Research Report

Effects of bevacizumab on neuronal viability of retinal ganglion cells in rats

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ABSTRACT

The aim of this study was to investigate the effects of single and repeated intravitreal injections of bevacizumab on various retinal layers focusing more on retinal ganglion cells (RGCs) in healthy rats. Male Wistar rats were treated with intravitreal injection of bevacizumab (4 μL) within right eye. Left eyes were injected with the same volume of balanced salt solution (BSS) and used as control. Ten rats received a single intravitreal injection and ten rats had three injections, with seven days time interval. Histological and immunohistochemical evaluations and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay were performed in order to find out if some degree of apoptosis could occur on RGCs. Histological and immunohistochemical analyses showed that bevacizumab induces neuronal loss compared to control eyes, after multiple injections. RGCs apoptosis after multiple treatments was demonstrated to occur by TUNEL, Annexin V and Bax assays. The loss of ganglion cells following repeated injections was confirmed and quantified by the decrease in RGC specific protein Brn3a measured by western blotting in ten additional rats. The present results need to be considered when multiple intravitreal injection of bevacizumab are performed to treat retinal diseases.

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Abbreviations: RGC, retinal ganglion cell; BSS, balanced salt solution; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; VEGF, vascular endothelial growth factor; AMD, age-related macular degeneration

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1. Introduction

Antagonists of vascular endothelial growth factor (VEGF) are new pharmacological agents for the treatment of several retina disorders such as age-related macular degeneration (AMD). The VEGF is an endothelial cell-specific angiogenic as well as a vasoparmerse factor which is involved in the choroidal neovascularization potentially leading to the formation of edema and reduction of visual acuity. The pharmacotherapy with intravitreal injections of the anti-VEGF antibody bevacizumab (Avastin®; Roche, Welwyn Garden City, UK) and ranibizumab (Lucentis®; Novartis, Horsham, UK) represents the first line therapy in AMD (Meyer and Holz, 2011). Ranibizumab is a Fab of the same antibody, with a half-life shorter than full length antibodies and it was developed for intraocular administration. Bevacizumab is a recombinant humanized monoclonal immunoglobulin G1 antibody, which was originally approved by the Food and Drug Administration for the use in patients with metastatic colorectal cancer. At present, bevacizumab is worldwide administered intravitreally off-label in a variety of retinal disorders such as AMD. Both ranibizumab and bevacizumab bind to all isoforms of human VEGF-A and inhibit its biological activity (Ferrara et al., 2004; Ferrara et al., 2006). Therapeutic doses of bevacizumab and ranibizumab are equally potent to neutralize VEGF (Meyer and Holz, 2011). Bevacizumab does not appear to be toxic in vitro and it does not modify cell viability in rat retinal ganglion cells (RGC) (Iríyama et al., 2007), rat adrenal medulla-derived PC12 cells (Cheng et al., 2009), human retinal pigment epithelial (Brar et al., 2009), RGC-5 cell lines (Brar et al., 2010) and corneal keratinocyte and endothelial cells (Yøerruek et al., 2007).

Several studies demonstrate the efficacy of anti-VEGF in the treatment of different retinal disorders (Blessler (2009); Kubota-Taniai et al., 2011; Iqbal et al., 2011). However, only a few studies aimed to analyze the potential toxicity of anti-VEGF agents on retinal cells. Most reports fail to describe toxicity on retinal cells at therapeutic concentrations (Lu and Adelman, 2009; Iandiev et al., 2011). Indeed, adverse reactions with anti-VEGF therapy have been reported in other tissues (Chan and Lin, 2007; Miura et al., 2010; Shima et al., 2008). For instance high concentrations of bevacizumab may produce a damage to human trabecular meshwork in primary cell cultures (Kahook and Ammar, 2010), and anti-VEGF agents decrease porcine retinal pigment epithelial cell barrier function (Miura et al., 2010). In line with this, bevacizumab may impede the protective effects of VEGF against oxidative stress in differentiated RGC-5 cell cultures (Brar et al., 2010). In addition, emerging studies show increased intraocular pressure, intraocular inflammation and systemic toxicity following intravitreal administration of anti-VEGF (Adelman et al., 2010; Pellé et al., 2011; Tseng et al., 2012). This evidence call for a more in-depth studies to elucidate potential detrimental effects of anti-VEGF agents in retinal cells by using specific markers such as Brn3a, neurofilament 200 and tubulin beta III-isoform. In fact, each of these proteins provide specific information; for instance, Brn3a is a protein expressed in the RGC nuclei which allows to assess the amount of cell loss (Nadal-Nicolás et al., 2009). In contrast, the neurofilament 200, which is expressed in the cell body and axon of RGCs, can be used for qualitative analysis of cellular degeneration (Petzold et al., 2009).

