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Mechanism of retinal ganglion cells death in secondary degeneration of the optic nerve☆

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ABSTRACT

In central nervous system injury, the secondary degeneration process is known to play a major role in determining the final extent of impairment. Here, we investigated the mechanism of retinal ganglion cell (RGC) death in secondary degeneration of the optic nerve using a unique model that allows morphological separation between primary and secondary degeneration. A partial transection model was applied unilaterally in 110 Wistar rat eyes. The rate of apoptosis was evaluated in primary and secondary degeneration over a period of 6 months using the Hoechst staining technique. The involvement of caspase 3 and members of the Bcl-2 family (Bax, Bad, Bcl-2 and Bcl-xl) was evaluated at multiple time points for 6 months after the injury by immunohistochemistry and RT-PCR. We found that in secondary degeneration of the optic nerve, RGCs died by apoptosis from day 3–6 months following the injury, peaking at 3 months (16.3% ± 2.5% apoptotic cells, $p < 0.01$). Both primary and secondary degeneration of the optic nerve resulted in caspase 3 activation, which was longer and more intense in the former. Similarly, both primary and secondary degeneration led to significant ($p < 0.05$) downregulation of the pro-survival genes Bcl-2 and Bcl-x-L and up-regulation of the pro-apoptotic genes Bax and Bad ($p < 0.05$), with a suggested delay in secondary degeneration. Thus, secondary degeneration of the optic nerve leads to RGC apoptosis over long periods in a similar mechanism as in primary degeneration.

1. Introduction

The mechanism of optic nerve damage in glaucoma is controversial and not fully understood. It is well accepted that retinal ganglion cells (RGCs) of glaucomatous eyes die by apoptosis (Kerrigan et al., 1997), and that many signaling pathways are involved in this process (Johnson et al., 2000; Levkovitch-Verbin et al., 2006, 2007; McKinnon et al., 2002). Gene expression analysis reveals that pro-apoptotic genes are still up-regulated in retinas from glaucomatous eyes long after intraocular pressure (IOP) has returned to baseline (Levkovitch-Verbin et al., 2006, 2007). Thus, the apoptotic process continues long after the primary detrimental factor is removed. Also, clinical studies have shown that patients can deteriorate even after IOP is significantly reduced, and that visual field defects may appear months after IOP reduction (McKinnon et al., 2008). It is possible that secondary degeneration contributes to that long-lasting process of ongoing RGC death and so may play an important role in glaucoma as well as open an additional window of opportunity for neuroprotective treatment (Schwartz and Yoles, 1999; Yoles and Schwartz, 1998). However, other factors, such as slower death of RGCs that are more resistant to the influence of increased IOP or the death of cells that had been only partially injured can also be influential in this complex chain of events.

In central nervous system (CNS) injury including spinal cord (Nevo et al., 2001), the secondary degeneration process is known to play a major role in determining the final extent of impairment. Injury from various primary lesions can lead to widespread damage to neurons beyond the initial injury site (Dusart and Schwab, 1994; Yao et al., 2001). In ischemic stroke, for example, the neurons in the core infarct die rapidly and release chemicals that stimulate a chain reaction called the “stroke cascade”. This reaction lasts for a considerable length of time and endangers neurons in a much larger area (the penumbra area) (Nishioka et al., 2006; Schaller et al., 2005). This phenomenon is also known as “secondary degeneration” and can, therefore, result in greater loss of tissue than that caused by the initial disorder. Moreover, it may continue for an extended period of time after termination of the primary event (Schwartz and Yoles, 1999; Yoles and Schwartz, 1998).

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Currently available models of experimental glaucoma cannot distinguish primary from secondary RGC death. We have developed a model for secondary degeneration in the optic nerve known as the partial transection model (Blair et al., 2005; Levkovitch-Verbin et al., 2001, 2003). In this model, the upper third of the intraorbital optic nerve is transected, causing initially primary degeneration of the upper optic nerve and of the corresponding RGC in the upper retina. We have shown that cutting the upper third of the optic nerve in primates and rats also causes significant but delayed axonal and RGC loss in the inferior uninjured area of the nerve and retina, resulting in secondary degeneration. This raises the possibility that some of the loss of RGCs in glaucoma and other optic neuropathies is also due to indirect or secondary degeneration (Moalem et al., 1999; Schwartz and Yoles, 1999; Yoles and Schwartz, 1998). If such is the case, secondary degeneration could be a substantial additive factor in glaucomatous damage and other optic neuropathies.

The partial transection model is unique because it allows morphologic separation between primary and secondary degeneration in the optic nerve and retina. In the current study, we used this model to characterize the mechanism of RGC death in primary and in secondary degeneration and to provide time frame of events. Specifically, we investigated whether RGCs die by apoptosis in secondary degeneration of the optic nerve and whether the caspase family and the bcl-2 family are involved in this process as seen in glaucoma.

