PERGAMON

Vision Research 42 (2002) 2333–2337

Vision Research

www.elsevier.com/locate/visres

Effects of anti-glaucoma medications on ganglion cell survival: the DBA/2J mouse model

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Received 2 April 2002; received in revised form 24 June 2002

Abstract

We studied whether several agents, approved or undergoing trials in human glaucoma, were effective in preventing ganglion cell loss in the DBA/2J mouse. Adult DBA/2J mice were treated with timolol, pilocarpine, brimonidine, dorzolamide, or NMDA-receptor antagonist memantine. Surviving retinal ganglion cells of treated and control mice were retrogradely labeled with fluorogold and counted after whole mount preparation. In treated mice, only memantine and timolol had significant effects on retinal ganglion cell survival ($P < 0.0001$, analysis of variance). Brimonidine was lethal to these mice, and these retinae were not analyzed further. The DBA/2J mouse represents a promising candidate for further experimentation in ocular hypertension.

Keywords: Ocular hypertension; Model; DBA/2J mouse; Retinal ganglion cell survival

1. Introduction

For the past three decades, pressure-induced glaucoma in the monkey has remained the primary animal model of human glaucoma. Recently, much interest has been shown in developing small animal models. Work by multiple groups has made impressive strides in developing a rat model of ocular hypertension (Levkovitch-Verbin et al., 2002; Morrison, Nylander, Lauer, Cepruna & Johnson, 1998; Neufeld, Sawada & Becker, 1999; Shareef, Garcia-Valenzuela, Salierno, Walsh & Sharma, 1995; Yoles, Wheeler & Schwartz, 1999). Given the wealth of knowledge about the mouse genome, as well as the plethora of murine transgenics, a mouse model would advance our understanding of the human condition.

Sheldon, Warbritton, Bucci, and Turturro reported in 1995 that a strain of DBA mice could develop glaucomatous changes under certain conditions, although they did not note optic nerve cupping (Sheldon, Warbritton, Bucci & Turturro, 1995). Simon John (Jackson Laboratories, Maine) has reported that the DBA/2J mouse develops essential iris atrophy, pigment dispersion, and glaucomatous changes (including cupping) over time (Chang et al., 1999; John et al., 1998). It was reported recently that pigment dispersion and iris stroma atrophy are caused by distinct mutations in genes encoding melanosomal proteins (Anderson et al., 2001a,b). However, although these investigators have measured the intraocular pressure in these animals, they have not explored whether conventional glaucoma therapy could retard the ganglion cell loss in these animals, nor have they attempted to quantify ganglion cell survival in this mouse strain.

The selection of suitable control animals for such experiments is problematic. One cannot be certain that the observed ganglion cell loss in an inherited model is truly pressure dependent. However, Williams and coworkers performed an exhaustive (252 mice) analysis of ganglion cell number as a function of genetic and environmental factors. They noted "The 82 youngest mice (<48 days old) had an average (ganglion cell) population of $59{,}124 \pm 686$ (SE), whereas the 84 oldest mice (>180 days old) had an average population of $58{,}545 \pm 1027$ (SE). Both groups contained a wide mix of strains. As expected from the non-significant difference between young and old groups, the correlation

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0042-6989/02/$ - see front matter
PII: S0042-6989(02)00188-8

coefficient across all cases between neuron number and age was close to zero'' (Williams, Strom, Rice & Goldowitz, 1996). They have generously posted the full data set on the web (http://www.nervenet.org/main/databases.html), which allows for an estimate of normal ganglion cell number across multiple murine strains.

Accordingly, we sought to determine whether pilocarpine, dorzolamide, timolol, brimonidine and memantine—all agents either approved or undergoing trials in human glaucoma—protect against ganglion cell loss in the DBA/2J mouse.

2. Methods

All experiments were carried out in accord with the ARVO statement for the use of animals in vision research. Adult DBA/2J mice were obtained from Jackson Laboratories (Bar Harbor, MA) at 2 months of age. These mice were housed in a 12 h light/dark cycle with water and food provided ad libitum. Beginning at the age of 8 weeks, either application of commercial eyedrops containing timolol (0.5%), pilocarpine (4%), brimonidine (0.2%), or dorzolamide (2%), or intraperitoneal injections with 5 mg/kg memantine twice a day was initiated.

After 6 months, control mice and mice of all treatment groups were deeply anaesthetized with choral hydrate (6 ml/kg body weight of a 7% solution), administered intraperitoneally. To determine RGC densities, cells were labeled retrogradely with the fluorescent tracer Fluorogold (Molecular Probes, Eugene, Oregon) by stereotaxic injections into both superior colliculi as described previously (Vorwerk et al., 1996).