In the present study, we investigated the effects of single or repeated intravitreal administrations of bevacizumab on various retinal layers of health rats focusing more on retinal ganglion cells. In detail, we measured the occurrence of retinal cell death and the involvement of apoptotic proteins.

2. Results

2.1. Histological evaluation

Following multiple injections, we consistently found the occurrence of bevacizumab-induced retinal damage, mostly within retinal ganglion cell layer. In particular, a single treatment with intravitreal bevacizumab produced a slight, not significant decrease in cell number compared with vehicle (Fig. 2). In contrast, multiple treatment with three intravitreal injections of bevacizumab causes severe RGC loss and a marked damage of inner and outer nuclear layer (Fig. 2). The severe retinal damage within RGC layer was homogeneous along a medio-lateral extent of 2.5 mm from the injection site. It is worth to be mentioned that vehicle alone when repeatedly injected induced a slight injury compared to single administration and non-injected retina (see also supporting Fig. S1).

Fig. 1 – Representative photo of the intravitreal injection performed on rats. The intravitreal administration was carried out by the Hamilton microlsyringe (25 μL) with 30 gauge needle by injecting 4 μL of bevacizumab or vehicle (BSS). The rats were sacrificed 48 h after the last injection.
The TUNEL staining was virtually absent in controls and increased only slightly following a single treatment with bevacizumab. In contrast, repeated bevacizumab administration produced an intense TUNEL staining which is consistent with robust apoptosis within RGCs. This extended also to the inner and outer nuclear layers (Fig. 3). Only a few TUNEL-positive cells were observed in the ganglion cells after repeated treatment with BSS (Fig. 3).

2.2. Western blot analysis

Western blot for the specific RGC protein Brn3a (Nadal-Nicolás et al., 2009) confirms what detailed by morphological analysis. In particular we could not observe any decrease of such a protein following single injection while a dramatic decrease of this RGC hallmark protein was quantified after multiple bevacizumab administration (Fig. 4).

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2.3. Immunohistochemical analysis

In addition to the protein Brn3a also beta-III tubulin and neurofilament 200 are expressed in RGCs being commonly used as markers for ganglion cells. Immunohistochemistry for anti-beta-III tubulin antibody further confirms that repeated treatment with bevacizumab produces significant RGCs loss compared with multiple BSS and single bevacizumab administration (\( p < 0.01 \), \( p < 0.001 \)) (Fig. 5). In line with this, repeated intravitreal administration of bevacizumab increases the expression of neurofilament 200 (a protein involved in neuronal derangement) within the RGC layer (Fig. 6).

Immunohistochemistry for Annexin V shows intense labeling within RGC, inner and outer nuclear layers following repeated bevacizumab administration. This is consistent with occurrence of robust apoptosis (arrows in Fig. 7). It is worth of note that single bevacizumab and multiple BSS administrations produce only a few Annexin V positive ganglion cells (Fig. 7, arrows).

Remarkably, when we used antibodies against Bax which is a reliable of apoptosis in the visual system we obtained comparable data thus adding further evidence on the occurrence of apoptosis (Fig. 8, arrows).

3. Discussion

Intravitreal bevacizumab is a common procedure used worldwide to treat retinal diseases, such as AMD and choroidal neovascularization. Clinical ocular side effects following this procedure, such as inflammation/uveitis, retinal tears/detachment, intraocular pressure elevation, ocular bleeding, ocular ischemia, are well known and widely reported in literature (Tolentino, 2011). Although, the retinal response to intravitreal bevacizumab injections remains unknown, a small sample of nine patients receiving intravitreal bevacizumab did not evidence retinographic alterations at 1 month post-operatively (Maturi et al., 2006). This result was confirmed by another study with a similar design but the follow up lasted 4 months (Ziemssen et al., 2008). These functional studies lack a morphological counterpart; in fact there is a lack of studies analyzing the retinal morphology following
multiple bevacizumab administrations, which would be useful to detect even slight alterations.

Therefore in the present study, we focused on morphological alterations following intravitreal bevacizumab in an animal model administered single and multiple bevacizumab administrations.

Our data indicate that retinal damage does occur after repeated injections. This consists of frank cell loss concomitant with occurrence of apoptotic cells and derangement of retinal cell layers. These phenomena are severe following repeated bevacizumab administration but do not occur following single injections. A slight damage is induced by the mechanical procedure which can be mimicked by multiple BSS injections. However, this mechanical damage is way lower than the damage observed following multiple administration of bevacizumab solutions. In detail, the mechanical procedure consisting of injecting a total volume of 4 μL at a flow rate of 1 μL/min per se might produce deleterious effects due to increased pressure on retinal cells as witnessed by comparisons between single and multiple vehicle injections (Supporting Information). Again, these non-specific likely mere mechanical effects, never reach the severity of the damage observed in bevacizumab-injected retina which is way more severe in all the analyses we carried out.

