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Enhanced survival of retinal ganglion cells is mediated by Müller glial cell-derived PEDF

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ABSTRACT

The death of retinal ganglion cells (RGC) leads to visual impairment and blindness in ocular neurodegenerative diseases, primarily in glaucoma and diabetic retinopathy; hence, mechanisms that contribute to protecting RGC from ischemia/hypoxia are of great interest. We here address the role of retinal glial (Müller) cells and of pigment-epithelium-derived factor (PEDF), one of the main neuroprotectants released from the glial cells. We show that the hypoxia-induced loss in the viability of cultured purified RGC is due to apoptosis, but that the number of viable RGC increases when co-cultured with Müller glial cells suggesting that glial soluble mediators attenuate the death of RGC. When PEDF was ablated from Müller cells a significantly lower number of RGC survived in RGC-Müller cell co-cultures indicating that PEDF is a major survival factor allowing RGC to escape cell death. We further found that RGC express a PEDF receptor known as patatin-like phospholipase domain-containing protein 2 (PNPLA2) and that PEDF exposure, as well as the presence of Müller cells, leads to an activation of nuclear factor (NF)-κB in RGC. Furthermore, adding an NF-κB inhibitor (SN50) to PEDF-treated RGC cultures reduced the survival of RGC. These findings strongly suggest that NF-κB activation in RGC is critically involved in the pro-survival action of Müller-cell derived PEDF and plays an important role in maintaining neuronal survival.

1. Introduction

The prevalence of retinal degenerative diseases increases, not only in the aging population in industrialized countries but worldwide. Hypertensive and ischemic retinopathies as well as glaucoma are ocular diseases that are associated with the loss of retinal ganglion cells (RGC). This leads to visual impairment and eventually results in blindness. Diabetic retinopathy and glaucoma are major causes of acquired blindness in industrial countries. It has been shown that impaired physiological cell metabolism of RGC and compromised cell-to-cell interactions may lead to programmed cell death (i. e., apoptosis) and to a limited retinal function (Guo et al., 2005; Quigley et al., 1995). Due to the neuronal origin and post-mitotic state of RGC, their demise cannot be functionally compensated in the postnatal human individual. Therefore, and given the complex pathology of most retinal diseases, previous treatment strategies have proved to be of little effect and remain unsatisfactory (Chung et al., 1999; Flammer et al., 2002; Grunwald et al., 1998).

Different mechanisms have been considered responsible for neuronal and RGC demise, such as activation of glutamate receptors, oxidative stress, nitric oxide (NO), pro-inflammatory factors, and deprivation of neuroprotective factors (Barber et al., 2011; Howell et al., 2013; Joo et al., 1999; Kern and Barber, 2008; Quigley, 1999; Rego et al., 1996; Sucher et al., 1997; Tezel and Wax, 2000). Neuroprotective properties for soluble mediators that are active in the retina have been demonstrated for nerve growth factor (NGF) (Skaper, 2008), vascular endothelial cell growth factor (VEGF) (Foxton et al., 2013; Jin et al., 2000; Kilic et al., 2006), ciliary neurotrophic factor (CNTF) (Mey and Thanos, 1993; Osborne et al., 2004; Wen et al., 2012), brain-derived neurotrophic factor (BDNF) (Marini et al., 2004; Mey and Thanos, 1993; Osborne et al., 2004), glial-derived neurotrophic factor (GDNF) (Osanai et al., 2010), insulin-like growth factor (IGF)-1 (Benarroch, 2012; Kermer et al., 2000; Seigel et al., 2000), EPO (Chang et al., 2013; Tsai et al.,

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2005; Zhong et al., 2007) and pigment-epithelim-derived factor (PEDF) (Sánchez et al., 2012; Unterlauft et al., 2013).

PEDF is a neurotrophic/-protective (Tombran-Tink and Barnstable, 2003) and anti-angiogenic (Dawson et al., 1999) 50-kDa glycoprotein that belongs to the serine protease inhibitor (serpin) family. PEDF occurs in the blood plasma (Petersen et al., 2003) and it is secreted in the eye by retinal pigment epithelium and Müller glial cells in considerable quantity. The neurotrophic/neuroprotective properties of PEDF are confined to its N-terminal region Ocular angiopathies such proliferative diabetic retinopathy and choroidal neovascularization may be accompanied by decreased intraocular PEDF levels (Holekamp et al., 2002; Spranger et al., 2001). The neurotrophic and neuroprotective properties of PEDF have been demonstrated in different conditions, mostly in neurotoxic milieus resulting from, for example, transient retinal ischemia (Ogata et al., 2001), glutamate excitotoxicity (Bilak et al., 1999), light exposition (Cao et al., 2001), and peroxide-mediated oxidative stress (Cao et al., 1999), and were also found in models of inherited retinal degeneration (Cayouette et al., 1999; Miyazaki et al., 2003).

Although the beneficial effect on neuronal survival provided by PEDF is well established, the intracellular signaling pathway(s) exploited during its neuroprotective actions have not been completely elucidated. It has been proposed that PEDF access multiple pathways such as the NF-κB/Rel (Pang et al., 2007; Yabe et al., 2001), p38 MAP kinase (Chen et al., 2006), ERK-1/-2 MAP kinase (Pang et al., 2007; Sánchez et al., 2012; Tsao et al., 2006), stress-activated phospho-kinase (JNK) (Konson et al., 2011), and phosphatidylinositol 3-kinase/Akt signaling cascades (Haribalaganesh et al., 2010).

