired cells. Moreover, overexpression of PTEN inhibits cell proliferation during eye development (13).

To our knowledge, the function of PTEN in the retina and optic nerve (ON) is far from complete. PTEN experimental models may provide ophthalmologists a good chance to study the molecular basis involved in ocular malformations or neurodegenerative processes, including ON hypoplasia, microphthalmos, and other pathologies that can lead to optic atrophy, like glaucoma.

In the present work, ON from heterozygous knockout PTEN mice were processed for light and electron transmission microscopes, and morphologic and morphometric analyses were performed.

METHODS

Animals and experimental design

All experiments were performed in accordance with EC Regulation (Nov/86). Normal and heterozygous (C57BL/6) PTEN mutant mice (PTEN+/-) were used. Mice were maintained in a laboratory room illuminated from 7 am to 7 pm, in standard conditions of temperature and humidity. Two study groups were established: 1) 14 26-week-old "wild type" mice (14 males) as the control group (C-G); 2) 14 26-week-old mutant heterozygotes (PTEN+/-) (14 males) (PTEN-G). They all had free access to food and water. The groups were fed with standard diet (Purina). Mice were classified and maintained in cages during the whole experiment until euthanasia (previously anesthetized with ether).

Morphologic and morphometric analysis

Light microscopy (LM). The right eyes and ONs were then enucleated and the optic nerves sectioned 1 to 3 mm behind the eyeball. Samples were fixed in 2% glutaraldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Standard dehydration of the samples was performed by embedding in epoxy resin (EPON 850). Semithin sections (1 μm) were cut on an ultramicrotome LKB. Sections were stained with 2% toluidine blue and examined using a LM (Leika) with image analysis system (Q-win). To assess the morphometric analysis of the ON, digital micrographs were taken, as reported (14). The ON cross areas were measured (Scion Image, Scion Corporation, PC Windows XP), excluding meningeal covering.

Transmission electron microscopy (TEM). Eyes were first examined under LM to determine areas of interest. Ultrathin sections of approximately 100 nm were then cut and collected on copper grids. Sections were stained with 4% uranyl acetate and lead citrate. Ultrathin sections were then evaluated by TEM and photographed at 12,000 X and 25,000 X magnification.

• *Axonal area measurement:* The axonal cross-areas were measured (Scion Image, Scion Corporation, PC Windows XP). In general, 453 axons from 700 μm² in the C-G and 316 from the same area in the PTEN-G were analyzed.

• *Total number of axons forming the ON:* By using the ON cross-sectional area measurement and the axonal

