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Neuroprotective and axon growth promoting effects of intraocular inflammation do not depend on oncomodulin or the presence of large numbers of activated macrophages

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Abstract

Retinal ganglion cells (RGCs) cannot regenerate their axons after injury and undergo apoptosis soon after an intraorbital injury of the optic nerve. However, RGCs reactivate their axonal growth program when inflammatory reactions occur in the eye, which enables them to survive axotomy and to regenerate lengthy axons into the lesioned optic nerve. Lens injury (LI) and zymosan injections can induce these beneficial processes and provoke also a strong accumulation of activated macrophages in the vitreous body. It has recently been suggested that macrophage-derived oncomodulin is the principal mediator of this phenomenon. We show here that oncomodulin is not significantly expressed in primary macrophages and that the intraocular levels of this protein do not increase after LI or zymosan treatment. Furthermore, greatly reducing the invasion of macrophages into the inner eye does not diminish the neuroprotective effects of LI, but rather increases axon regeneration into the optic nerve. Axon regeneration is correlated with the activation of retinal astrocytes and Müller cells. Our data suggest that intraocular inflammation mediates its main beneficial effects through factors other than oncomodulin and that the underlying mechanism might be independent of the presence of activated macrophages.

Keywords: Retina; Optic nerve; Axon regeneration; Retinal ganglion cells; Macrophages; Oncomodulin; Zymosan; CNS

Introduction

The visual system has been long regarded as a model of regenerative failure and neuronal degeneration in the mature central nervous system (CNS). Like most CNS neurons, mature retinal ganglion cells (RGCs) fail to regenerate injured axons beyond the injury site. Instead, more than 90% of all RGCs undergo apoptosis within 14 days after an intraorbital optic nerve crush (ONC) (Mey and Thanos, 1993; Berkelaar et al., 1994; Fischer et al., 2000). We and others have recently shown that inflammatory reactions in the vitreous body markedly delay the degeneration of axotomized RGCs. These beneficial effects can be provoked either by a lens injury (LI) or by intravitreal injections of the proinflammatory yeast wall extract zymosan (Fischer et al., 2000, 2001; Leon et al., 2000; Yin et al., 2003). Most RGCs enter into a robust regenerative state between 3 and 4 days after ONC and LI, which enables them to extend axons from retinal explants at higher rates, and to regenerate lengthy axons into a peripheral nerve graft or injured optic nerve (Fischer et al., 2000, 2001, 2004a,b; Yin et al., 2003; Lorber et al., 2005; Pernet and Di Polo, 2006). Regeneration in the lesioned optic nerve is strongly increased when LI is combined with a gene therapeutic approach that makes growth cones less sensitive to myelin or glial scar inhibitors, whereas blocking inhibitory signaling pathways alone is not sufficient to produce strong regeneration (Fischer et al., 2004a,b). Similarly, combining the treatment of sensory ganglia with zymosan and modification to chondroitin–sulfate proteoglycans results in robust and functional regeneration of sensory axons through the dorsal root entry zone (Steinmetz et al., 2005). Thus, understanding the molecular mechanisms controlling this

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regenerative switch may reveal novel ways to improve axon regeneration in other CNS pathways.

The inflammatory reactions induced by LI or zymosan affect several types of retinal cells. For instance, Müller cells and astrocytes respond by strongly increasing their expression of glial fibrillary acid protein (GFAP), which is a characteristic indicator of inflammation and injury in the CNS (Leon et al., 2000; Pernet and Di Polo, 2006). However, most attention has been focused on the activation and invasion of macrophages into the vitreous body and injured lens. There is no direct evidence that activated macrophages are functionally essential to switch RGCs into a regenerative state following LI or zymosan injection *in vivo*. Although there are published data indicating that intravitreal injections of activated primary macrophages do not improve regeneration or increase growth-associated protein 43 (GAP-43) expression in RGCs (Leon et al., 2000), and that lens-derived factors might also contribute to these effects without activating macrophages (Lorber et al., 2002, 2005), the term "macrophage activation" is generally used as a synonym for inflammatory reactions that positively influence regeneration (Leon et al., 2000; Yin et al., 2003, 2006; Fischer et al., 2004a,b; Pernet and Di Polo, 2006). The calcium-binding protein oncomodulin has been identified as the principal active factor of macrophage-cell-line-conditioned medium, which itself promotes axon outgrowth from dissociated RGCs in culture in the presence of elevated intracellular cAMP levels (Yin et al., 2006). Intravitreal coadministration of oncomodulin with these drugs that elevate intracellular cAMP reportedly enhances axon regeneration into the optic nerve. This led to the hypothesis that oncomodulin is also the principal mediator of the beneficial effects of intraocular inflammation (Yin et al., 2006).