![Fig. 6](image1.png) Retinal cells were detected by using a neuronal marker. Anti-neurofilament 200 antibody evidences a decrease of immunoreactivity in the single treatment of bevacizumab compared to vehicle. In the repeated administrations of bevacizumab was evident an attempt of nerve restore but not visible after repeated injections with vehicle. Scale bar 100 μm. Neurofilament 200 immunostaining is shown at higher magnification in the insert (scale bar 10 μm).

![Fig. 7](image2.png) Representative immunostaining of apoptotic cell by using Annexin V antibody. Anti-Annexin V staining shows a marked immunoreactivity (arrows) in the eye treated with single and multiple bevacizumab; the repeated administration of vehicle produced a mild positive staining not evident after a single treatment. Scale bar 100 μm.
In fact, cell loss occurring after vehicle injection is not accompanied by apoptosis which characterizes instead bevacizumab-induced cell death. These latter findings are consistent along various experimental procedures such as TUNEL, Annexin V and Bax, which converge to witness for apoptotic cell death. In particular, Bax protein appears a good marker to stain apoptosis in the retina since Bax is up-regulated following optic nerve transection and Bax expression occurs early during retinal ganglion cell (RGC) apoptosis (Goldenberg-Cohen et al., 2011). The amount of cell loss induced selectively by bevacizumab is substantiated by quantitative densitometry of western blotting for a RGC-specific marker, Brn3a (Nadal-Nicolas et al., 2009). Moreover, our results show a bevacizumab-induced increase of neurofilament 200 expression after repeated injections. This is in line with a loss of RGCs since those heavy neurofilaments as well as the Brn3a protein are considered as markers for RGC degeneration (Parrilla-Reverter et al., 2009; Petzold et al., 2009).

All retinal layers are deranged after bevacizumab treatment, even though ganglion cell layer is mostly affected. For instance, severe apoptotic damage in the inner and outer nuclear layers after multiple injections of bevacizumab occurs. Therefore, the present study indicates that bevacizumab may potentially induce cell toxicity for retinal cells. In order to produce such a detrimental effect bevacizumab needs to be administered chronically following a pattern which reproduces what happens in patients. When we administered a single dose of bevacizumab (0.025 mg/μL) we could not find any noticeable cell loss. Indeed, this confirms what reported in previous studies administering bevacizumab in single injection at doses ranging between 0.025–0.0375 mg/μL (Iriyama et al., 2007; Thaler et al., 2010; Iandiev et al., 2011). In other words, the present study was the first to analyze the effects of repeated bevacizumab injections (following the same temporal pattern used in patients). Therefore, it is likely that the key point for the lack (Iriyama et al., 2007; Iandiev et al., 2011) or the occurrence of bevacizumab toxicity is represented by single (Iriyama et al., 2007; Iandiev et al., 2011) compared with multiple (present study) drug injections.

One might argue that an inter-species comparison based merely on the adjustment of dose and on the volume of vitreous is rather simplistic. Even considering this point at present we could not find any better adjustment even considering the size of the molecular target (the retinal cells). At the same time, rodents are known to be fast drug metabolizers compared with humans who would render our data underestimated.

Again, in human patients multiple bevacizumab administrations are protracted over much longer periods (months) which may lead to higher cumulative doses. Several studies exploring the potential toxicity of bevacizumab were carried out in vitro by using cultured retinas (Lüke et al., 2007) or cultured ocular cell lines (Spitzer et al., 2006), such as PC12 cells (Cheng et al., 2009), which are not supposed to depend on blood supply for their survival. If one considers the complexity of the impairment of blood vessels formation underlying retinal damage, the in vitro model is inadequate to investigate these effects. Moreover, in dealing with cell cultures it is critical to choose an appropriate cell line. In fact PC12 cells appear to be inapt to mimic retinal cells and they possess high aberrancy (Fornai et al., 2007). The toxicity in the rat retina involves human beings much more than safety in PC12 cells.

We have to admit that disclosure of potential toxicity in rats does not necessarily imply analogous risks in humans but certainly it requires an in depth investigation to rule out the occurrence of similar effects in human patients.

In summary, our results evidence that intravitreal repeated injection of bevacizumab in health rats provide a severe toxicity to several retinal layers. Although some clinical trials demonstrate that bevacizumab is beneficial for the treatment of AMD without severe adverse effects, the long term safety needs to be firmly established.

