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Research article

Müller glia activation by VEGF-antagonizing drugs: An *in vitro* study on rat primary retinal cultures

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ABSTRACT

The effects of the anti-Vascular Endothelial Growth Factor (VEGF) drugs ranibizumab and aflibercept were studied in Müller glia in primary mixed cultures from rat neonatal retina. Treatment with both agents induced activation of Müller glia, demonstrated by increased levels of Glial Fibrillary Acidic Protein. In addition, phosphorylated Extracellular-Regulated Kinase 1/2 (ERK 1/2) showed enhanced immunoreactivity in activated Müller glia. Treatment with aflibercept induced an increase in K^+ channel (Kir) 4.1 levels and both drugs upregulated Aquaporin 4 (AQP4) in activated Müller glia. The results show that VEGF-antagonizing drugs influence the homeostasis of Müller cells in primary retinal cultures, inducing an activated phenotype. Upregulation of Kir4.1 and AQP4 suggests that Müller glia activation following anti-VEGF drugs may not depict a detrimental gliotic reaction. Indeed, it could represent one of the mechanisms able to contribute to the therapeutic effects of these drugs, particularly in the presence of macular edema.

1. Introduction

Vascular Endothelial Growth Factor (VEGF) plays a pivotal role in increasing retinal blood vessel growth and inducing macular edema. For this reason, VEGF-counteracting drugs that inhibit VEGF activity have been increasingly used in the therapy of Age-related Macular Degeneration (AMD), to counteract choroidal vascularization, and Diabetic Retinopathy (DR), to contrast Diabetic Macular Edema (DME). These innovative therapeutic agents have drastically reduced disease progression and sensibly increased visual acuity in AMD patients, an outcome considered unreachable just a few years ago, and had statistically superior efficacy to laser in DME (Regnier et al., 2014).

In the last decade, several drugs have been developed to inhibit ocular pathologic neovascularization by blocking VEGF. Among them, ranibizumab (Lucentis®), a high-affinity Fab fragment of a monoclonal antibody that neutralizes all forms of VEGF-A, is the most commonly used. It is approved for the 'wet' form of AMD, DME, macular edema following central retinal vein occlusion and choroidal neovascularization in pathologic myopia. More recently, aflibercept (Eylea®), a recombinant fusion protein consisting of portions of human VEGF receptors 1 and 2 extracellular domains fused to the Fc portion of human IgG1, has been registered for AMD, DME and macular edema following central retinal vein occlusion. Recently, results from an exploratory study have shown that ranibizumab reduced the risk of DR progression in patients with DME, suggesting that counteracting VEGF may exert beneficial effects on the development of DR (Ip et al., 2012).

Several studies have been conducted to investigate the effects of anti-VEGF drugs in retinal cells, both *in vitro* and *in vivo*. Most studies have focused on the effects on endothelial cells, analyzing the influence on cell proliferation, differentiation and apoptosis (Costa et al., 2009; Deissler et al., 2008) or to retinal ganglion cells, to evidence possible neurotoxic effects (Schnichels et al., 2013; Thaler et al., 2010). However, only a few studies have analyzed

Abbreviations: VEGF, Vascular Endothelial Growth Factor; GFAP, Glial Fibrillary Acidic Protein; ERK 1/2, Extracellular-Regulated Kinase 1/2; AQP4, Aquaporin 4; Kir4.1, K^+ channel 4.1; AMD, Age-related Macular Degeneration; DR, Diabetic Retinopathy; DME, Diabetic Macular Edema; DIV, day-*in vitro*.

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the influence of anti-VEGF drugs on retinal glial cells, which have a pivotal role in the maintenance of retinal homeostasis and could be modulated by a growth factor, such as VEGF, that has a pleiotropic role in nervous tissue (Guo et al., 2010; Kaempf et al., 2008).

Here, we analyze the effects of ranibizumab and aflibercept, on Müller glia in mixed primary cultures of neonatal rat retina.

2. Material and methods

2.1. Primary retinal cultures

Primary retinal cultures were obtained from newborn Wistar rat at day 1 (partially modified from Malchiodi-Albedi et al., 1998 and Matteucci et al., 2011). Briefly, after dissociation in trypsin, retinal cells were seeded onto poly-L-lysine-coated cell culture plates for Western Blot (WB) analysis or glass coverslips for immunocytochemistry, and grown in Minimum Essential Medium with 10% FCS (Life Technologies, Monza, Italy), giving rise to a mixed glial–neuronal cell population.

2.2. In vitro treatment

At day *in vitro* (DIV) 5, retinal cultures were exposed to clinically relevant concentrations of ranibizumab (0.125 mg/ml) or aflibercept (0.25 mg/ml) for 2, 4 or 6 days (Schnichels et al., 2013; Klettner and Roider, 2008). In order to keep to the extent possible constant the levels of anti-VEGF drugs during the treatment, at DIV 7 and 9, half of the cell culture medium was changed with fresh medium containing or not ranibizumab or aflibercept.

