

Frank Schuettauf · Robert Rejdak · Michal Walski
Malgorzata Frontczak-Baniewicz · Michael Voelker
Georgios Blatsios · Kei Shinoda · Zbigniew Zagorski
Eberhart Zrenner · Pawel Grieb

Retinal neurodegeneration in the DBA/2J mouse – a model for ocular hypertension

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Abstract Mice of the DBA/2J strain spontaneously develop complex ocular abnormalities, including glaucomatous loss of retinal ganglion cells (RGC). In the present study ultrastructural features of retinal neurodegeneration in DBA/2J mice of different age (3, 6, 8 and 11 months) are described. By 3 months, RGC apoptosis characterized by electron-dense karioplasm and cytoplasm of ganglion cells was observed. The occurrence of apoptotic ganglion cells peaked at the age of 6 months. Past this age, necrosis characterized by swelling and electron-rare cytoplasm appeared to be the prevailing form of cell death. Müller glia activation increased with age, but there were no signs of leukocyte infiltration. At 8 and 11 months, signs of neoangiogenesis were found both at the ultrastructural level and in clinical examinations. In these older animals myelin-like bodies, most probably representing the intracellular aggregates of phospholipids in irreversibly injured cells, were also seen. Photoreceptor cells were not affected at any age. Our observations suggest that retinal degeneration in the DBA/2J mice does not involve recruitment of blood-borne inflammatory/phagocytosing cells, and that apoptosis is gradually replaced by necrosis as the predominant pathway of RGC death. Retinal degeneration in 3- to 11-month-old DBA/2J mice partially resembles human pigment dis-

persion syndrome and pigmentary glaucoma with characteristic anterior segment changes and elevation of intraocular pressure. However, neovasculogenesis and myelin-like bodies are observed during aging. Therefore, the DBA/2J model requires judicious interpretation as a glaucoma model.

Keywords Ocular hypertension · Model · DBA/2J mouse · Apoptosis · Electron microscopy

Introduction

Despite recent advances in therapeutical approaches, there is a continuous need to develop novel, less toxic and/or more active pharmacological treatments for neurodegeneration [5]. A prerequisite for a new drug to enter clinical trials is the assessment of its safety and efficacy in animal models of disease.

In glaucoma, the current therapeutical approach aims at lowering the intraocular pressure (IOP). However, there is evidence to show the importance of neuroprotective strategies to stop progression of the disease [22, 32]. Pre-clinical assessment of anti-glaucoma drugs has been traditionally performed on monkeys with artificially increased IOP (see [11, 42] for recent examples). However, work with rodents was found to be less expensive and, therefore, it can be applied to larger numbers of animals. Currently rats with IOP elevated chronically by various surgical interventions [9, 21, 25] are being employed as small-animal surrogate models of human glaucoma. Both rat and primate models may reproduce certain aspects of the pathomechanism of human glaucoma, but in neither does the retinal ganglion cell (RGC) loss develop naturally.

Spontaneous glaucoma can occur in several animal species and breeds [10, 37]. To our knowledge they have not been extensively used for preclinical testing of anti-glaucoma medication, most likely due to the big variability in the extent of pressure elevation, and other ocular abnormalities.

Recently, a genetically determined murine pigmentary glaucoma in the DBA/2J strain has been described. These

