

Review

Morphological and functional changes in experimental ocular hypertension and role of neuroprotective drugs

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Summary. Glaucoma is a neurodegenerative disease characterized by progressive loss of retinal ganglion cell axons and their cell bodies in the retina. Elevated intraocular pressure (IOP) is considered to be the major risk factor associated with the development of this neuropathy. Randomized controlled clinical trials have demonstrated that in some patients the disease progresses, even after lowering the IOP. Several researchers have devised ways to induce elevated IOP in the rat eye with the aim of impeding the flow of aqueous humour out of the eye. Chronic ocular hypertension in rats induces morphofunctional changes in the optic nerve head and retina. Death of ganglion cells is thought to follow an apoptotic pathway. Changes have also been reported in neuronal and non-neuronal cells, levels of cyclooxygenase, and nitric oxide synthase, endothelin 1 and brain derived neurotrophic factor. Other mechanisms include intracellular electrolyte imbalance, microglial phagocytosis and elevated glutamate levels. Neuroprotection is the treatment strategy by preventing neuronal death. Hypotensive drugs (β -blockers, α -agonists and prostaglandins), Ca^{++} channel blockers, NMDA antagonists and nitric oxide synthase inhibitors have been used as neuroprotective drugs in experimental models of glaucoma.

Key words: Experimental glaucoma, Retina, Optic nerve head, Ganglion cell death, Neuroprotection

Introduction

Glaucomatous optic nerve disease is a chronic disease characterized by visual field loss, cupping of the optic nerve head and irreversible loss of retinal ganglion cells (Osborne et al., 1999a). Epidemiologic studies have

shown increased intraocular pressure to be the most frequent finding in human glaucoma (Armaly et al., 1980). Glaucoma is the second leading cause of visual loss and around 1% of the world population have primary open angle glaucoma (Quigley, 1996).

The clinical features of glaucoma are well described but the mechanisms that produce optic nerve damage and ganglion cell death remain to be elucidated (Kerrigan et al., 1997). Selective death of retinal ganglion cells is the hallmark of glaucoma and direct mechanical pressure, indirect ischemia or blockage of axonal transport at the lamina cribrosa may account for the damage (Quigley et al., 1981; Osborne et al., 1993). The excavated optic nerve head in glaucoma is thought to be caused by death of the ganglion cells and subsequent loss of their axons (Quigley et al., 1981).

In experimental and human glaucoma, large ganglion cells of a type related to the magno-cellular pathway are particularly susceptible (Quigley, 1995). Weber et al. (1998) described an initial degeneration of the dendritic arbor of the ganglion cells followed by a shrinkage of the cell soma, and Kalloniatis et al. (1993) reported no apparent selective loss of ganglion cells of a particular size or type. These results are based on histology specimens where the diameter of the axonal cells and ganglion cell somata were measured. Other reports have questioned such data due to the difficulties involved in identification of different types of ganglion cells in pathological tissues (Morgan, 1994).

However, in contrast, scotopic, and electrophysiology tests fail to support the data for selective ganglion cell death in glaucoma (Graham et al., 1996). Eyes with advanced glaucoma showed no loss of outer retinal neurons, as the cell counts were indistinguishable from non-glaucoma eyes (Kendell et al., 1995). Further studies found no loss of cones in experimental monkey glaucoma eyes (Wyganski et al., 1995). Histochemical studies of other monkey glaucoma showed no alterations in neurons other than ganglion cells (Vickers et al., 1995). We have observed in rats with experimental

ocular hypertension that the photoreceptor outer segments became edematous with fragmentation and disorganization of the membranous system (Fig. 1). In the mouse, ocular hypertension induces a preferential loss of upper optic nerve axons (Mabuchi et al., 2004).

Pathological studies of human glaucoma optic nerve have demonstrated that the changes of the extracellular matrix consist of several types of collagen and basement membrane materials, as well as elastin (Hernández, 2000). Immunohistochemical analysis of a hypertonic saline rat model documented accumulations of collagen, types I and III as well as type VI, and laminin in the optic nerve head, replacing normal axon bundles (Johnson et al., 1996).

The role of non-neuronal cells in glaucoma is partially unknown but it is well-established that the astrocytes and microglia protect the integrity of the retina during the development period as well as throughout its lifetime (Wang et al., 2000; Yuan and

Neufeld, 2001). These glial cells also intervene in acute situations to repair small traumas and protect against imbalances (Meril, 1994). Astrocytes are reported to perform functions like wrapping around ganglion cell axons, contacting other glial cells to form adherent junctions and also playing a role in potassium buffering (Lan et al., 1995).

Naskar et al. (2002) reported in a rat model of glaucoma activation of intraretinal microglia associated with degeneration of the retinal ganglion cells. Microglia represent the intraretinal phagocytic cells that play an important role in the development of the retina (Thanos, 1992). These cells respond rapidly within hours of elevation of intra-ocular pressure in the retina (Wang et al., 2000) and induce the release of glutamate (Osborne et al., 1993), a substance that participates in the possible pathophysiology of glaucoma.

The Müller cells do not express significant amounts of GFAP in normal retinas but GFAP-positive cells have been demonstrated in glaucoma (Fig. 2). This expression can be used as an indicator of most if not all acute and chronic neuroretinal diseases (Tanihara et al., 1997).

It is now accepted that normalization of pressure is necessary but in some cases insufficient. Many glaucoma patients continue to progress after the intra-ocular pressure has been restored and kept within the normal range (Brubaker, 1996). This suggests that this factor is not the only reason for the optic nerve damage and ganglion cell death (Yoles and Schwartz, 1998). For this reason, treatment should not only be aimed at ocular pressure, it should also include neuroprotective therapy (Schwartz, 2001).