Two days after the superior colliculus was injected with Fluorogold, animals were sacrificed by chloral hydrate overdose. Retinae were dissected, flat-mounted on nitrocellulose filters (Sartorius, pore size 60 μm) and fixed in 2% paraformaldehyde for 30 min. Cells were imaged under fluorescence microscopy. Three areas per retinal quadrant at three different eccentricities of one-sixth, one-half, and five-sixths of the retinal radius were counted (Klöcker, Cellerino & Bähr, 1998). Labeled cells were thereby counted in 12 distinct areas of 62,500 μm^2 each in each retina. Brimonidine, administered topically, was lethal to these mice, and these retinae were therefore not analyzed further.

To determine the age-dependency of retinal ganglion cell loss, the number of RGC of additional untreated mice, and mice treated with either timolol or memantine was quantified after 4 and 9 months using the same techniques described above.

We could not replicate the intraocular pressure measurement technique described by John (John, Hagaman, MacTaggart, Peng & Smithes, 1997; John et al., 1998). Therefore, IOP measurements in these mice are not provided.

3. Statistical analysis

A two-way nested analysis of variance (ANOVA) mixed model with 12 replicates for each eye was used to assess differences in retinal ganglion cell count and density (RGC/mm^2) between eyes treated with pilocarpine, dorzolamide, memantine, and timolol compared to DBA/2J controls over the first 6 months of life. Treatment effects were evaluated by the *F*-ratio and post-hoc

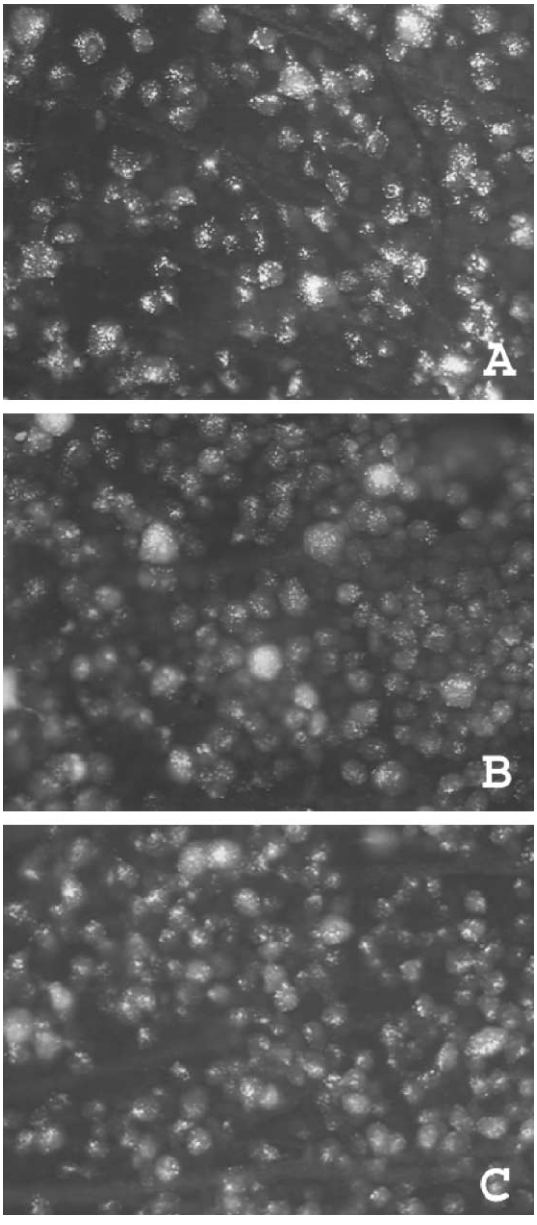

Fig. 1. High power view of retrogradely labeled retinal ganglion cells from DBA/2J mice at 6 months of age: (A) untreated, (B) treated with timolol, (C) treated with memantine.

Tukey method in ANOVA using a Bonferroni correction to adjust the significance level for multiple group comparisons among the four different treatments (Sokal & Rohlf, 1995). Therefore, two-tailed values of $P < 0.0125$ (0.05/4) were considered statistically significant. RGC counts and densities were tested for normality using the Kolmogorov–Smirnov test and no significant skewness or departures from a Gaussian-shaped distribution were found (Armitage & Berry, 1994). Therefore, continuous data are expressed in terms of the mean and standard deviation (SD) for control and treatment groups. Data analysis was performed using the general linear models procedure in the SPSS software package with group as a fixed factor and eye as a random factor (version 10.1, SPSS Inc., Chicago, IL).