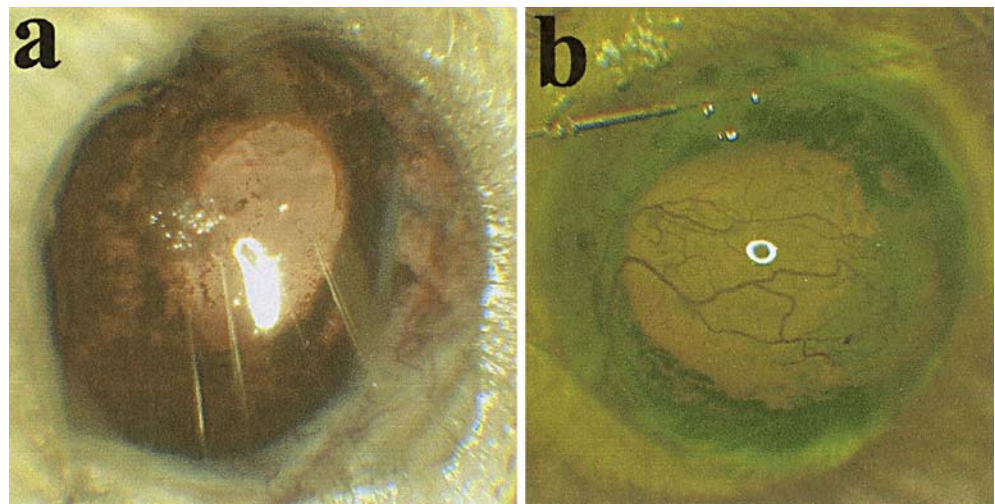
F. Schuettauf (✉) · R. Rejdak · M. Voelker · G. Blatsios
K. Shinoda · E. Zrenner
Experimental Ophthalmology, University Eye Hospital,
Roentgenweg 11, 72076 Tuebingen, Germany
Fax: +49-7071-295777,
e-mail: fschuettauf@uni-tuebingen.de

R. Rejdak · Z. Zagorski
Tadeusz Krwawicz Chair of Ophthalmology
and 1st Eye Hospital, Medical University, Lublin, Poland

M. Walski · M. Frontczak-Baniewicz
Laboratory of Cell Ultrastructure, Medical Research Center,
Polish Academy of Sciences, Warsaw, Poland

P. Grieb
Laboratory of Experimental Pharmacology,
Medical Research Center, Polish Academy of Science,
Warsaw, Poland

Fig. 1 Photograph of the anterior segment of DBA/2J mice. **a** An 11-month-old mouse representing the most common severity of this age. Unusual central and peripheral corneal opacities, vessel invasion, shallow anterior chamber, wide spread iris atrophy and slit-like transillumination defects, pigment dispersion on the anterior surface of the lens, diffuse posterior synechias and peripheral anterior synechias are seen. **b** A 10-month-old mouse, the same eye of Fig. 2c. Extensive corneal vascularization and an atrophic iris are seen. Relative large dilation could be kept due to preceding atropine instillation



mice develop progressive ocular abnormalities consisting of pigment dispersion, iris transillumination, iris atrophy and anterior synechias. By 9 months the IOP is elevated in most of the DBA/2J mice, and glaucomatous changes, including RGC loss, optic nerve atrophy and optic nerve cupping are evident. John et al. [16] noted that the DBA/2J mice may represent a useful model to study mechanisms of RGC death and to evaluate strategies of neuroprotection for glaucoma. We studied the dynamics of RGC loss in these mice [36]. A progressive reduction in the number of RGC, to 60% at the age of 9 months was observed. We also found that this ganglion cell loss model is responsive to pharmacological treatments; the RGC loss was blocked by the glutamate antagonist memantine given intraperitoneally, and by the β -blocker timolol applied as eye drops.

The aim of the present study was to describe clinical and ultrastructural features of retinal neurodegeneration in the DBA/2J mice of different ages. These data could be helpful to assess the relevance of this spontaneous murine ocular disease to human glaucoma.

Material and methods

Animals

Animals were treated in compliance with the guidelines of animal care of the European Community and the Association for Research in Vision and Ophthalmology. Breeder pairs were obtained from Charles River (Sulzfeld, Germany). Locally bred female DBA/2J mice were held under specific pathogen-free conditions at room temperature and a 24-h light/dark cycle. Acidified water and a special chow (ssniff M, from Sniff Spezialdiäten, Soest, Germany) were supplied ad libitum. Age-matched control retinas were collected from mice of the C57/BL6 strain, which do not display any ocular abnormalities.

Clinical examinations

Anterior segments were examined with a slit-lamp biomicroscope (Haag-Streit, Switzerland). Photographs were taken using a fundus camera (Kodak Megaplug Model 4.2i, Tokyo, Japan). Fluorescein fundus angiography (FFA) was performed in a standard manner [14] under ketamine (150 mg/kg) and xylazine (15 mg/kg) anesthe-

sia. Pupils were dilated with 2.5% phenylephrine and 1% cyclopentolate, and the cornea was kept moist. Mice were intraperitoneally injected with 10% sodium fluorescein at a dose of 0.005 ml/g body weight. Fundus fluorescent images were obtained with a scanning laser ophthalmoscope (Rodentstock Instrument, Munich, Germany) using argon laser (488 nm, 1 mW) as an exciter, and were recorded on S-VHS videotape. In three mice, 1% atropine eye drops were administered once a week for more than 2 weeks before FFA to maintain pupil dilation.