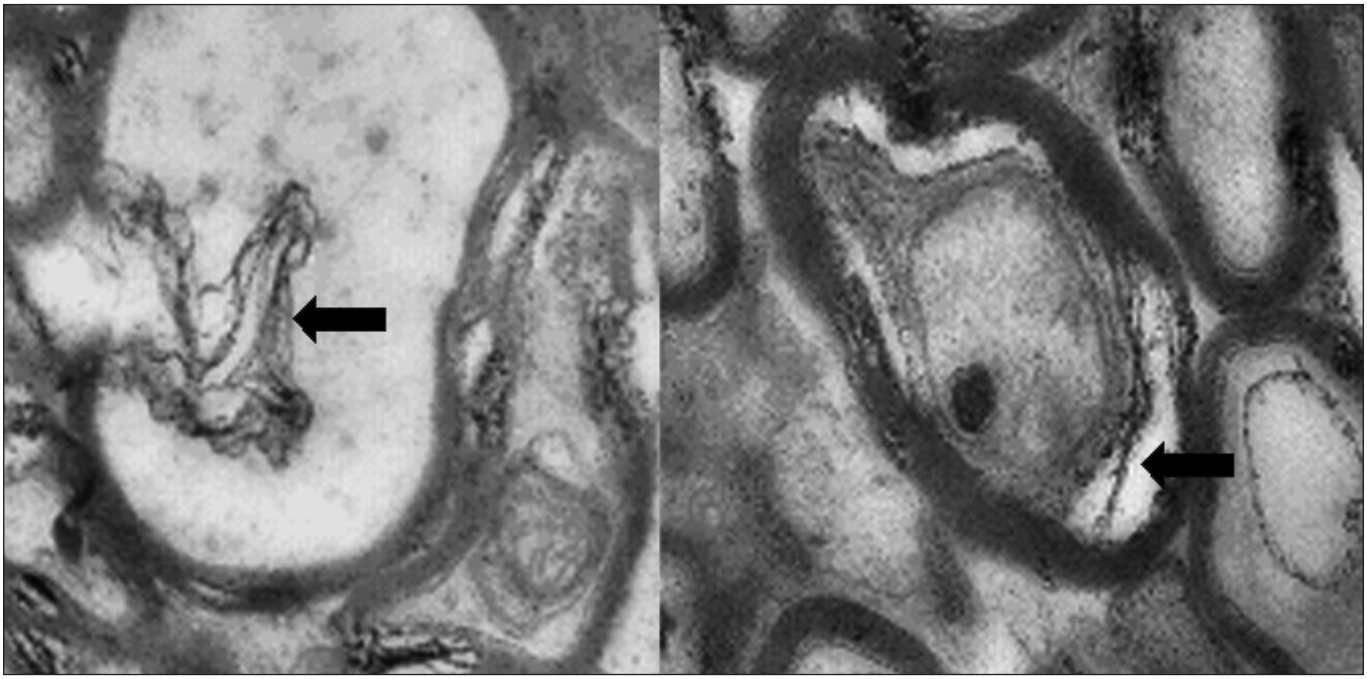

Fig. 2 - *Left: Axons with myelin sheath alterations (arrows). Ultrathin 0.5 µm 12,000 x. Right: Image suggesting an intra-axonal degeneration. Axolema swelling. Axoplasma vacuolation (arrow). Ultrathin 100 nm 10,400 x.*

count, an estimation of the total number of axons compounding the ON was calculated.

• *Myelin sheaths and axonal degeneration recording:* We counted the total number of normal/impaired axons in the electron micrographs with similar magnification and study area obtained from the two groups. The number of impaired axons and myelin was expressed as a percentage of the total number of counted axons.

• *Densitometry of the axoplasmic material:* In order to assess the possible differences regarding the electron lucent or dense axoplasms in both groups, we performed a densitometric analysis (Scion Image, Scion Corporation) comparing 100 axons from both groups (C-G and PTEN-G).

• *The myelination index:* Is expressed as the difference between the myelin thickness and the axon diameter. The results were assembled in four different subgroups depending on the axon diameter, as we previously described (15). First subgroup was formed by axons with a mean diameter <1 µm (SG1), second subgroup with an axon diameter between 1 µm and 1.5 µm (SG2), third subgroup with an axon diameter between 1.5 and 2 µm (SG3), and the fourth subgroup with an axon diameter >2 µm (SG4).

Western blot analysis

The left optic nerves were cryopreserved (–85ºC) until the Western blot and immunoblotting techniques were performed, as described before (16). Lysates were resolved on 12.5% Nu-PAGE gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Membranes were probed with antibody against glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP), which are elemental structural proteins of the optic nerves. Densitometric studies of the membranes were then performed.

Statistical analysis

Data were subjected to statistical analysis with SPSS, version 11.5. Student's t-test and comparison of proportions were performed.

RESULTS

ON morphometric study

Data of the ON cross-sectional area are shown in Figure 1 (results are expressed as mean ± SE). Mean cross sectional areas were higher in PTEN-G than in C-G (164.795 $\mu m^2 \pm 3.645$ versus 125.209 $\mu m^2 \pm 17.277$ μm^2, $p<0.01$).

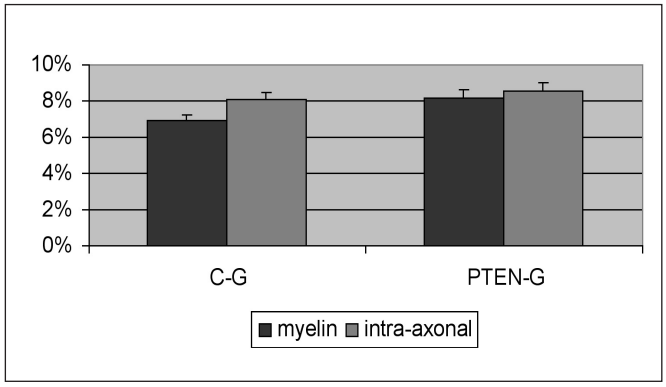

Fig. 3 - *Percentage of axons with myelin sheath (p=0.57) and intra-axonal degeneration (p=0.89).*

The axonal cross-sectional areas obtained from the TEM photographs revealed higher values for the axons of PTEN-G when compared with C-G (1.21 $\mu m^2 \pm 1.29$ versus 0.8 $\mu m^2 \pm 0.95$ μm^2, $p<0.01$).

In the estimation of the total number of axons within the ON, PTEN-G showed a higher number of axonal profiles than C-G (81.028±129 versus 79.101±241), although this difference was not statistically significant (p=0.4).

Morphologic study

Myelin sheath degeneration (Fig. 2, left). The total number of axons studied both in C-G and in PTEN-G was 420. The percentage of axons with profiles of myelin sheath degeneration was 6.9% in C-G and 8.18% in PTEN-G (Fig. 3). The statistical analysis failed to demonstrate a statistical difference between the two groups (comparison of proportions test, p=0.57).

