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Programmed cell death-1 is expressed in large retinal ganglion cells and is upregulated after optic nerve crush

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ABSTRACT

Programmed cell death-1 (PD-1) is a key negative receptor inducibly expressed on T cells, B cells and dendritic cells. It was discovered on T cells undergoing classical programmed cell death. Studies showed that PD-1 ligation promotes retinal ganglion cell (RGC) death during retinal development. The purpose of this present study is to characterize PD-1 regulation in the retina after optic nerve crush (ONC). C57BL/6 mice were subjected to ONC and RGC loss was monitored by immunolabelling with RNA-binding protein with multiple splicing (Rbpms). Time course of PD-1 mRNA expression was determined by real-time PCR. PD-1 expression was detected with anti-PD-1 antibody on whole mount retinas. PD-1 staining intensity was quantitated. Colocalization of PD-1 and cleaved-caspase-3 after ONC was analyzed. Real-time PCR results demonstrated that PD-1 gene expression was significantly upregulated at day 1, 3, 7, 10 and 14 after ONC. Immunofluorescent staining revealed a dramatic increase of PD-1 expression following ONC. In both control and injured retinas, PD-1 tended to be up-expressed in a subtype of RGCs, whose somata size were significantly larger than others. Compared to control, PD-1 intensity in large RGCs was increased by 82% in the injured retina. None of the large RGCs expressed cleaved-caspase-3 at day 5 after ONC. Our work presents the first evidence of PD-1 induction in RGCs after ONC. This observation supports further investigation into the role of PD-1 expression during RGC death or survival following injury.
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1. Introduction

Retinal ganglion cell (RGC) death is a final common pathway in many optic neuropathies including glaucoma (Sucher et al., 1997), optic nerve trauma (Levkovitch-Verbin et al., 2001) and ischemic optic neuropathies (Levin and Louhab, 1996), which is responsible for irreversible visual loss. In addition to any primary damage to RGCs during an initial stress, a variety of other processes such as neurotrophin delivery failure (Quigley et al., 2000), glutamate excitotoxicity (Bai et al., 2013), oxidative stress (Tezel, 2006) and glia dysfunction (Neufeld and Liu, 2003) act to initiate a cascade of cellular events leading to delayed, secondary RGC death. Multiple intracellular signaling pathways are involved in the secondary damage period, including phosphatidylinositol 3-kinase (PI3K) and MAPK/ERK pathways (Kikuchi et al., 2000). These undergo modulation and may be responsible for transducing an apoptotic signal from the cell surface to the nucleus, resulting in cell death. A plethora of investigations have focused on the secondary RGC cell death aimed at uncovering therapeutic strategies to prevent long term RGC loss. However, these efforts are not uniformly successful and treatment for RGC degenerative diseases remains challenging. This gives impetus to continued investigation and understanding of potential upstream molecular mechanisms that correspond to increased or decreased RGC cell death to provide potential targets for the development of efficient treatment for these vision-threatening diseases.

Programmed cell death-1(PD-1) is a key negative coreceptor which regulates the T cell response and plays an important role in tolerance and immunity (Keir et al., 2008). The PD-1 gene was discovered in 1992 when investigators observed significant expression of PD-1 mRNA in T and hematopoietic progenitor cells

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upon induction of classical programmed cell death (Ishida et al., 1992). Subsequent experiments have confirmed that the ligation of PD-1 with one of its ligands controls downstream events. In the immune system, PD-1 is a critical immunoregulator which delivers inhibitory signals and suppresses the immune response through engagement with its ligands PD-L1 (B7-H1) or PD-L2 (B7-DC) (Francisco et al., 2010). Upon activation, PD-1 is inducibly expressed on T and B cells, and ligation of PD-1 acts to dampen downstream signal transducers including PI3K/Akt (Keir et al., 2008) and ERK (Saunders et al., 2005) via recruitment of phosphatase SHP2 (p-SHP2) (Yokosuka et al., 2012), eventually leading to the reduction of T cell proliferation and cytokine production. PD-1 ligation is also important in tumor biology, wherein high expression of PD-L1 is observed in many tumor cells. The PD-1-PD-L1 interaction plays a crucial role in preventing tumor immunosurveillance by inhibiting tumor-infiltrating CD8 T cells (Okazaki et al., 2013), either through direct induction of CD8 T cell apoptosis (Dong et al., 2002), or alternatively by mediating activated dendritic cell apoptosis (Park et al., 2014). These findings strongly indicate that PD-1 may function to deliver apoptotic signals in the immune system.

Our laboratory previously reported that PD-1 is constitutively expressed in retinal ganglion cells (RGCs), an unanticipated observation since PD-1 was previously believed to function exclusively within immune system (Chen et al., 2009a). Concordant with its functions in the immune system, we further demonstrated that PD-1 and its partner(s) play an important role in RGC death during retinal development (Chen et al., 2009b; Sham et al., 2012). It is known that pathological RGC degeneration may share the same mechanisms as those in physiological RGC loss; therefore, we investigated whether PD-1 is involved in RGC apoptosis in pathological settings. In this present study, we examined the expression and regulation of PD-1 following optic nerve crush (ONC) in a mouse model and observed a strong induction of PD-1 expression in a subset of RGCs.