Electron microscopy

Retinas were collected from mice at the ages of 3, 6, 8 and 11 months. Animals were killed by CO₂ intoxication, and the eyes were enucleated immediately. Following hemisection of the eye along the ora serrata, the cornea, lens and vitreous body were removed. Eyecups were immersion-fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 20°C for 20 h and postfixed in 1% OsO₄ and 0.8% K₄FeCN₆. After dehydration in a series of ethanol and propylene oxide, tissue specimens were embedded in Spurr resin. Ultrathin (50 nm) sections were examined with a JEM 1200EX electron microscope.

Results

Clinical examinations

Slit-lamp examinations revealed several anterior segment abnormalities (Fig. 1): corneal opacity, corneal vessel invasion, keratic precipitates, shallow anterior chamber, pigment dispersion, iris atrophy, ectopic pupil, posterior synechias, peripheral anterior synechias, and cataract, most of which have been reported previously [3, 16]. The severity

Table 1 Clinical examinations. Semiquantitative assessment of the prevalence of anterior segment and vasculature changes in retinas of DBA/2J mice of different age (N/A not applicable)

Age	3 months	6 months	8 months	11 months
Anterior segment changes	–	+	++	+++
Ischemic retinal changes	N/A	–	++	++
Retinal neovascularization	N/A	–	–	+(+)

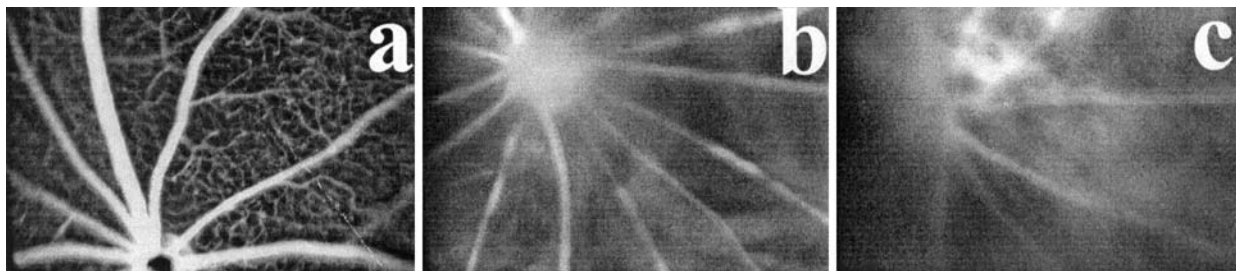


Fig. 2 Fluorescein angiograms of DBA/2J mice. **a** A 6-month-old mouse, taken in the early venous phase. All retinal arterioles and venules are fully filled with dye and the entire retinal capillary architecture can be distinguished. No obvious abnormalities are observed. **b** An 8-month-old mouse, taken in the late venous phase with suboptimal quality due to corneal opacity and ectopic pupil, demonstrating abnormal blood vessel pattern with multiple segmental attenuations. Non-perfusion areas are seen but no obvious leakage is found, suggesting an intraretinal ischemic stage. **c** A 10-month-old mouse, taken in the late venous phase at the same session as Fig. 1b. Corneal opacity does not allow optimal visualization of the fundus. Several hyperfluorescent spots with fuzzy borders associated with widespread non-perfusion area are observed throughout the retina. A leakage from the optic disc is also found (new vessels of disc). Ultrastructural evaluation of these areas demonstrated retinal neovascularization (see Fig. 6)

and incidence of these conditions increased with age. By 8 months, the pupil failed to dilate, which made fundus visualization difficult. Between 6 and 11 months, only 4 out of 11 mice could be sufficiently dilated for FFA recordings (Table 1). Two (6 and 8 months old) showed normal retinal vasculature (Fig. 2a). A better quality was obtained in the 6-month-old animal, which had clearer media. The quality of recording was acceptable in another 8-month and a 10-month-old mouse. In these mice, obvious ischemic changes were found. These included abnormal major retinal vessels showing segmental attenuations and diffuse areas of non-perfusion (Fig. 2b, 8 months old). Multiple leakage due to neovascularization were also detected in one eye (Fig. 2c, 11 months old). None of the mentioned changes of the anterior segment or retinal vasculature were observed in age-matched control mice (data not shown).