Experimental glaucoma in rats

Several methods exist to produce glaucoma in rats with chronically increased pressure, optic nerve

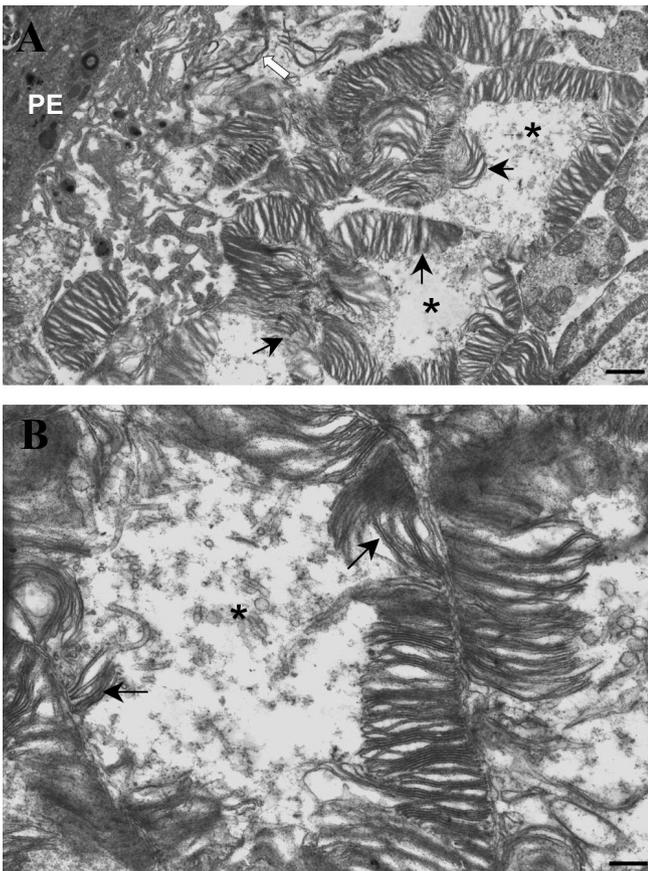


Fig. 1. (A). Degeneration of the photoreceptor outer segments and formation of some laminar bodies (white arrow) near the pigment epithelium (PE). Photoreceptor outer segments became oedematous (asterisks) with disorganisation of the membranous discs (arrow). Scale bar: 1.8 μm . (B). Oedema (asterisk) and disorganisation of the membranous discs (arrows). Scale bar: 5 μm .

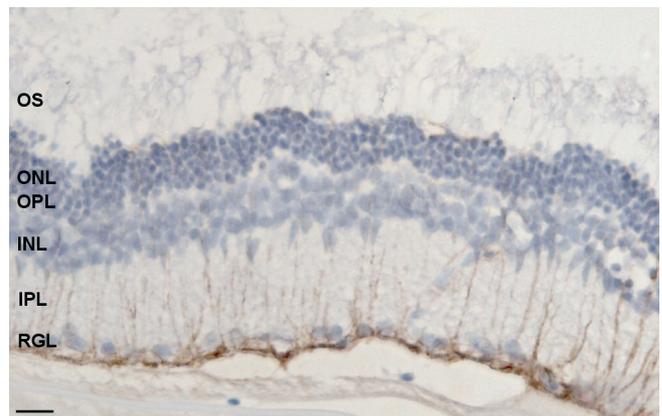


Fig. 2. GFAP-stained retinal sections obtained from elevated intraocular pressure. Immunostaining of the Müller cells after elevations of IOP. GFAP immunoreactivity is markedly increased and labelled processes located in the IPL are thin and tortuous. Scale bar: 10 μm .

Experimental glaucoma and neuroprotection

degeneration and retinal ganglion cell loss (Table 1). In one method, a hyperosmotic solution is injected into the limbal venous plexus and the aqueous veins sclerosed with hypertonic saline solution. In rats, the aqueous humor can cross the trabecular meshwork into Schlemm's canal and then enter into a venous plexus through numerous channels. This plexus is drained by multiple radial episcleral veins.

The procedure consists of exposing the aqueous vein under 25x40 magnification. A microneedle is inserted into the vessel lumen. After cannulation, a volume of 50 ml filtered hypertonic saline solution is injected (Morrison et al., 1997).

Johnson et al. (1996) observed that of 20 animals, nine had elevation of intraocular pressure following a single injection, while subsequent injections raised intraocular pressure in seven others and one eye became hypotense. In the remaining animals subsequent injections were sufficient to raise intraocular pressure. In another study, Johnson et al. (2000), using the same method in 22 experimental eyes, obtained a mean intraocular pressure in the experimental eyes of 36 ± 8 mmHg compared with 19.5 ± 2.8 mmHg in the untreated eyes. Elevated intraocular pressure by limbal hypertonic saline injection or laser coagulation produced similar results in rats (McKinnon et al., 2002).

Intraocular pressure can also be elevated by cauterizing three of the four trunks formed by limbal derived veins (Shareef et al., 1995). Following surgery, intraocular pressure rose to approximately 22 mmHg (Sawada and Neufeld, 1999) or more than 30 mmHg (Mittag et al., 2000). The procedure increases intra-

ocular pressure for at least 6 months without retreatment. During this time, intra-ocular pressure was maintained at a level almost 1.7 (Neufeld et al., 1999), 1.6 (Sawada and Neufeld, 1999) or 2.2 (Ahmed et al., 2001) fold higher compared to the contralateral eye. In our experience (Diaz et al., 2005), after this surgical procedure, the mean intra-ocular pressure increased to 35.5 ± 1.06 mmHg, 1.25 fold compared to the control group, and three months later the intraocular pressure remained significantly elevated (Fig. 3).

