Experimental Eye Research 160 (2017) 1–10

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Cellular stress response in human Müller cells (MIO-M1) after bevacizumab treatment

Monique Matsuda, MSc, PhD [a], Paloma Gava Krempel, PhD [a], Mônica Valeria Marquezini, MSc, PhD [b], Alfred Sholl-Franco, MSc, PhD [c], Amanda Lameu [d], Mário Luiz R. Monteiro, MD, PhD [a, *, 1], Nádia Campos de Oliveira Miguel, MSc, PhD [e, 1]

[a] Laboratory of Investigation in Ophthalmology (LIM-33), Division of Ophthalmology, University of São Paulo Medical School, Brazil
[b] Laboratory of Experimental Air Pollution, University of São Paulo Medical School & Pro-Sangue Foundation, São Paulo, Brazil
[c] Laboratório de Neurogênese, Programa de Neurobiologia, Instituto de Biofísica Carlos Chagas Filho, UFRJ, Rio de Janeiro, Brazil
[d] Pharmacy College, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil
[e] Program of Cell and Developmental Biology, Institute of Biomedical Sciences, Federal University of Rio de Janeiro (UFRJ), Brazil

ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form
30 March 2017
Accepted in revised form 14 April 2017
Available online 15 April 2017

Keywords:
Bevacizumab
Anti-VEGF
Müller cells
Retina
Caspase-3
Beclin-1
GFAP
Vimentin

ABSTRACT

Bevacizumab, an anti-vascular endothelial growth factor (VEGF) agent, is widely used in the treatment of retinal vascular diseases. However, due to the essential role Müller cell derived-VEGF plays in the maintenance of retinal neurons and glial cells, cell viability is likely to be affected by VEGF inhibition. We therefore evaluated the effect of bevacizumab-induced VEGF inhibition on Müller cells (MIO-M1) *in vitro*. MIO-M1 cells were cultured for 12 or 24 h in media containing bevacizumab at 0.25 or 0.5 mg/mL. Controls were cultured in medium only. Cell viability was determined with the trypan blue exclusion test and MTT assay. Caspase-3, beclin-1, glial fibrillary acidic protein (GFAP) and vimentin content were quantified by immunohistochemistry. Gene expression was evaluated by real-time quantitative PCR. Treatment with bevacizumab did not reduce MIO-M1 cell viability, but increased metabolic activity at 24 h (0.5 mg/mL) and induced apoptosis and autophagy, as shown by the increased caspase-3 levels at 12 h (0.25 and 0.5 mg/mL) and the increased beclin levels at 24 h (0.5 mg/mL). Caspase-3 mRNA was upregulated at 12 h and downregulated at 24 h in cells treated with bevacizumab at 0.25 mg/mL. Bevacizumab treatment was also associated with structural protein abnormalities, with decreased GFAP and vimentin content and upregulated GFAP and vimentin mRNA expression. Although bevacizumab did not significantly affect MIO-M1 cell viability, it led to metabolic and molecular changes (apoptosis, autophagy and structural abnormalities) suggestive of significant cellular toxicity.

1. Introduction

Over the past few years, several authors have evaluated the ability of intravitreally administered antiangiogenic drugs to inhibit neovascularization in vascular retinopathies in adults and newborns (Gunther and Altaweel, 2009; Miller, 2016). One such drug, bevacizumab, commercially known as Avastin (Genentech, Inc., San Francisco, California, USA), is currently used to prevent angiogenesis, mainly through the inhibition of all vascular endothelial growth factor (VEGF) isoforms, and has proven to be highly efficacious in the treatment of patients with proliferative diabetic retinopathy, neovascular age-related macular degeneration, central retinal vein occlusion, idiopathic choroidal neovascularization, and retinopathy of prematurity (Spaide and Fisher, 2006; Gunther and Altaweel, 2009; Russo et al., 2009; Wu et al., 2011).

VEGF is an essential molecule in the maintenance of retinal neurons and glial cells (Saint-Geniez et al., 2008). VEGF inhibition results in failure of choroidal development and visual function loss during vascular development, influencing vessel remodeling, stabilization, and differentiation of endothelial cells (Saint-Geniez et al., 2006). Furthermore, VEGF exerts a critical role in neurogenesis, with significant neurotrophic, neuroprotective, and instructive functions in the nervous system (Jin et al., 2000), including the retina (Saint-Geniez et al., 2008).

* Corresponding author. Av. Angélica 1757 conj. 61, 01227-200, São Paulo, Brazil.
E-mail address: mlrmonteiro@terra.com.br (M.L.R. Monteiro).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.exer.2017.04.005
0014-4835/

The concern that bevacizumab might be harmful to the retina has led researchers to evaluate its possible side effects through cell viability assays and the identification of molecules involved in cell death, proliferation and cell reactivity. While several experimental studies have failed to demonstrate any retinal toxicity from intravitreal injections of bevacizumab (Bakri et al., 2006; Feiner et al., 2006; Manzano et al., 2006; Zayit-Soudry et al., 2011), others have described ultrastructural abnormalities, including increased apoptotic activity (Saint-Geniez et al., 2008), increased glial cell reactivity (Fusco et al., 2012), and changes in retinal function (Myers et al.). In addition, newborn rat retinal explants exposed to bevacizumab have been shown to undergo molecular changes such as increased vimentin content, decreased GFAP (Miguel et al., 2012), and decreased neurocan mRNA levels (Krempel et al., 2014), potentially interfering with neuron and glial cell maturation early in the retinal development.