Electron microscopy

In DBA/2J mice, numerous ultrastructural alterations, signs of cellular degeneration and tissue rebuild were observed, some of them evident already in the retinas of 3-month-old animals. Electron micrographs are arranged in the order of ascending age (Figs. 3, 4, 5, 6). A semiquantitative summary of the ultrastructural findings is compiled in Table 2.

Two morphologically distinct types of RGC death were distinguished. In the first, particularly frequent in the retinas of 6-month-old mice, homogenization of the nucleoplasm and cytoplasm were found together with dilation of the perinuclear cisternae and endoplasmic reticulum (Fig. 3a, b). Although no heterochromatin segregation, which is considered characteristic for typical apoptosis, was found, we interpret this picture as indicating the morphological

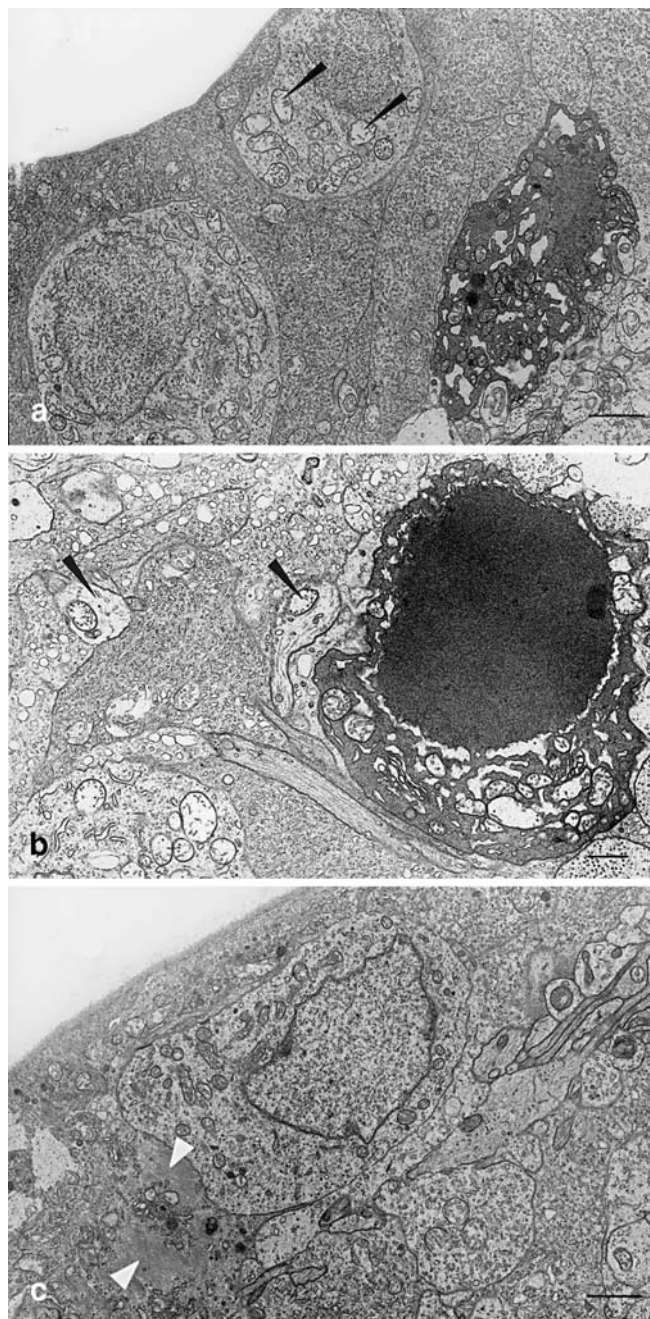


Fig. 3 Retinal section from a 6-month-old DBA/2J mouse. **a** Two retinal ganglion cells with swollen mitochondria (*arrowheads*) and a third one with an electron-dense structure indicating apoptotic type of death. **b** An apoptotic retinal ganglion cell with a characteristic electron-dense nucleus, fragmentation of perikaryal part and swollen axon (*arrowheads*). **c** Activated Müller cell (*arrowhead*) in the vicinity of ganglion cell. Bars **a**, **c** 2 μ m; **b** 1 μ m