Although these methods lead to retinal ganglion cell death in substantially shorter periods than in human glaucoma, similarities between rat and human eyes show the relevance of rat ocular hypertension to chronic glaucoma in humans.

According to previous reports (García-Valenzuela et al., 1995; Schwartz, 2001), apoptosis of retinal ganglion cells begins to occur within 7 days of elevating intraocular pressure. It is estimated that in this period, between 36% (Neufeld et al., 1999) and 40% (Sawada and Neufeld, 1999) of the retinal ganglion cells in the peripheral retina are lost. Ahmed et al. (2001) described a survival of 68.4% at 5 weeks and Naskar et al. (2002) and Díaz et al. (2005) a loss of 40% and 35% retinal ganglion cells after 2.5 months and three months of intra-ocular pressure elevation, respectively.

In some cases after the cauterization of three episcleral veins, the intra-ocular pressure gradually returned to that of the contralateral eye over 20 weeks, possibly because of restoration by growth of new vessels. This can be avoided by subconjunctival 5-FU injections. With this glaucoma experimental model, the retinal ganglion cell loss appears to be primarily focal (Mittag et al., 2000).

The cautery method produces less injury than would be expected and damage in this model may be due to

Table 1. Models of experimental elevated intraocular pressure in rats.

Authors	Glaucoma model
Shareef et al., 1995 Laquis et al., 1998 Sawada and Neufeld, 1999 Mittag et al., 2000	Cauterizing three trunks of the limbal veins
Morrison et al., 1997 et al., 2000	Injection of hypertonic saline Johnson solution
Ueda et al., 1998	Laser photocoagulation and ink injection in anterior chamber
Wijano et al., 1999 Wolde-Mussie et al., 2001	Laser photocoagulation of the limbal and episcleral veins
Levkovitch-Verbin, 2002	Diode laser treatment of the trabecular meshwork
Levkovitch-Verbin, 2002	Diode laser treatment of the trabecular meshwork and episcleral veins
Grozdanic et al., 2004	Injection of indocyanine green dye in the anterior chamber and diode laser treatment
Benozzi et al., 2002 Moreno et al., 2005	Intracameral injection of hyaluronic acid
Thanos and Naskar, 2004	Mutant rat with glaucoma

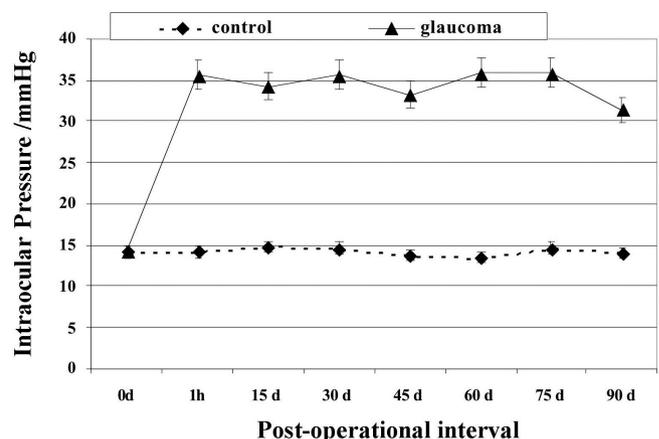


Fig. 3. Comparison of the intraocular pressure between control and experimental eyes, after cauterization of three episcleral veins. Measurements were made each two weeks. Data are mean \pm SEM of the results in each group.

ischemia (Goldblum and Mittag, 2002; Grozdanic et al., 2003). This is supported by Bayer's observation, where a-wave amplitude was diminished with little retinal ganglion cell loss (Bayer et al., 2001). The authors suggest that the involvement of the outer retina may be due to choroidal insufficiency.

Laser photocoagulation of limbal and episcleral veins 0.5-0.8 mm from the limbus also resulted in an increased intraocular pressure (Wijano et al., 1999). An increase of 2-fold (WoldeMussie et al., 2001) or 2.2-fold (Wheeler et al., 2001; Siu et al., 2002) caused ganglion cells loss of 29.8% and 44.34% after 5 and 8 weeks, respectively.

Previous studies (Garcia-Valenzuela et al., 1995; Laquis et al., 1998), where the animals received two laser treatments on separate days, showed cell loss of approximately 37% and 44% after 6 and 10 weeks of experimental glaucoma.

Ueda et al. (1998) enhanced the laser uptake by ink injection into the anterior chamber before treatment and Levkovitch-Verbin et al. (2002) developed a translimbal diode laser treatment to the trabecular meshwork. In all treated eyes, a moderate intra-ocular pressure increase was found with significant optic nerve damage that begins at 1 week (Martin et al., 2002).

A combination using a diode laser with a wavelength of 532 nm to the trabecular meshwork and episcleral vein was reported. All the eyes had elevated intraocular pressure during the first 48 hours after laser treatment. With this method, 50%-60% of retinal ganglion cells died within six weeks (Levkovitch-Verbin et al., 2002).

Grozdanic et al. (2004) induced an elevation of the intra-ocular pressure by a combined injection of indocyanine green dye into the anterior chamber and diode laser treatment. The increase in intra-ocular pressure reached maximal values 14 days after surgery.