4. Results

The DBA/2J mice showed a significant age-related decline in retinal ganglion cell number through 9 months $F(1,24) = 88.11$, $P < 0.0001$. At 4 months, DBA/2J mice had 89 ± 12.8 cells per field ($n = 4$; mean $\pm$ SD); 75.6 ± 14.3 cells at 6 months, ($n = 10$), and 40.0 ± 9.7 per field at 9 months ($n = 12$).

At 6 months, eyes treated with pilocarpine had 76.3 ± 5.1 ($n = 8$) cells surviving while those treated with dorzolamide had 86.4 ± 7.7 ($n = 8$). Animals injected with memantine had 105.6 ± 10.6 ($n = 8$) cells surviving and those treated with timolol had 105.5 ± 12.9 cells surviving ($n = 8$) (Fig. 1). Memantine and timolol were similarly protective against ganglion cell loss at 9 months (Fig. 3). Other drugs were not tested beyond 6 months (illustrated in Figs. 2 and 3).

ANOVA revealed highly significant differences between the treatment groups and control mice, $F(4,47) = 12.93$, $P < 0.0001$. Tukey's post-hoc method indicated that the loss of ganglion cells over the first 6 months of life was significantly reduced in mice receiving memantine and timolol compared to DBA/2J controls ($P < 0.0001$ for each). However, no significant differences were detected in RGC survival between controls and pilocarpine ($P = 0.98$) or dorzolamide ($P = 0.32$) treatment groups (Table 1).

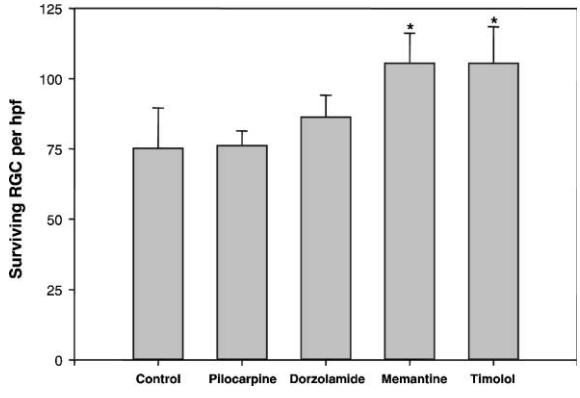

Fig. 2. Retinal ganglion cell survival at 6 months in the DBA/2J mouse. Values represent the mean $\pm$ SD. (*) indicates significantly higher RGC survival at 6 months of life for mice receiving memantine and timolol compared to DBA/2J controls ($P < 0.0001$ for each) by ANOVA. No significant differences were detected between controls and pilocarpine ($P = 0.98$) or dorzolamide ($P = 0.32$).

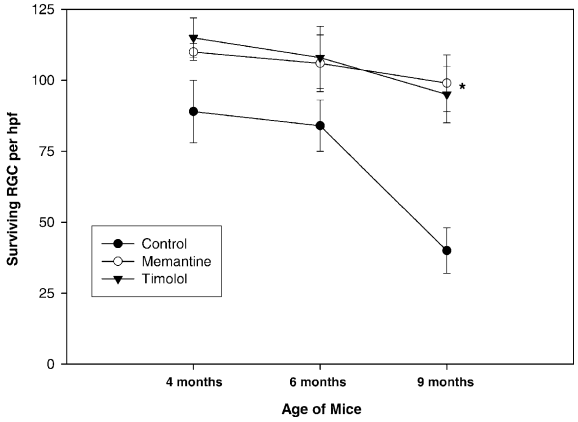

Fig. 3. Retinal ganglion cell survival in DBA/2J mice as a function of age ($F(1,24) = 88.11$, $P < 0.0001$) after treatment with timolol or memantine.

Table 1
RGC survival in DBA/2J mice at 6 months

Group	Number of eyes	RGC/HPF
Control	10	75.3 ± 14.3
Pilocarpine	8	76.3 ± 5.1
Dorzolamide	8	86.4 ± 7.7
Memantine	8	105.6 ± 10.6[a]
Timolol	8	105.5 ± 12.9[a]

Shown are mean number of RGC per high power field $\pm$ SD.

[a] Indicates significantly higher survival of retinal ganglion cells for mice treated with memantine and timolol compared to DBA/2J controls ($P < 0.0001$ for each) by ANOVA. No significant differences were detected between controls and pilocarpine ($P = 0.98$) or dorzolamide ($P = 0.32$).