2. Materials and methods

2.1. Animals

Eight to ten week old C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). All animal experiments were carried out with approval of the University of California, Los Angeles, Chancellor's Animal Research Committee, and in compliance with the ARVO Statement for the Use of animal in Ophthalmic and Vision Research.

2.2. Optic nerve crush surgery

The animals were anesthetized by inhalation of isoflurane (0.8% in oxygen). ONC injury was performed according to a protocol described previously (Nadal-Nicolas et al., 2009). Briefly, after animals were anesthetized, a conjunctival incision was made on the temporal side of the globe, the optic nerve was exposed intraorbitally by blunt dissection, with care to avoid damage to the optic nerve blood supply. Crush was applied approximately 2 mm behind the globe for 3 s with a self-closing forceps (Fine Science Tools, Foster City, CA) and the unoperated fellow eye served as controls. During the procedure, both eyes were treated with an ophthalmic lubricating ointment to protect the cornea. Antibiotic ointment was applied to the ocular surgical site for 3 days after crush.

2.3. Tissue preparation

Eyes were enucleated immediately after euthanasia and a small pinhole was applied on the dorsal cornea as a mark of retinal orientation. The globes were postfixed in 4% paraformaldehyde for an hour at room temperature. For whole mount staining the entire retina of each animal was carefully dissected en bloc and washed multiple times in phosphate buffered saline (PBS). For immunofluorescence staining, each whole retina was incubated with specific antibodies in a glass scintillation vial (Fisher Scientific, Pittsburgh, PA). For vertical sections, paraformaldehyde fixed, frozen vertical retina sections of 7 μm thickness were prepared from adult C57Bl/6 mice, 3 and 7 days post ONC injury. Control vertical retina sections were obtained using the uninjured fellow eye.

2.4. Immunofluorescence staining

For whole mount staining, blocking was performed for 1 h at room temperature with blocking buffer (20% fetal bovine serum [FBS] with 2% goat serum, 0.5% bovine serum albumin [BSA], and 1% PBS-Triton X-100 in phosphate buffered saline [PBS]), retinas were incubated overnight at 4 °C with a primary antibody: rat anti-mouse PD-1 (1:200; clone 29F. 9A2, rat IgG2a, k, gift from Dr. Gordon J. Freeman), rabbit anti-Rbpms (1:500; gift from Dr. Joseph Caprioli), rabbit anti-cleaved-caspase-3 (1:400; Cell signaling, Beverly, MA) in primary diluent (2.5% blocking buffer, 1% PBS-triton X-100). After 4 washes in washing buffer (2.5% blocking buffer, 1% PBS-triton X-100), the retinas were incubated with secondary AlexaFluor 594 goat-rat IgG antibody or AlexaFluor 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 in secondary diluent (2.5% blocking buffer, 1% PBS-triton X-100) overnight at 4 °C. After 4 washes, whole retinas were mounted flat with ganglion cell side up on a glass microscope slide (Fisher Scientific, Pittsburgh, PA) and were air dried overnight. Nuclei were counterstained with Hoechst 33342 (Thermo Scientific, Rockford, IL, USA), tissues were mounted and imaged.

Vertical sections were incubated at 4 °C overnight with the following primary antibodies: anti-mouse PD-1 (clone 29F.1A12, rat IgG2a) and rabbit anti-RBPMS. The following secondary antibodies were used for incubation for 1 h at room temperature: AlexaFluor 488 goat anti-rabbit IgG and AlexaFluor 594 goat anti-rat IgG (Life Technologies, Carlsbad, CA). Nuclei were counterstained with 1 μM DAPI (Life Technologies, Carlsbad, CA).

Antibody blocking experiments were performed using a recombinant mouse Pd-1-Fc fusion protein (R&D Systems, Minneapolis, MN) in 10-fold molar excess to the rat anti-mouse PD-1 (clone 29F.1A12, rat IgG2a) antibody. A non-specific protein was used as control. The fusion or control protein and antibody combination was incubated together for 2 h at room temperature. The retina sections were incubated at 4 °C overnight with the fusion protein–antibody mixture, and stained with the secondary antibodies and DAPI as described.

2.5. RGC quantification

Whole mount retinas were evaluated using the Confocal Laser Scanning microscope (Olympus FluoView™ FV1000, Tokyo, Japan), with a 40× objective and the RGCs were identified by Rbpms staining and quantified. Three sampling fields (371 × 371 μm^2) were acquired per retinal quadrant from central, middle and periphery area, respectively, and concordant regions were randomly sampled for each specimen. Rbpms positive cells were counted manually in each field using the public domain Java image processing software Image J (NIH). The cell number counted from a total of 12 photographs per retina were totaled and the mean RGC density (number of cells per mm^2) was obtained for statistical analysis.