The experimental increase of ocular pressure can be induced by intracameral injection of hyaluronic acid (Benozzi et al., 2002). With this method, Moreno et al. (2005) observed 40% ganglion cell death at ten weeks.

Thanos and Naskar (2004) documented a mutant rat with glaucoma. The new mutant resembles human inherited buphthalmos with intra-ocular pressure between 25-45 mmHg.

These models of experimental glaucoma in rats have become more useful since the development of the Tonopen Tonometer with its small appplanation tip, which greatly facilitates the measurement of intra-ocular pressure in this animal (Barkana and Belkin, 2004).

Apoptosis

Apoptosis is a program of genetically controlled death that cells undergo in different diseases (Kerr et al., 1972). In this program, the cell requires energy and participates actively in self-elimination (programmed cell death). This programmed process is essential for cell number homeostasis in the organism. It is widespread during nervous system development and is involved in

several neurodegenerative diseases such as glaucoma.

Since the first reports of apoptosis (Hamburger and Levy-Montalcini, 1949), much has been learned about programmed cell death, including the elucidation of the molecular mechanisms involved. The exact progression of nuclear and cytoplasmic alterations has only been inferred from histological studies, and even the time required for the execution of the death program remains unknown (Cellerino et al., 2000).

From embryonic differentiation (Meier et al., 2000) to aging (Zhang and Herman, 2002), apoptosis was until recently an undervalued mechanism. Apoptosis may begin at a particular moment of cell life and represents a final event in numerous diseases, not only in ophthalmology (Nickells and Zack 1996), but also in practically all biomedical disciplines (Vaux and Korsmeyer, 1999).

Cell death by apoptosis is a defense mechanism against cell injury (Samali et al., 1996). When a cell is damaged, one choice is to repair the injured cell and another to commit suicide. We suspect that when the injury is slight, the cell attempts repair. But when the injury is moderate or severe the solution is to eliminate the damaged cell by apoptosis and to build a new cell (Tempestini et al., 2003).

Apoptosis is a slower, subacute process characterized by cell degradation rather than disruption (Tatton et al., 2001). It produces plasma membrane blebbing, caspase activation, nuclear chromatic condensation and the formation of nuclear bodies. No evidence exists to suggest that ganglion cells die in glaucoma by necrosis.

The basis for suggesting that the death of ganglion cells in experimental glaucoma and open angle glaucoma is by apoptosis is controversial. Osborne et al. (1999b) reported that there is no conclusive evidence of apoptosis and the authors that support this theory do so because of lack of demonstration of cell necrosis. Recent studies have demonstrated that the number of Tunel-positive ganglion cells in glaucoma patients is few, though it is nevertheless greater than in control retinas (Kerrigan et al., 1997). Guo et al. (2005) reported that retinal ganglion cell apoptosis in experimental glaucoma correlates strongly with elevated intraocular pressure and is associated with changes in specific extracellular matrix components in the ganglion cell layer.

Cyclooxygenase-2, an enzyme associated with inflammation, is not increased in glaucomatous tissues (Neufeld et al., 1997a), endorsing the idea of apoptosis in the retinal ganglion cells. The idea that all neuron death is apoptotic or necrotic may not be valid, although it seems a good approximation in certain cases (Clarke, 1998).

Ganglion cell death in glaucoma

For many years, two theories have been postulated to explain the causes of retinal ganglion cell death in glaucoma. The alteration of the vascular perfusion or the

compression of the cribriform plates may be responsible for the glaucomatous pathology in the optic nerve head that leads to loss of axons and retinal ganglion cells (Quigley and Anderson, 1976; Maumenee, 1977). Changes have been described in the structure of lamina cribrosa (Hernández and Pena, 1997), in the amount of cyclo-oxygenase (Neufeld et al., 1997a), in transforming growth factor- β 2 (Pena et al., 1999) and in the levels of nitric oxide synthase in the optic nerve of experimental ocular hypertension and glaucoma patients. The problem is to determine whether such changes represent cause or effect (Osborne et al., 1999b).

The vascular supply of the optic nerve head is complex and an insult may cause ischemia or hypoxia to the ganglion cell axons (Ernest, 1976). Raised pressure may result in an alteration of the blood supply to the optic nerve head, inducing ischemia and affecting the normal function of the ganglion cell. Flammer and Orgul (1998) postulated that there is some evidence to support the idea of a perversion in the blood supply of the optic nerve head in glaucoma patients. Components that may be affected include axon ganglion cells, astrocytes and microglia (Hernández, 2000). Structural changes at the optic nerve head may be apparent before total loss of ganglion cell function, supporting the opinion that the initial insult occurs at the optic nerve head and that the death of the ganglion cell soma is not the cause of the glaucomatous cup (Osborne et al., 1999b).

After an ischemic insult to the optic nerve head, astrocytes may become reactive. The astrocytes of the optic nerve head have the capacity to communicate with retinal astrocytes and Müller cells (Newman, 2001) via gap junctions (Hernández, 2000). These cells respond to ganglion cell death by migrating, filling the spaces occupied by axons, phagocytosing cell debris and synthesizing new extracellular matrix components. They also release potential toxins, matrix metalloproteinases and tumor necrosis factor alpha (Yan et al., 2000), nitric oxide (Neufeld, 2004; Vidal et al., 2006) and glutamate (Araque et al., 1999). N-methyl-D-aspartate (NMDA) receptors that could mediate glutamate injury are on the ganglion cell body. Substances released from stressed astrocytes and Müller cells may indirectly affect the ganglion cell body. A true neuroprotector substance may protect not only the axon and cell body of the ganglion cell, but also the functioning of Müller cells, astrocytes and microglia (Osborne et al., 1999c).