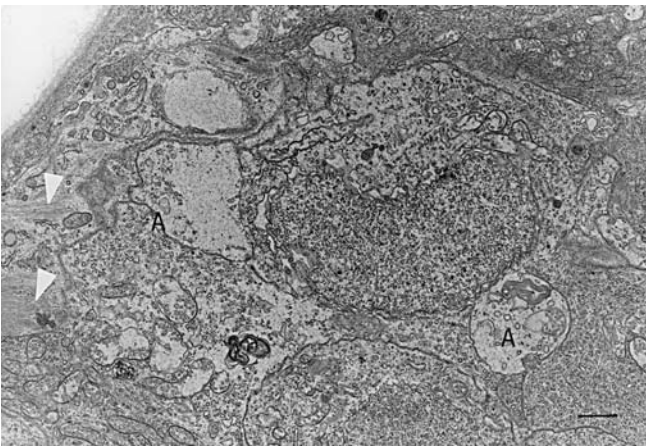


Fig. 4 Retinal section from an 8-month-old DBA/2J mouse. Axonal necrosis (A) and Müller glia activation (arrowheads)

signs of apoptosis on the basis of the electron-dense appearance of the dying cells. These ultrastructural changes were also found in retinas collected from 9- and 11-month-old mice (Fig. 5a), although their occurrence gradually diminished with age, and were replaced by the necrotic type of RGC death. The necrotic ganglion cells and their axons were characterized by progressive cytolysis; both were electron rare. The swollen organelles were dispersed in electron-lucent cytoplasm. The intensity of RGC degeneration peaked at 6 months. We observed the greatest loss of RGC and their axons in the retinal sections from 8- and 11-month-old mice. Many of the axons were characterized by aggregation of intramembranous particles of plasma membranes and the formation of myelin-like bodies (Fig. 5c). Swollen axons were found in the vicinity of ultrastructurally altered RGC (Fig. 5b).

Müller cells with ultrastructural signs of enhanced activation, including the presence of fibrillae (presumably made of glial acidic fibrillary protein, GFAP) (Fig. 3c) were found in retinas of 3- and 6-month-old mice along with the apoptotic ganglion cells. Similar morphological features of Müller cells were also visible at 8 and 11 months, but at that time necrosis of RGC prevailed, and the swelling of axons in the vicinity of RGC was very significant (Fig. 4). Activated Müller cells at the age of 11 months presented longer processes with more numerous microfilaments (Fig. 5b).

In the retinas from 6-month-old and older mice capillary vessels with high hypertrophic endothelium and narrow vessel lumen were observed. Tight junctions between endothelial cells were elongated. The basement membrane which envelops these vessels was blurred and thickened. These morphological features indicated non-sprouting angiogenesis (Fig. 7).

No ultrastructural alterations in the photoreceptor cells were found (Fig. 6). The discs remained well arranged and devoid of any structural anomalies. Age-matched control mice did not show any of the changes observed in DBA/2J mice.