The importance of studying the effect of bevacizumab on isolated glial cells lies in the fact that Müller cells provide nutritional support and protection for neurons by producing neurotrophic factors, growth factors (including VEGF) and cytokines (Wang et al., 2015; Saint-Geniez et al., 2008). Müller cells convert substrates, regulating the blood-retinal barrier and the synaptic communication in the central nervous system (Bringmann et al., 2006). It is also important to further investigate the role of bevacizumab on retinal fibrosis, a finding that has been reported in patients with retinal diseases treated with repeated bevacizumab injections (Batman and Ozdamar 2010). Fibrosis has been considered a significant side effect of bevacizumab and can contribute to scar formation and the development of tractional retinal lesions leading to visual loss (Arevalo et al., 2008; Batman and Ozdamar 2010, Zhang et al., 2015). While in many patients with vascular retinal diseases repeated injections of anti-VEGF are efficient at preserving vision in some retinal fibrosis may be a significant complication leading to permanent visual loss. (van der Reis et al., 2011; Zhang et al., 2016). Reactive gliosis is produced by retina-specific Müller-glial cells and astrocytes (Bringmann et al., 2006) However, to our knowledge, no other study has evaluated the effect of anti-VEGF agents on isolated human Müller cells. Since Müller cells may have a significant role in fibrosis and are one of the main cell types producing VEGF (Wang et al., 2015), the purpose of the present study was to evaluate the cytotoxic effect, if any, of bevacizumab on human Müller cell *in vitro*.

2. Materials and methods

2.1. Materials

Dulbecco Modified Eagle Medium (DMEM), penicillin-streptomycin, TRIzol® and SuperScript Vilo cDNA Synthesis Kit were obtained from Invitrogen (Life Technologies, Carlsbad, CA). Bevacizumab was purchased from Genentech, Inc. (Avastin®). MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and dimethyl sulfoxide (DMSO), HEPES, sodium pyruvate and sodium bicarbonate were obtained from Sigma-Aldrich Quimica SA (Sintra, Portugal). RNeasy Mini Kit and Rotor Gene SYBR Green PCR Kit were purchased from Qiagen (São Paulo, Brazil). Primary antibodies against caspase-3 (Anti-active + pro-Caspase 3 antibody ab1384) and beclin-1 (rabbit anti-beclin 1 polyclonal antibody ab15417) were purchased from Abcam Plc. (Cambridge, UK). Primary antibodies against GFAP (anti-GFAP: C-19 clone) and vimentin (anti-vimentin C-20 clone) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Goat secondary antibody to rabbit IgG - H&L (DyLight® 488) (ab96899) and blue DAPI stain for nuclear labeling were obtained from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade.

2.2. Cell culture

Human Müller cells (MIO-M1) (Limb et al., 2002), courtesy of Dr. G. A. Limb, Moorsfield Institute of Ophthalmology, University of London, UK, were cultured in media containing DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin-streptomycin, 1 mM sodium pyruvate, 44 mM sodium bicarbonate and 25 mM HEPES. Cell viability and gene expression were determined by culturing 5×10^3 and 4×10^4 cells/well on plates (TPP, Switzerland) with a growth area of 0.33 cm^2 and 3.8 cm^2, respectively. For immunocytochemistry, MIO-M1 cells were seeded on chamber slides with a growth area of 0.7 cm^2 (Nalge Nunc International, Rochester, NY, USA) and maintained for 48 h before bevacizumab treatment. The cells were then exposed for 12 or 24 h to bevacizumab diluted in culture media at two concentrations (0.25 mg/mL and 0.5 mg/mL), corresponding to sub- and supra-clinical doses. The concentrations were estimated by dividing the dose of bevacizumab most commonly used in the treatment of retinal disease in humans (1.5–2.5 mg per eye) by the volume of the eye. In preliminary tests, the concentration of 0.5 mg/mL was found to be adequate. Based on this rationale we decided to use both 0.25 and 0.5 mg/mL concentrations in the current study.

2.3. Cell viability assays

2.3.1. Trypan blue dye exclusion test

To determine the number of dead (blue staining) and live (clear cytoplasm) cells, MIO-M1 cells were transferred to a 96-well plate for adherence during 48 h. Then bevacizumab at 0.25 or 0.5 mg/mL was added in the experimental groups and not in controls, followed by incubation for 12 or 24 h. The cells were subsequently detached from the plate surface using a 0.25% trypsin-EDTA solution (Sigma-Aldrich) for 3 min at 37 °C. Fetal bovine serum (v:v) (Gibco by Life Technologies) was added to inhibit trypsin activity. The supernatant was centrifuged and the pellet was diluted in fresh media. A 10-μL aliquot of MIO-M1 cell suspension was diluted in 0.4% trypan blue stain (v:v) (Gibco by Life Technologies) and counted in a Neubauer chamber. The percentage of viable cells was calculated by dividing the number of live cells by the total number of cells, multiplied by 100. Three replicates were performed for each condition.

2.3.2. MTT assay

After 48 h of adhesion in 96-well plates, the MIO-M1 cells were cultured in the presence of bevacizumab for 12 or 24 h. A 5% MTT solution diluted in PBS was added to the medium in the last 4 h of culture, at 37 °C. After incubation, the cells were immersed in DMSO for 30 min, protected from light. The optical density was measured at 540 nm wavelength (Spectra Max 190, Molecular Devices; San Francisco, CA) and cell viability was expressed as the percentage of cells relative to the average number of untreated cells. Three replicates were performed for bevacizumab-treated and untreated cells.

2.4. Immunofluorescence

MIO-M1 cells were seeded on chamber slides for confocal analysis (Nalge Nunc International, Rochester, NY) until attaining 80–90% confluency (48 h), followed by the addition of bevacizumab at the times and doses described above. After incubation, the slides were fixed with 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 10 min at room temperature, followed by washing in 0.01 M PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and treatment for 60 min with a blocking solution (10% bovine serum albumin) diluted in PBS (BSA-