The compression of the axons at the optic nerve head or lamina cribrosa is thought to impair retrograde axonal transport. This transport enables survival of the ganglion cell body. Anderson and Hendrickson (1974), using radioactive probes, demonstrated blockage of axonal transport in the ganglion cells at the level of the sclera in the optic nerve head. Quigley et al. (1991) pointed out that the site of injury is the collagenous tissue beam opposite the sclera.

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that is retrogradely axonally transported back to the ganglion cell body where it influences

neuronal survival and growth (Cassacia-Bonnet et al., 1999). Recent studies in animal glaucoma models with increased ocular pressure have shown a decrease in radiolabeled BDNF axonal transport to the ganglion cell body (Pease et al., 2000). Moreover, when BDNF is injected into the superior colliculus it reduces ganglion cell death (Ma et al., 1998).

One of the areas of great interest in glaucoma is what stimulates the ganglion cell death (Nickells, 1999). Apoptosis can be activated by a variety of stimuli, but neurons are programmed to die by two primary effectors: deprivation of neurotrophic factors (Oppenheim, 1991) and elevated concentrations of excitatory aminoacids, such as glutamate (Lipton and Rosenberg, 1994).

The direct detrimental effect of glutamate on retinal ganglion cells is observed by exposing the retina to high glutamate concentrations in vitro (Levy and Lipton, 1990), in animal models where glutamate is injected intravitreally (Sisk and Kuwabara, 1985), in the vitreous of glaucoma patients (Dreyer et al., 1996), in experimental glaucoma (Adachi et al., 1998) and during hypoxia (Neal et al., 1994). In contrast, a recent study in a monkey model of experimental glaucoma found only normal levels of glutamate in the vitreous (Carter-Dawson et al., 2002).

Neurons contain excitatory and inhibitory receptors, but in physiological conditions these receptors cannot be overstimulated because of appropriate homeostatic mechanisms (Osborne et al., 1999c). Excitatory receptors are abundant in ganglion cells (Brandstatter et al., 1994). Glutamate-induced excitotoxicity occurs when extracellular glutamate levels are increased, either due to increased release or decreased uptake from the synapse. The destructive effect of glutamate on ganglion cells is due to its interaction with ionotropic glutamate receptors. High concentrations of glutamate activate several types of cell receptors, including NMDA, which allows a large influx of calcium (Osborne et al., 1999c). Excessive amounts of Ca^{++} lead to an activation of a complex cascade of nucleases, proteases and lipases attacking cell constituents and producing free radicals, and possibly the activation of nitric oxide synthase leading to increased nitric oxide production and the formation of toxic peroxynitrite and stimulation of protein kinase (Budd et al., 2000; Naskar and Dreyer, 2001). The death of ganglion cells may be by an overactivation of glutamate receptors and the hypoxia the cause of the increase in extracellular glutamate (Siliprandi et al., 1992; Osborne et al., 1993, 1999c). Although hypoxia has been repeatedly mentioned, definitive proof remains elusive (Osborne et al., 1999c).

In human glaucoma there is an excessive production of nitric oxide by astrocytes and microglia at the optic nerve head with cluster of NOS-1 and NOS-2 positive astrocytes (Neufeld et al., 1999; Neufeld, 2004), and accumulation of organelles (principally, mitochondria) in optic nerve axons (Hollander et al., 1995). The production of superoxide by mitochondria in excess of

nitric oxide could yield copious amounts of peroxynitrite capable of neurodestruction (Neufeld et al., 1999).

Increased levels of nitric oxide synthase have been detected in astrocytes of the lamina cribrosa and optic nerve head in human glaucoma patients (Neufeld et al., 1997b) and in an animal model of glaucoma (Franco-Bourland et al., 1998). In rats whose extraocular veins were cauterized to produce chronic ocular hypertension, expression of NOS-2 was increased in optic nerve head astrocytes (Shareef et al., 1999; Vidal et al., 2006) (Fig. 4).

In the optic nerve of rats with elevated intraocular pressure, the levels of NOS-1 and NOS-2 are increased in eyes within 28 days (Neufeld et al., 1999) and three months of chronic, moderate elevated intraocular pressure. The total nitric oxide level in retinas of laser-treated rats with experimental glaucoma was significantly increased compared with controls (Siu et al., 2002).

A recent report demonstrated that glaucomatous optic neuropathy was not associated within a significant change in the expression of NOS-2 in the retina or optic nerve. In most cases of experimental glaucoma, occlusion of the limbal vessels may induce damage such as ischemia, which may confound the conclusion that NOS-2 is critical to glaucoma or ocular hypertension-induced retinal ganglion cell loss (Pang et al., 2005).

Nitric oxide has dual actions in NMDA receptor-mediated neurotoxicity (Kashii et al., 1996). A low concentration of nitric oxide appears to protect retinal neurons by inhibiting NMDA-channel activity, but overproduction of nitric oxide interacting with oxygen radicals leads to the death of retinal neurons in NMDA neurotoxicity (Adachi et al., 1998).

All these studies suggest a potential role for NOS-2 in pressure-induced optic nerve damage, but it has not been corroborated in recent studies using other models (Morrison et al., 2001).