Here we show that primary macrophages do not express oncomodulin at significant levels even when they are activated. We provide evidence that LI or zymosan treatments do not significantly increase oncomodulin levels in the inner eye and that macrophage depletion does not compromise the neuroprotective and axon-growth-promoting effects of LI. These data suggest that oncomodulin is not the principal factor mediating the beneficial effects of intraocular inflammation and that these effects are mainly mediated by a macrophage independent mechanism.

Materials and methods

Preparation of clodronate liposomes

Forty milliliters of clodronate liposomes or control liposomes were prepared by applying freeze–thawing and filter extrusion to soy phosphatidylcholine (4.0 g; Epikuron 200, Lukas Meyer, Hamburg, Germany), cholesterol (0.6 g; Fluka, Buchs, Switzerland) and D,L-α-tocopherol (0.02 g; Merck, Darmstadt, Germany) at a molar ratio of 1:0.3:0.01. The dry lipid mixture was solubilized in a physiologic phosphate buffer (20 mM, pH 7.4) supplemented with 230 mM mannitol and 2.64 g of clodronate (clodronic acid disodium salt tetrahydrate, $CH_2Cl_2Na_2O_6P_2 \times 4H_2O$; Bioindustria LIM, Novi Ligure, Italy). The resulting multilamellar vesicles were freeze–thawed using three cycles of liquid nitrogen and water at 40 °C, followed by repetitive (5–10×) filter extrusion through 400-nm membranes (Nuclepore, Sterico, Dietikon, Switzerland) using a Lipex™ extruder (Lipex Biomembranes, Vancouver, Canada). Nonencapsulated clodronate was removed by dialysis (Spectrapore tube, 12–14 mol. wt. cut-off). The liposome size and homogeneity were routinely measured with a laser-light-scattering particle sizer (Nicomp 370, Santa Barbara, CA). Prepared small unilamellar clodrolip liposomes contain clodronate at approximately 20 mg/ml in phosphate-buffered saline (PBS; 67 mM, pH 7.4) and have a mean diameter of 135 nm (SD=55 nm). Control liposomes were prepared accordingly except for the addition of clodronate.

Optic nerve crush, lens injury and administration of zymosan and liposomes/clodrolip

Adult female Sprague–Dawley rats weighing 220–250 g were used in all the experiments except for retinas from adult Fisher rats being used to generate RNA for real-time polymerase chain reaction (PCR) analysis. Rats were anesthetized by an intraperitoneal injection of ketamine (60–80 mg/kg) and xylazine (10–15 mg/kg). A 1.0- to 1.5-cm-long incision was made in the skin above the right orbit, and the optic nerve was surgically exposed under an operating microscope. The dura was opened longitudinally, and the nerve was crushed 1 mm behind the eye for 10 s using a jeweler's forceps, avoiding injury to the central retinal artery. The vascular integrity of the retina was verified by funduscopic examination. Sham surgery was performed on animals using the same protocol except without injuring the optic nerve. These animals were used to test whether sham surgery alone caused macrophage or microglia activation in the eye. LI was induced through a retrolenticular approach, puncturing the lens capsule with the narrow tip of a microcapillary tube; inflammation was enhanced by injecting 10 μl of PBS intravitreally after removing the same volume of liquid from the anterior chamber of the eye. Intraocular inflammation was induced by intravitreally injecting 65 μg of zymosan suspended in 10 μl of PBS. Fifteen microliters of either control liposomes or clodrolip (20 mg/ml) were intravitreally injected at the same time of LI, ONC or LI+ONC. Injections were repeated after 3 days in order to maintain intraocular concentrations high. Retinas were isolated after 1, 2, 5 or 7 days and used to generate either RNA or protein lysates, or to prepare explant cultures. To quantify numbers of macrophages and microglia in the eye, we prepared the following groups: untreated animals, sham operated animals, animals that were subjected to ONC, LI, ONC+LI, ONC+intravitreal injection of control liposomes, ONC+intravitreal injections of clodrolip, ONC+LI+intravitreal injections of control liposomes, ONC+LI+intravitreal injections of clodrolip, ONC+intravitreal injection of zymosan, ONC+LI+intraperitoneal injections of liposomes, ONC+LI+intraperitoneal injections of clodrolip.

Animals that were used for studying axon regeneration in the optic nerve or RGC survival received ONC or ONC+LI and additionally three intravitreal injections of PBS, control liposomes