Müller glial cells are PEDF producers. They radially traverse the neuroretina, ensheath the somata and dendrites of RGC and form a 'cellular scaffold' for other retinal cell populations with which they make up intimate intercellular contacts. Müller cells are essential for controlling fundamental processes of cell–cell communication and tissue homeostasis in the retina. The cells take up and metabolize glutamate or they secrete neuroprotective compounds such as the antioxidant, glutathione, or soluble mediators, among them are survival-promoting cytokines. Thus, Müller cells are able to protect retinal neurons from a variety of pathologic external influences (Bringmann et al., 2006; Newman and Reichenbach, 1996), but they also regulate angiogenesis and neovascularization in the retina (Bai et al., 2009; Eichler et al., 2004b; Stone et al., 1995). Regeneration of injured RGC is supported by Müller-cell derived neurotrophic/-protective factors (Hauk et al., 2008), among them are most likely VEGF (Eichler et al., 2004b; Foxton et al., 2013; Kilic et al., 2006), CNTF (Wen et al., 2012), IGF-1 (Benarroch, 2012) and PEDF (Lange et al., 2008; Unterlauft et al., 2013). Selective ablation of Müller cells was shown to compromise the blood-retinal barrier and leads to retinal neovascularization and photoreceptor apoptosis (Shen et al., 2012).

In spite of the obvious and numerous physiological functions of retinal glial (Müller) cells, their particular impact in various pathological conditions has not sufficiently been clarified. There is mounting evidence that Müller cells play a key role in diabetic retinopathy, demonstrating reduced glutamate uptake and modulating aberrant blood vessel formation through secretion of angiogenesis-related mediators (Barber et al., 2011). The present study provides further evidence for neuroprotective actions of Müller-cell derived PEDF in the retina. We have also addressed intracellular signaling molecules that may operate in RGC under conditions in which PEDF of glial origin exerts its pro-survival effects on RGC.

2. Materials and methods

2.1. Materials

The antibodies used during this study are summarized in Table 1. If applicable, appropriate normal control IgG from rabbit or mouse serum was included. SN50 and SN50M were from Merck Millipore, all other reagents were purchased from Sigma–Aldrich (Taufkirchen, Germany) unless stated otherwise.

2.2. Immunoisolation and culture of mouse RGC

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the German Law on Protection of Animals. Primary RGC were isolated from postnatal mouse retinae as described previously (Unterlauft et al., 2013). Briefly, seven days old mice were sacrificed and retinae were dissected and incubated for 45 min in Dulbecco's phosphate buffered saline (D-PBS) supplemented with 160 U/ml papain and 200 U/ml DNase. The retinal tissue was then sequentially triturated in D-PBS containing 0.2% bovine serum albumin (BSA, fraction V) and 650 U/ml DNase. Cells were pelleted, resuspended in D-PBS/0.2% BSA, passed through a 20-μm nylon mesh and added to immunopanning plates.

Immunopanning was performed using two subtraction plates coated either with goat anti-rabbit IgG or anti-mouse IgM/anti-Thy1.2 to remove anti-macrophage antibody-targeted microglial cells followed by positive selection of RGC. Purified RGC were then plated on poly-D-lysine (MW 40 kDa; 5 μg/ml)-coated glass coverslips (10 mm in diameter) at 600 cells/mm^2 and cultured for seven days (37 °C, 5% CO_2, 95% humidity) in Neurobasal medium/0.01% BSA containing 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate, 2 mM glutamine, 60 μg/ml *N*-acetyl-L-cysteine, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 100 μg/ml BSA, 40 ng/ml triiodothyronine, 100 μg/ml holotransferrin, 250 μM dibutyryl cyclic AMP, 5 μg/ml insulin, 62 ng/ml progesterone, B-27 (1:50), 50 μM D-mannose, and 10 μM forskolin. Neurobasal medium was

Table 1
Targets, concentrations and source of applied primary antibodies.

Target molecule	Target cell	Clone	Concentration/dilution	Manufacturer
Thy 1.2	RGC	F7D5	30 μg/ml	Serotec, Düsseldorf, Germany
not specified	Macrophages	Polyclonal	1:80	Accurate Chemical & Scientific Corp., Westbury, NY
NF-κB p65/NLS[a]	RGC	12H11	1 μg/ml	Merck Millipore, Darmstadt, Germany
NF-κB p65/phospho-serine 536	RGC	polyclonal	1 μg/ml	Signalway Antibody LLC, College Park, MD
Caspase-3[b]	RGC	5A1E	1:200	Cell Signaling Technology, Inc., Danvers, MA
Neurofilament H	RGC	SMI-32	1:1000	Covance, Emeryville, CA
GFAP	Glial cells	G-A-5	5 μg/ml	Sigma–Aldrich, Taufkirchen, Germany
PEDF receptor/PNPLA2 (TSS 2.2)	RGC	polyclonal	10 μg/ml	R&D Systems, Wiesbaden, Germany

[a] Nuclear localization sequence.
[b] Large fragment of activated caspase 3 resulting from cleavage adjacent to asparagine 175.