Neuroprotection

Treatment for glaucoma attempts to lower ocular pressure (Brubaker, 2003; Goldberg, 2003). Nevertheless, all glaucoma specialists have had patients who have a worsening visual field with ocular pressure in the range that we can catalogue as normal. Is ocular pressure really the villain, then? Patients with a mean ocular pressure near 13.3 mmHg had no important changes in their visual field, although one group of patients had a worse visual field, but this was similar to the percentage that improved (AGIS Investigators, 2000). In another study, 20% of the patients with a 30% reduction in pressure progressed at 3 and 5 years. The evolution of the visual field was better than in the control group, in which 40% and 60% progressed at 3 and 5 years (Collaborative Normal Tension Glaucoma, 1988). This means that patients can have a worse evolution, independently of the intra-ocular pressure.

Probably, when the primary injury and the damage

to neurons begin, changes in the extracellular environment are produced (Siesjo, 1988). This allows for a self-perpetuating destructive cascade that remains active long after the initial or primary insult has abated. This concept provided a possible explanation for the fact that in some patients progression of glaucomatous damage continues with an ocular pressure in the range of normal (Yoles and Schwartz, 1998).

The strategy of treating a disease by preventing neuronal death is termed neuroprotection. In the context of glaucoma, this definition precludes the use of the term for ocular hypotensive drugs and limits it to modalities which, by interacting with neuronal processes, minimize the progress of glaucomatous neuropathy (Kaushik et al., 2003; Barkana and Belkin, 2004).

β -blockers

β -blockers, whether selective -betaxolol- or non-selective -timolol-, have some activity as calcium-channel blockers, reducing the excessive influx of calcium into the stressed cells (Setoguchi et al., 1995; Chidlow et al., 2000). Of all the β -blockers used in the treatment of glaucoma, betaxolol is probably the most effective calcium-channel blocker (Yu et al., 1999). In animal studies, the topical application of betaxolol reached the back of the eye, protecting the ganglion cells from various types of insults (Osborne et al., 1999c).

Timolol and betaxolol interact directly with L-type calcium channels (Melena et al., 2001), but betaxolol is more effective than timolol at reducing sodium and calcium influx into brain synaptosomes (Chidlow et al., 2000; Osborne et al., 2004). This effect could explain the apparent vasodilating activity of betaxolol. However, the potency of betaxolol in reducing calcium influx into ganglion cells may not be sufficient to use it as a true

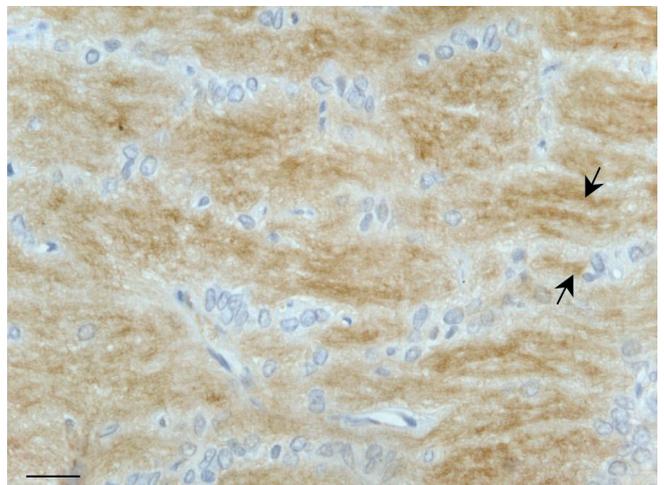


Fig. 4. NOS-2 positive cells and processes (arrows), presumably reactive astrocytes in the lamina and postlamina region of the ONH with elevated intraocular pressure. Scale bar: 10 μ m.

neuroprotector.

Timolol suppresses aqueous formation and increases retinal blood flow by blocking the β -adrenergic receptor in rats (Tan et al., 2002). We have demonstrated (Diaz et al., 2005) that topical treatment with timolol, started two weeks after experimental ocular hypertension, protects from loss of ganglion cells. The number of neurons in the retinal ganglion layer, counted by immunohistochemical labeling with Neu-N, showed 423 ± 11 and 283 ± 10 neurons/mm² in normal and elevated intraocular pressure models, respectively. After treatment with timolol the number of neurons was 331 ± 10 cells/mm² (Fig. 5).

From a clinical point of view, numerous trials have demonstrated that betaxolol provides greater protection of the visual field compared with timolol, independently of the lowering of the ocular pressure (Drance, 1998). Betaxolol has been reported to increase the blood flow velocity in the optic nerve head similar to other Ca⁺⁺-channel blockers (Araie and Muta, 1997). Gross et al. (1999) reported that it can block glutamate activity preserving the ganglion cells of the retina.

Topical nipradilol 0.25%, a non-selective β -blocker with nitroglycerin-like vasodilating activity, reduces ocular pressure by decreasing the aqueous outflow rate and probably also by increasing uveoscleral outflow (Schuman et al., 1994). In rabbits, its intra-ocular pressure lowering effect is greater than that of 0.5% of timolol (Kanno et al., 1998). No neuroprotective effect has been demonstrated.

Ca⁺⁺-channel blockers

Several researchers have reported beneficial effects of Ca⁺⁺-channel blockers such as flunarizine, flupirtine, lomerizine, nifedipine, and verapamil (Jensen, 1995; Toriu et al., 2000). They neutralize glutamate NMDA receptors-induced intracellular Ca⁺⁺ influx.

Flunarizine, a potent Ca⁺⁺-channel blocker, induces survival of ganglion cells after 14 days of optic nerve transection in mice (Eschweiler and Bahr, 1993).