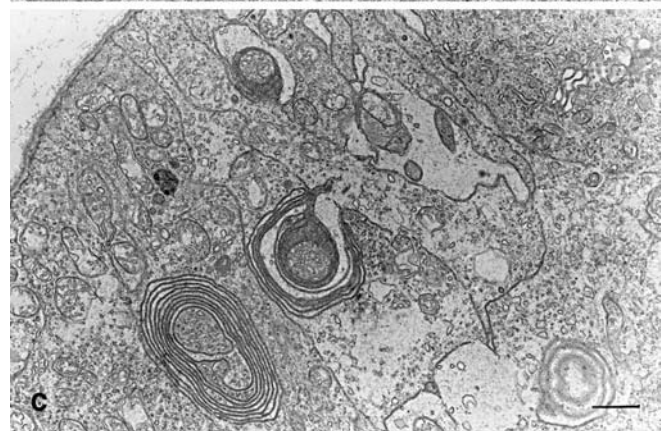
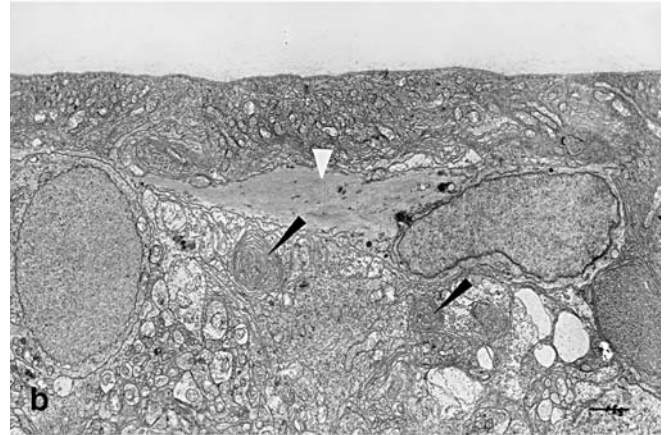
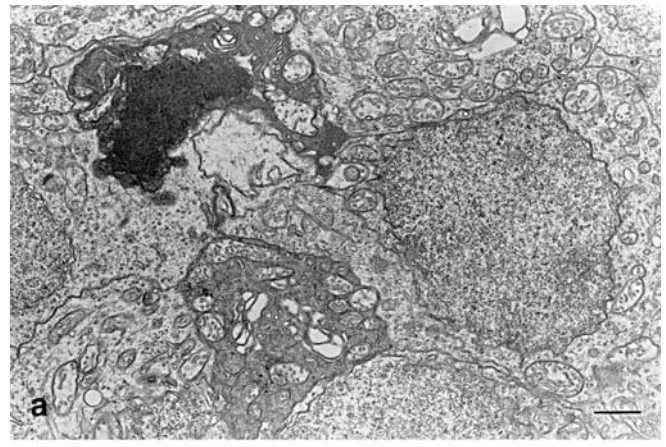


Fig. 5 Retinal section from an 11-month-old DBA/2J mouse. **a** Cells with signs of apoptosis. **b** A Müller cell probably rich in GFAP-like filaments (white arrowheads) surrounded by swollen axons, and a myelin-like body (black arrowhead). **c** Myelin-like bodies in degenerating axons of ganglion cells. Bars **a, c** 1 μm ; **b** 2 μm

Discussion

Both direct [19, 29, 31, 40, 44] and indirect [20] evidence exists that in human glaucomatous eyes RGC death occurs via the apoptotic process. These findings are potentially important because apoptotic cell death may be a feasible target for pharmacological interventions [40]. Signs of RGC apoptotic cell death are also evident in animal surro-

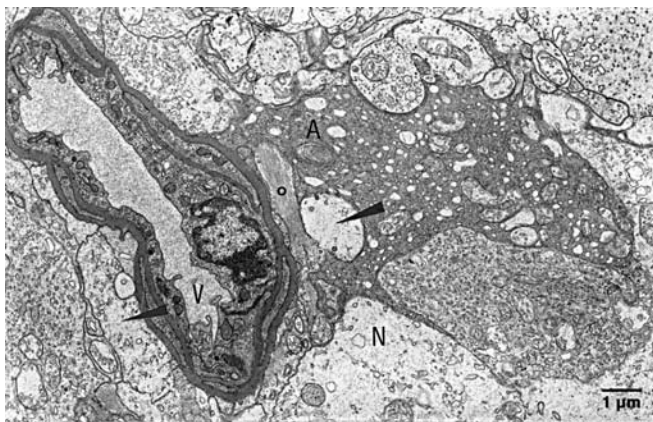


Fig. 6 Retinal section from an 8-month-old DBA/2J mouse. A young capillary vessel (v) with high and hypertrophic endothelium. In its vicinity apoptotic (A) and necrotic (N) retinal ganglion cells, and activated Müller cell with GFAP-like filaments (o)

Table 2 Semiquantitative assessment of the prevalence of ultrastructural alterations in retinas of DBA/2J mice of different age (RGC retinal ganglion cell)

Age	3 months	6 months	8 months	11 months
RGC apoptosis	++	+++	++	+
RGC necrosis	–	+	++	+++
RGC axon degeneration	+	+	++	+++
Müller glia activation	+	++	+++	+++
Neoangiogenesis	–	–	+	+
Myelin-like bodies	–	–	+	++