The axonal degeneration signs like swelling, vacuolation, and inclusion bodies were examined using TEM (Fig. 2, right). The percentage of axons with intra-axonal degeneration was 8.09% in C-G and 8.57% in PTEN-G (Fig. 3). Statistical analysis using the comparison of proportions test showed no significant difference (p=0.89) between the two groups.

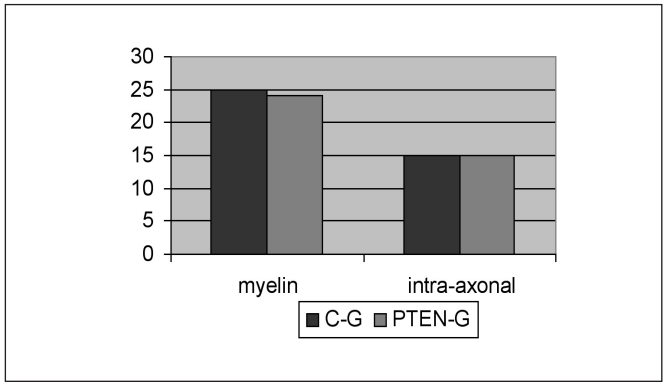

Fig. 4 - *No differences were noticed in the Western blot quantitative results (densitometric units).*

Densitometry of the axoplasmic material: Quantification of densitometry failed to demonstrate a statistical difference between C-G and PTEN-G (paired t-test, p=0.89).

The myelination index failed to demonstrate a statistical difference when comparing C-G with PTEN-G in the SG1, SG2, SG3, and SG4 subgroups (Student's t-test, p=0.35, p=0.93, p=0.98, and p=0.87).

Western blot analysis: The transference to nitrocellulose membranes and incubation of the antibodies showed no difference in the band expression of the studied proteins. Quantification of densitometry demonstrated that the comparison between relative densitometric units of protein expression from both groups failed to find any difference (Fig. 4).

DISCUSSION

The proteolipid phosphatase PTEN plays a significant role in programming apoptosis, as well as in regulating cell size, cell adhesion, cell migration, and cell differentiation (2, 3, 11).

The PTEN dephosphorylates, and thus deactivates, PIP3, a major kinase involved in cell survival. In a variable proportion of many human tumors PTEN is downregulated or absent, activating PIP3 signaling. In this situation of PTEN downregulation, tumor cells are less responsive to apoptosis (3-5).

A wide range of disorders affecting PTEN mutant mice have been described (6). Cell size abnormalities in PTEN-/+ Drosophila eyes have been reported (13), suggesting that similar alterations should be expected in rodent models. We have attempted to characterize the ON in genetically targeted PTEN-/+ mice using morphologic and morphometric approaches. This is a preliminary step for further programmed research about the role of PTEN gene in the pathogenic mechanisms of retinal and ON diseases.

As described herein, statistically significant quantitative differences in ON size and axonal areas were found when comparing with the control ON. No pattering alterations in the myelin structure or intra-axonal alterations were present. Furthermore, no alterations in the GFAP or MBP ex-

pression could be demonstrated. Similar changes have been described in rat pups exposed to methamphetamine during pre- and postnatal development (17), in which the developing ON showed anomalies in size without noticeable anatomic disturbances. On the whole, our results strongly suggest that PTEN-/+ mice show ON hypertrophy pattern with cytoarchitectural preservation.

Other authors (18) had previously described in PTEN-/+ mice an inherited deficit in cell apoptosis, leading to gradual hypertrophy and cell accumulation. This abnormality is reminiscent of phenotypes which have been described in mice deficient in FAS signaling (19) and would be responsible for the association between PTEN mutation and multiorgan neoplasia. However, this deficiency has only been demonstrated in endometrial, breast, and lymphoid tissues (8). In the present study we have not found a significant increase in the total number of axons in the optic nerve as would be expected in an apoptotic deficient state, but we did find one in the diameter of each axon. This condition cannot be explained by the action of the PTEN mutation on the apoptotic control, but it is more plausibly explained taking into account the action of PTEN in the insulin receptors.

From a molecular point of view, Bohni et al (20) described an antagonic function of PTEN with PI3K, an insulin receptor with the role of activating cell membrane and controlling insulin signaling pathway, in order to perform the regulation of cell size without affecting pattern formation (21, 22). Consistent with the role of PTEN as a negative regulator of the insulin pathway, in our PTEN model, the effects of a positive stimulus on the insulin receptor were linked to the cellular and subcellular morphologic and morphometric data, which were noted under light and transmission electron microscopy.

Interestingly, retinal and central nervous system hamartomas, often found in carriers of PTEN mutations (23), could not be detected in our mice, because their tissue morphology was always well preserved.

In summary, heterozygous PTEN knockout mice showed hypertrophic ON but not ultrastructural abnormalities.

None of the authors have a proprietary interest.

Reprint requests to:
E. López-Sánchez, MD
Gran Vía Germanías 45 p-31
Valencia 46006, Spain
elopsan@ono.com

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