Flupirtine is a triaminopyridine compound that has

been used as a non-opioid analgesic with muscle relaxant properties (Friedel and Fitton, 1993). It has been found to protect against ganglion cell death, blocking excessive activation of NMDA receptors, preventing intracellular calcium overload into neurons in animals with transient elevation of intraocular pressure (Nash et al., 2000).

Lomerizine is a new synthesized Ca⁺⁺-channel blocker and a potent anti-migraine drug (Hara et al., 1998). It reduces retinal damage *in vitro* and *in vivo* and perhaps may be useful as a therapeutic drug against retinal neurotoxicity in experimental intraocular hypertension (Toriu et al., 2000).

Kittazawa et al. (1989) found a visual improvement in a number of patients treated with nifedipine. Mikheyteva et al. (2004) demonstrated that mechanisms involving adrenalin and calcium are involved in glaucoma and that the use of verapamil 0.25% is not effective in the early stages of experimental glaucoma in rabbits.

A beneficial improvement of Ca⁺⁺-channel blockers was postulated by one clinical study in patients with normal-tension glaucoma, demonstrating a decrease in glaucoma progression relative to controls, although this effect is still the subject of debate (Netland et al., 1993).

Nitric oxide synthase inhibitors

Nitric oxide (NO) is a potent vasodilator that physiologically regulates blood pressure (Moncada, 1992). In the central nervous system it is a neurotransmitter and neuromodulator. NO is a physiologic mediator that is used by rods, bipolar cells, amacrine cells and ganglion cells. It can also be neurotoxic. Glutamate neurotoxicity is mediated in part by excess nitric oxide (Dawson et al., 1993).

The mediator is synthesized enzymatically by nitric oxide synthase (NOS), which has three isoforms referred to as NOS-1, -2 and -3. NOS-1 is found in neuronal tissues like the retina and the central nervous system and referred to as brain or neuronal NOS (Koistinaho and Sagar, 1995). NOS-3 is found in endothelial cells and in

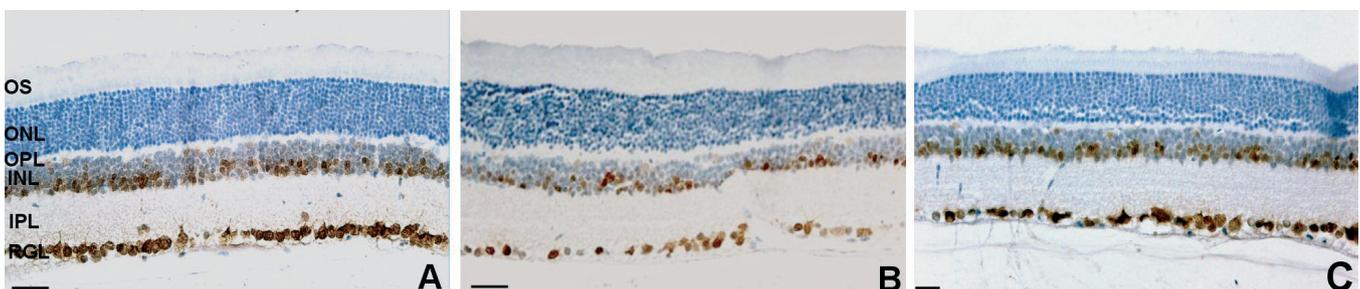


Fig. 5. Representative photomicrographs of NeuN-stained retinal sections obtained from normal (A), elevated intraocular pressure (B) and timolol treated eyes (C). The monoclonal antibody NeuN labelled almost all the neurons in the RGL and some neurons in the INL. (OS: outer segment; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGL: retinal ganglion layer) Scale bars: 25 μ m. (Diaz F. et al., *Histol. Histopathol* 20, 1077-1084, 2005)

nonvascular cells and referred to as endothelial NOS (Lamas et al., 1992), and NOS-2 is an inducible form of the enzyme not found under normal conditions. Nitric oxide synthesized by NOS-2 is the most important component of inflammation and degeneration and becomes cytotoxic (Benz et al., 2002).

The three isoforms of NOS are present in increased amounts in the optic nerve of patients with primary open angle glaucoma (Neufeld et al., 1997b), and in the retina of experimental glaucoma (Siu et al., 2002).

In a rat model of glaucoma, Neufeld (2004) postulated that aminoguanidine, an inhibitor of NOS-2, prevents the loss of retinal ganglion cells. In histological specimens, the untreated eyes lost a mean of 36% of their ganglion cells whereas in the treated eyes only 10% were lost (Neufeld et al., 1999).

Following experimental transient retinal ischemia produced by elevation of the intraocular pressure above systolic blood pressure, approximately 50% of the ganglion cells disappeared at two weeks. Treatment with SC-51 during this period following ischemia prevented the loss of ganglion cells. In the untreated rats 55,000 ganglion cells degenerated compared with 17,000 in the treated rats. SC-51 was more potent than aminoguanidine, producing substantially more neuroprotection (Neufeld et al., 2002).

NMDA-antagonists

One promising treatment in neuroprotective therapy is to block the glutamate receptor NMDA with antagonists, providing neuroprotection by preventing excessive calcium influx (Bullock, 1995). These receptors are absent in the white matter.

MK-801 is a NMDA-receptor blocker that protects retinal cells in a variety of models of retinal ischemia. Joo et al. (1999) showed that treatment with intravitreal injections of MK-801 prevents early neuronal degeneration after ischemia induced by increasing intraocular pressure. mRNA and protein levels of p53, the tumor suppressor gene known to induce apoptosis, were increased in the retinal areas undergoing apoptosis one to three days after ischemia injury.