2.3. Immunofluorescence

Primary retinal cultures grown on glass coverslips were fixed for 25 min in 4% paraformaldehyde in PBS, 0.12 M sucrose, and immunolabeled with the following primary antibodies: polyclonal anti-Glial Fibrillary Acidic Protein (GFAP) 1:500 in PBS, monoclonal anti-Rhodopsin (Rhod) 1:100 in PBS, polyclonal anti-Microtubule Associated Protein 2 (MAP2) 1:500 in PBS and polyclonal anti-K^+ channel 4.1 (Kir4.1) 1:100 in PBS from Millipore (Billerica, MA); monoclonal anti-Aquaporin4 (AQP4) 1:100 in PBS and monoclonal anti-cellular retinaldehyde-binding protein (CRALBP) 1:100 in PBS from Santa Cruz Biotechnology (Dallas, TX); monoclonal anti-vimentin (Vim) 1:500 in PBS and polyclonal anti-γ-aminobutyric acid (GABA) 1:1000 in PBS from Sigma–Aldrich (St.Louis, Mo); polyclonal anti-pERK1/2 1:100 in PBS from Cell Signaling Technology (Danvers, MA). Retinal cultures were counterstained with Hoechst 33258 and observed at an Eclipse 80i Nikon Fluorescence Microscope (Nikon Instruments, Amsterdam, Netherlands), equipped with a VideoConfocal (ViCo) system.

2.4. Electrophoresis and western blot analysis

Retinal cultures grown on plastic plates were washed with ice-cold PBS, then ice-cold freshly prepared RIPA buffer was added to the well and cells were dislodged using a sterile, disposable cell scraper. Lysates were placed on ice for 30 min, then centrifuged at 16,000 g for 15 min at 4 °C, and supernatant fractions were stored at −80 °C. Samples were dissolved in 4x Laemmli sample buffer and boiled for 5 min. The protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins (30 μg) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes at 35 V overnight. The membranes were blocked at room temperature in 3% BSA and incubated overnight at 4 °C with the following primary antibodies: polyclonal anti-GFAP from Chemicon (Temecula, CA), polyclonal anti-Kir4.1 from Millipore, monoclonal anti-AQP4 from Santa Cruz Biotechnology, polyclonal anti-pERK1/2 from Cell Signaling Technology, and monoclonal anti-β actin from Calbiochem – Oncogene Research Products (Cambridge, MA). The membranes were washed and incubated with the appropriate peroxidase-labeled secondary antibody (Bio-Rad, Hercules, CA) for 1 h at room temperature. After extensive washes, the immunoreactive bands were detected by chemiluminescence coupled to peroxidase activity (ECL kit, Thermo Scientific, Rockford, IL) and imaged with a ChemiDoc XRS system.

3. Results

3.1. Characterization of rat neonatal retinal cells in culture

In our culturing conditions, rat neonatal retinal cells grow as a mixed cell population, composed of both glial and neuronal cells (Fig. 1). They form cords composed of Müller glial cells immunostained for CRALBP (Fig. 1C) and Vim (Fig. 1D first panel), and a heterogeneous population of neurons composed from larger neurons, with a rich neuritic tree positive for the MAP2 (Fig. 1A, red) and the inhibitory neurotransmitter GABA (Fig. 1B) antibodies, which identifies a subpopulation of retinal ganglion cells, amacrine cells and horizontal cells; photoreceptors are also present, they are labeled for rhodopsin and form rosette-like aggregations (Fig. 1A, green).

Exposure to both anti-VEGF drugs did not induce modifications in primary retinal culture composition and morphology (data not shown).

3.2. Both ranibizumab and aflibercept upregulates GFAP in Müller glia in vitro

To better characterize the effects of both agents on Müller glia, first of all we analyzed the expression of GFAP, a marker of macroglial activation. The retinal cultures were exposed to ranibizumab (0.125 mg/ml) or aflibercept (0.25 mg/ml) for 2, 4 or 6 days. Western blot analysis showed that treatment with ranibizumab for 4 and 6 days and with aflibercept for 2 and 4 days induced upregulation of GFAP, reaching significant difference with control cultures (Fig. 2). Immunocytochemical analysis (Fig. 3A) confirms that the treatment with both ranibizumab and aflibercept (4 days) induced Müller glia activation, as shown by increased positivity for GFAP, while control cultures were nearly negative.

3.3. Effects of treatment with ranibizumab and aflibercept on ERK1/2 activation

In *in vivo* (Fischer et al., 2009; Nakazawa et al., 2008) and *in vitro* (Matteucci et al., 2014) models of retinal neurotoxicity, ERK1/2 activation has been shown to trigger neuroprotective pathways in activated Müller glia. To analyze if ERK1/2 pathway was involved in anti-VEGF-drug-induced Müller cell activation, primary retinal cultures were immunolabelled for phosphorylated ERK1/2 (Fig. 3B). In comparison to control cultures, Müller glia that had been exposed to ranibizumab or aflibercept for 96 h exhibited a stronger immunoreactivity for pERK1/2, with respect to control cultures (Fig. 3B). However, WB analysis only showed a trend towards increased pERK1/2 level, without reaching significant difference (Fig. 3C,D).

3.4. Effects of treatment with ranibizumab and aflibercept on Kir4.1 and AQP4

In gliosis, key factors in the physiology of Müller glia, such as inward rectifying Kir4.1 and AQP4 are downregulated or change