2. Methods

2.1. Animals

Adult Wistar rats (375–425 g) were used in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research in protocols approved and monitored by the Animal Care Committee of the Tel-Aviv University School of Medicine. The animals were housed with a 14-h light and 10-h dark cycle with standard chow and water *ad libitum*.

2.2. Partial optic nerve transection

The animals ($n = 110$) were anesthetized with intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg) and given topical proparacaine 1% eye drops. The conjunctival 12 o'clock position was marked unilaterally by a light cautery burn. An incision in the superior conjunctiva was made and the eye was gently retracted outward with forceps, exposing the nerve behind the eye. The dural sheath was opened with fine scissors and the superior pole of the nerve was re-identified relative to the cautery mark. A specially designed diamond knife was then used to incise the optic nerve to a depth of one-third of its diameter. The knife was similar to that previously used for radial keratotomy in human eyes, with a blade that could be advanced in 10-μm increments from a twist-handle and a single metal guard that limited penetration as measured on the scale. Typically, the blade was set at a depth of 100 μm. The conjunctival incision was self-closing. Sulfacetamide 10% ophthalmic ointment was applied at the end of surgery.

Each operated eye was inspected ophthalmoscopically to insure patency of blood flow to the eye.

2.3. Enucleating the eyes for cryopreservation and RT-PCR

Before enucleation, the 12 o'clock position on the globe was marked by a light cautery burn. Following enucleation, the anterior segment was removed and relaxing incisions were made to the posterior segment to assist in keeping the orientation of the superior point. The enucleated eyes (both eyes of each rat) were cryopreserved for immunohistochemistry and Hoechst staining, keeping the orientation of their superior and inferior regions. Cryosections were prepared separately for the upper third of the retina that represents the primary degeneration area and for inferior third of the retina that represents the secondary degeneration area and for corresponding areas in the control fellow eyes.

For PCR, the upper and lower thirds of the retina of both eyes in each rat were separated from the globe and immediately frozen separately. The central area of the retina was not used for any analysis.

2.4. Histology of optic nerves

Histology of optic nerves was performed ($n = 15$) 2 weeks after the injury to reassure the feasibility of the model and depth of transection. After enucleating the eyes together with the attached optic nerves and separating the anterior segment, a relaxing incision was made in a superior point in order to mark the superior area. The optic nerves and the attached sclera were immersed in paraformaldehyde 4% and were sent to the HA Quigley Laboratory of Johns Hopkins University, Baltimore, MD for evaluating the depth of transection. After fixation, many 1-mm thick portions of the optic nerve were removed with razor slits marking the superior (1 slit) and nasal (2 slits) meridian for orientation after sectioning. This was post-fixed in 1% osmium tetroxide, dehydrated in alcohol and embedded in epoxy resin. One-micron sections were stained with 1% toluidine blue.

2.5. Hoechst staining for apoptosis

To investigate whether RGCs die by apoptosis in secondary degeneration, we used the Hoechst 33342 dye that stains the condensed chromatin of apoptotic cells. The animals ($n = 29$) were sacrificed at different time points up to 6 months after partial transection and both eyes were cryopreserved. Cryosections 10-μm thick from superior and inferior areas of both eyes, at a similar distance from the optic nerve, were collected and stained with a cell-permeable form of bisbenzimide (Hoechst 33342) at a concentration of 4 μg/ml for 30 min at 37 °C. The cells were visualized under an ultraviolet fluorescent microscope, and the number of apoptotic cells with condensed and fragmented chromatin was counted in each section, 8 sections per eye, 4 sections for each superior and inferior areas. In addition, the total number of nuclei in the RGC layer was counted and the proportion exhibiting apoptosis was calculated per eye. The counting process was done by a masked observer. We refer here to the RGC nuclei as those identified by their presence in the RGC layer and by their size and morphology. It is possible that some of the cells identified in the RGC layer were amacrine cells.

2.6. Immunohistochemistry

Both eyes of each animal ($n = 34$) were enucleated and cryopreserved in sucrose/OCT (Sakura Finetek, USA Inc.; Torrance, CA). Cryosections 10-μm thick from superior and inferior areas of both eyes, at a similar distance from the optic nerve, were collected separately onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at −80 °C before immunolabeling. At least eight sections from each eye (4 sections from each superior or inferior areas) were examined. The sections were stained with cleaved caspase 3 (Asp175) antibody (Cell Signaling Technology, Inc., Beverly, MA). Following incubation, the sections were washed and stained with goat anti-rabbit IgG (H + L) Biotin Conjugate (Zymed Laboratories, Inc., California, USA), followed by ABC solution-Vectastain