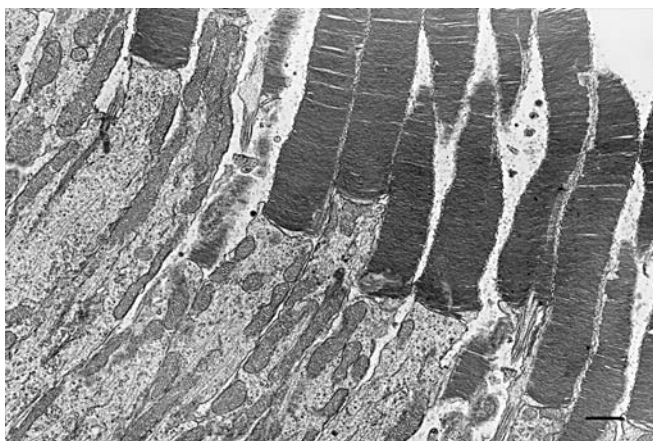


Fig. 7 Retinal section from an 8-month-old DBA/2J mouse showing ultrastructurally unchanged photoreceptors. Bar 1 µm

gate models of glaucoma such as acutely elevated IOP [13, 24, 43] and optical nerve axotomy [8, 33].

In several murine strains, the number of RGC has been found relatively stable over the lifespan [45]. However, as already mentioned in the introduction, the number of RGC in the DBA/2J mice decreased significantly over the first 9 months of life, and the RGC-sparing effect of some medications (including timolol) was evident already by

the age of 6 months [36]. These observations are somewhat surprising because, according to Savinova et al. [35], IOP values in the DBA/2J mice at the age of 2–6 months are approximately 12 mmHg (during the day) and 14 mmHg (during the night), i.e., about 30% lower than those found in a number of other murine strains that do not develop glaucoma or any other ocular abnormality (including, *inter alia*, the CBA/CaJ mice with the highest IOP values among all strains tested, ≥ 20 mmHg). The RGC loss in the DBA/2J mice of 6 months of age or younger may, therefore, precede the rise in IOP.

Results of the present study indicate that in the DBA/2J mice apoptosis appears to be the predominant pathway of RGC death, but only up to the age of 6 months, i.e., over the period of normal IOP. Past this age, RGC necrosis starts to prevail, which is compatible with the hypothesis that when IOP becomes increased, necrosis gradually replaces apoptosis as the predominant pathway of the RGC death. In contrast to our results, Stittsworth et al. [39], who used TUNEL staining in 2- to 12-month-old DBA/2J mice, did not find evidence for DNA fragmentation, and suggested that a mechanism other than apoptosis dominates RGC death. The disagreement with our findings might be explained by the fact that the time window for detecting TUNEL-positive cells is very narrow, while the process of apoptotic cell death is visible by electron microscopy over much longer period of time. Given the relatively low number of dying RGC per day, TUNEL staining might be not sensitive enough to detect apoptosis in this model. Similar argumentation has recently been raised by Uysal et al. [41], who failed to detect apoptosis by TUNEL staining in temporal lobe specimens obtained from patients suffering from intractable epilepsy, although they did detect increased Bax expression and activation of caspases.

The ocular disease in DBA/2J mice has been reported to resemble a combination of the pigment dispersion syndrome (PDS) and the iridocorneal endothelial syndrome, showing several anterior segment abnormalities. Therefore, it may most closely resemble human PDS and pigmentary glaucoma (PG). In human PDS the pigment released from the iris is carried to the trabecular meshwork, where it may reside either benignly (i.e., not affecting intraocular pressure), or malignantly (i.e., causing rise of IOP and PG) [2]. However, glaucomatous field changes in PDS may occur despite low measured IOP, possibly because pressure spikes, which are not measurable but sufficient to inflict nerve damage, may occur with stress, or exercise [1]. Subsequently to the nerve damage, ganglion cell loss may occur through excitotoxicity. Essentially, a similar mechanism may also be involved in the early stage of PDS in the DBA/2J mice. This hypothesis could explain the rescue of RGC by treatment with timolol [36], which is considered as a IOP-lowering drug. However, a recent report indicates that timolol may also have a direct neuroprotective effect on retinal cells [12].