Riluzole is a drug that protects against the deleterious effect of cerebral ischemia and antagonizes glutamate excitotoxicity inhibiting its release. Ischemia for 30 minutes caused a reduction of a and b waves on the electroretinogram. Systemic and topical treatment with this drug recovers the reduced a and b waves after defined reperfusion times (Ettaiche et al., 1999).

Another NMDA-antagonist, memantine, is undergoing testing in a placebo-controlled prospective, randomized, multicenter trial in USA. Memantine, in a rat model of retinal ischemia induced by elevation of intraocular pressure and in experimental glaucoma in primates, is safe and effectively reduces ganglion cell loss when given systemically (Hare et al., 2004), as well as protecting ganglion cells in a model of chronic elevation in vitreal glutamate (Vorwerk et al., 1996).

In seeking potential drugs for neuroprotection, it is

important to avoid compounds that block receptors that mediate important functions, or at least to use antagonists like MK-801 whose affinity toward such receptors is lower (Yoles and Schwartz, 1998).

Adrenergic agonist

One available drug, used in eyedrops to lower ocular pressure -brimonidine- has been demonstrated to have neuroprotective effects (Lafuente et al., 2001; Vidal-Sanz et al., 2001). Brimonidine is a well-tolerated, selective alpha-2-adrenergic agonist. Allergic reactions are infrequent (Katz, 2002). The mechanism to lower ocular pressure is to reduce aqueous production and increase aqueous uveoscleral outflow (Greenfield et al., 1997).

Recent studies support the effects of brimonidine on optic nerve degeneration in a rat model. The injection of a single dose of brimonidine into the intraperitoneal space produces a significant increase in ganglion cell survival rate (Yoles et al., 1999). Topical brimonidine delayed rat ganglion cell death caused by acute retinal ischemia (Wheeler et al., 1999).

Gao et al. (2002) found an increase in the endogenous BDNF expression in ganglion cell death after a single low-concentration dose of intravitreal brimonidine. These results suggest that brimonidine neuroprotection may be mediated through up-regulation of BDNF in the ganglion cells.

Pretreatment with a single dose of brimonidine can completely prevent the early phase of retinal ganglion cell loss after 90 minutes of retinal ischemia induced by ligation of the ophthalmic vessels. Donello et al. (2001) postulated that brimonidine treatment prevents an increase in the vitreal content of glutamate and aspartate, suggesting a possible mechanism of neuroprotection by hyperpolarization.

Alpha-2 agonist receptors are located in the inner rat retina by immunohistochemistry. Brimonidine is neuroprotective in the lasered chronic hypertensive rat model, reducing ganglion cell loss over three weeks from 33% to 15% (Wheeler et al., 2001).

Brimonidine neuroprotection can occur by different mechanisms. Eti et al. (1999) documented an antiapoptotic effect of brimonidine when it was injected together with rauwolfscine, an α -adrenoreceptor antagonist. A minimal effect of brimonidine, as compared with a control, on the intraocular pressure in experimental ocular hypertension indicates that its neuroprotective effect is independent of its effect on ocular pressure.

Ahmed et al. (2001) reported that intraperitoneal brimonidine in an experimental model of ocular hypertension by cauterization of three episcleral veins had a neuroprotective effect, at least by an anti-apoptotic pathway due to the increased expression of two antiapoptotic genes bcl2 and bcl-xl (Lai et al., 2002) and secondarily by inhibition of the extracellular accumulation of aspartate and glutamate (Donello et al., 2001).

Prostaglandin

Latanoprost, a prostaglandin PG F₂ α -analogue, has a potent lowering effect on intraocular pressure in patients with glaucoma through the improvement of uveoscleral outflow (Hejkal and Camras, 1999). Kudo et al. (2006) postulate that latanoprost stimulates the prostaglandin F₂ α -receptors in retinal ganglion cells and leads to a neuroprotective effect against experimental glaucoma. Intravitreal administration of latanoprost has a neuroprotective effect on rat retinal ganglion cell damage induced by either intravitreal administration of NMDA or optic nerve axotomy.

The effective dose of latanoprost for retinal ganglion cell neuroprotection against NMDA administration is one hundred times more than against axotomy, indicating that the mechanism of retinal ganglion cell damage by NMDA could be different from that of axotomy. Drago et al. (2001) reported the neuroprotective effect of the intraperitoneal injection of latanoprost on the retina damaged by ischemia-reperfusion in vivo.

In conclusion, the most documented factor for glaucomatous damage continues to be high intra-ocular pressure. All the drugs used in glaucoma attempt to lower this pressure. Yet, a proportion of cases continue to suffer glaucomatous damage despite reducing the intraocular pressure to normal levels (Brubaker, 1996). Glaucoma is considered to be a neurodegenerative disease. Retinal ganglion cells are the most susceptible to the damage caused by increased intraocular pressure (Yücel et al., 2003). New medical therapies are being investigated as a strategy to reduce neuronal death of the ganglion cells. Antiglaucoma drugs currently used for patients, such as timolol (Goto et al., 2002), betaxolol (Endo et al., 2002), brimonidine (Vidal-Sanz et al., 2001; Wheeler et al., 2001), latanoprost (Kudo et al., 2006), and unoprostone (Melamed, 2002), have been reported to possess neuroprotective profiles for retinal neurons in experimental glaucoma. However, this effect has not been shown in human glaucoma. Further research is needed to find a substance that protects from ganglion cell death in glaucoma.

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