5. Conclusions

The DBA/2J mouse represents a promising candidate for further experimentation in ocular hypertension, inasmuch as the ocular hypertension develops spontaneously, and leads to ganglion cell loss in a time dependent fashion. We were able to show this continuous loss of retinal ganglion cells during a period of 9 months. Strikingly, the significant age-dependent loss of RGC is observed in 6 and 9 month old mice, at a relatively early stage of the anatomical changes, especially if compared to another proposed mouse model of ocular hypertension where changes are observed much later and in a slower fashion (Anderson et al., 2001a).

Moreover, DBA/2J mice are drug responsive. The time-dependent loss of RGC can be inhibited by either

conventional anti-glaucomatous therapy with β-blockers or injections with an NMDA-receptor antagonist. The aqueous suppressant, timolol was effective in preserving ganglion cell function. Timolol reduces aqueous production, therefore it might be more suitable to a model with blocked trabecular meshwork if compared to medications increasing the aqueous outflow through the trabecular meshwork (pilocarpine). Otherwise, dorzolamide, the other of the tested medications reducing aqueous production, did not alter RGC survival.

It has been suggested that toxic levels of glutamate might contribute to the ganglion cell loss seen in glaucoma (Dreyer, Zurakowski, Schumer, Podos & Lipton, 1996). This raises the possibility that a glutamate antagonist might be efficacious in the management of this disease. In this model, the glutamate antagonist, memantine, was able to block ganglion cell loss. Memantine is known to be effective in rescuing ganglion cells from chronic low dose exposure to glutamate (Vorwerk et al., 1996). Other groups have also demonstrated that memantine can protect retinal ganglion cells against other injuries (Lagreze, Knorle, Bach & Feuerstein, 1998; Osborne, 1999).

In contrast, we did not observe a rescue of ganglion cells after pilocarpine treatment. It has been previously reported that pilocarpine might be toxic to ganglion cells under certain conditions (Vorwerk et al., 1999). Furthermore, it is not known whether the anterior segment of the DBA/2J mouse has either receptors or the ciliary muscle necessary for pilocarpine to effectively lower IOP. Consequently, it is not surprising that pilocarpine was ineffective in this experimental model.

Dorzolamide was not statistically effective at retarding ganglion cell loss. As for the other pressure lowering agents, there is no knowledge if a suitable receptor for dorzolamide is present in the DBA/2J mouse. Also, a recent publication shows that β-blockers are more effective in blocking voltage-dependent calcium channels in the rat retina compared to dorzolamide and pilocarpine (Melena, Stanton & Osborne, 2001). This might influence the protecting ability of dorzolamide in the DBA/2J mouse model as well.

Given our inability to measure intraocular pressure in these mice, as well as our limited knowledge of murine aqueous dynamics, it is difficult to assign any protective effect of these agents to their conventional mechanisms as postulated in the human eye. Nonetheless, it is encouraging that the DBA/2J mouse loses ganglion cells in a time dependent fashion faster than one might predict in a "normal" mouse eye. One cannot rule out the possibility that the ganglion cell loss seen in these mice is unrelated to the elevated IOP reported by John et al. (1998). However, the aqueous suppressant, timolol was effective in preserving ganglion cells. Future work will be undertaken to determine the effect of the tested medications on intraocular pressure.

The fact that brimonidine was lethal to DBA/2J mice is slightly concerning, since this medication is in frequent clinical use. Although an excellent safety profile of the drug was described (Angelov et al., 1996), there are reports on different severe side effects in infants (Berlin et al., 2001; Enyedi & Freedman, 2001; Korsch, Grote, Seybold & Soditt, 1999; Walters & Taylor, 1999). It has been suggested that brimonidine should be used with caution in young children because of a potential depression of the central nervous system (Enyedi & Freedman, 2001). This would stand in accordance with our results in DBA/2J mice, which might be different to those in control mice.

The DBA/2J mouse model proved to be a candidate for further experimentation in ocular hypertension inasmuch as the DBA/2J disease process develops spontaneously and leads to retinal ganglion cell loss in a time dependent fashion, which can be inhibited by IOP-lowering or glutamate receptor blocking agents. Hopefully, future research will establish whether lowering the IOP in this model can achieve good results with respect to the preservation of ganglion cells.

Acknowledgements

Supported by NIH grant EY 10009-09. The authors appreciate the helpful comments of Alan Laties, MD.

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