According to Mo et al. [26], the eyes of DBA/2J mice exhibit defects of the normal immunosuppressive ocular microenvironment, which make it unable to support ante-

rior chamber-associated immune deviation. These defects result in the loss of immune privilege and allow infiltration of inflammatory leukocytes into the anterior chamber and their accumulation within the iris. However, the anterior chamber inflammation apparently does not spread to the retinal tissues, since we were unable to find blood-borne macrophages or any other evidence of blood-retinal barrier disruption in the retinas of the DBA/2J mice of any age. Similarly, Garcia-Valenzuela and Sharma [7] did not find macrophages invading the retina beyond the nerve fiber layer following optic nerve axotomy in the rat. In contrast, following acute rise of IOP in rats, Wang et al. [43] found macrophages infiltrating throughout the retinas, and Naskar et al. [27] saw dying RGC surrounded by activated microglia. These features were not encountered in the present study.

In human primary open-angle glaucoma, some authors have reported swelling and loss of photoreceptors [28], whereas others have not seen such phenomena [18]. The involvement of photoreceptors has also been observed in monkey [28] and rat [43] models of surgically evoked glaucoma in which IOP was increased abruptly. Our data indicate that in the DBA/2J mice photoreceptor cells are not affected, at least until 8 months of age. Retina-derived microglial cells have been shown to be capable of inducing photoreceptor cell death *in vitro* [34]. However, a possibility that photoreceptor degeneration in glaucoma is somehow mediated by the abrupt rise of IOP, leading to the opening of blood-retinal barrier and infiltration of retina by blood-borne phagocytic cells, should be considered.

A prominent feature of retinal degeneration in the DBA/2J mice revealed by the present study is the activation of Müller cells. This is vastly different from pictures seen in some other ocular pathologies. For example, in the retinas of rats infected by the Borna virus, Müller cells showed only a moderate alteration, while a marked increase in the number of microglial cells and also monocytes were seen accompanying the degeneration of both retinal ganglion cells and photoreceptors [17].

Structures known as myelin-like bodies or myelin figures were frequently seen inside the dying cells in retinas from DBA/2J mice aged 9 and 11 months. Structures of this appearance may be formed as artifacts of tissue fixation for EM [38]. However, according to Cotran et al. [4], they may also represent large intracellular aggregates composed of phospholipids, which are a characteristic feature of irreversibly injured cells. Artifacts of tissue fixation are not a likely explanation here because neighboring cells had well-preserved mitochondrial cristae. Formation of myelin-like bodies may be a consequence of relative paucity of “professional” phagocytes (microglia and monocytes) around dying retinal ganglion cells. In this situation, the main task of clearing the debris presumably depends upon Müller cells, which are capable of phagocytosis [6, 23]. However, these “non-professional” phagocytes clear cells undergoing apoptosis slowly and reluctantly [30], leaving the time necessary to form myelin-like structures inside the dying retinal cells.

Lastly, in the present study morphological signs of non-sprouting retinal neovascularization were found in mice of the age 6 months and older, and this was confirmed by FFA. According to the current concept, both physiological and pathological growth of new blood vessels in the retina is driven by hypoxia-induced expression of vascular endothelial growth factor (VEGF), but the pathological type of the process associated with expression of a specific VEGF isoform (VEGF₁₆₄), which leads to a subsequent influx of inflammatory cells to the sites of neovascularization [15]. Considering the above, neovascularization seen in the retinas of DBA/2J mice seems to resemble physiological neovascularization or “revascularization” rather than pathological neovascularization. This neovascularization stands in contrast to human glaucoma. Further studies regarding the mechanism of the development of vascular changes in these mice are needed.

We conclude that retinal degeneration in 3- to 11-month-old DBA/2J mice partially resembles human PDS and PG with characteristic anterior segment changes and elevation of IOP. The destruction of RGC does not involve recruitment of blood-borne inflammatory/phagocytosing cells. Consequently, photoreceptors are spared and Müller cells must be engaged in clearing RGC debris. However, in contrast to human PG, neovasclogenesis and myelin-like bodies are observed during aging. Therefore, the DBA/2J model requires judicious interpretation as a glaucoma model, since glaucomatous changes are accompanied by other features of retinal degeneration.

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