4. Experimental procedures

4.1. Animals

Twenty adult male Wistar rats (Harlan, S. Pietro al Natisone (UD) Italy), weighting 350–400 g, were recruited. The rats were...
housed for one week in paired cage, at light/darkness cycles of 12 h and with free access to food and beverage. Experiments were performed in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with the Italian law on animal care no. 116/1992 and the EEC/609/86. All efforts were made to reduce the number of animals used.

4.2. Experimental procedure

Before intravitreal injection all the rats underwent general anesthesia, by intraperitoneal injection of ketamine (40 mg/Kg) and xylazine (8 mg/Kg). Eyes were administered according to four groups: group 1 received intravitreal single-dose of bevaczumab; group 2 receive a single injection of vehicle; group 3 receive a repeated administration of bevaczumab; group 4 received repeated injection of vehicle following the same pattern. In this way, within single rat, one eye was injected with bevaczumab while the contralateral eye received vehicle. This experimental protocol was in depth planned and discussed since it allowed: (1) to avoid sacrificing an unnecessary number of rats, (2) to reduce inter-individual variability both in the retina structure and pharmacological effects of bevaczumab solutions.

By assuming rat vitreous volume as 60 μL and human vitreous as approximately 4 mL, the dose of 0.1 mg bevaczumab (4 μL, 25 mg/mL) was assumed as equivalent to the therapeutic dose given to humans with the approximation due to species-dependent variations in the kinetics. Hamilton microsyringe (25 μL) with a 30 gauge needle was used to inject intravitreally 4 μL, at a flow rate of 1 μL/min of bevaczumab solution (Avastin® Roche, Welwyn Garden City, UK) (Fig. 1). Intravitreal injection was performed in the right eye 1 mm behind the limbus. The left eye received the same amount (4 μL) of vehicle (balanced salt solution, BSS Plus, Alcon, Fort Worth, TX) and it was used as control. The balanced salt solution corresponds to what used as control in all previous studies which used either BSS or other saline-based solutions with no significant difference in term of cell loss compared with our study (Rowley et al., 2004; Karawa et al., 2008; Arraes et al., 2009; Thaler et al., 2010; Iandiev et al., 2011; Myers et al., 2012).

Rats were sacrificed 48 h after the last treatment, eyes were enucleated immediately and the anterior segment and vitreous body were rapidly removed (within 10 min) to fix completely the posterior segment.

4.3. Histology and immunohistochemistry

The posterior segment of the eye containing the retina, was dissected out and immediately placed in a Carnoy solution containing ethyl alcohol (60%), acetic acid (10%), and chloroform (30%), and 20 h later, it was placed in 70% ethanol until paraffin inclusion. The retina was cut into 10 μm serial sagittal sections (through the medio-lateral extent) and used for histological and immunohistochemical analysis. Sections were deparaffinised and processed for staining with haematoxylin and eosin (H&E).

In order to investigate whether the intravitreal injection of bevaczumab could induce the RGCs apoptosis, tissue sections underwent to terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) test (Promega Corporation, Madison, USA) using a kit for in situ cell death detection with fluorescein to be visualized at fluorescence microscopy.

The potential loss of RGCs was also detected with immunohistochemistry. The deparaffinised tissue sections were incubated overnight with monoclonal mouse antibody anti-tubulin, beta-III isoform (TUJ1) (1:100; Millipore, Temecula, CA, USA), with rabbit antibody anti-Annexin V (1:50; Abbottic, Resnova, Italy), with monoclonal mouse anti-neurofilament 200 (phosphorylated and non-phosphorylated) (1:300; Sigma Aldrich, Milan, Italy), with polyclonal rabbit anti-Bax (1:100; Millipore) and then for 1 h with secondary biotin-coupled anti-mouse (1:200; Vector Laboratories, Burlingame, CA) and anti-rabbit (1:200; Vector Laboratoires). Control staining was performed without the primary antibodies (see also supporting Fig. S2 for Annexin V and Bax).

4.4. Cell density

Beta-III tubulin quantification was obtained by counting of beta-III tubulin positive cells showing an intense cytoplasmic immunoreactivity, in 280 μm distanto medio-lateral serial sections of the group treated with bevaczumab vs. vehicle group (single and repeated treatment). Data are expressed as the number of beta-III tubulin positive cells per mm².

4.5. Western blot

Rat retinal tissue was homogenized and lysed on ice with RIPA buffer containing protease and phosphatase inhibitors cocktail (Calbiochem, Merck Millipore, Italy) for 30 min. Lysates (50 μg) was separated on 10% SDS-PAGE at 100 V for 1 h and transferred to a nitrocellulose membrane for blotting (Sigma Aldrich). The membrane was incubated for 2 h in blocking buffer (TBS, 0.05% Tween-20 and 2.5% nonfat milk) and then overnight with mouse anti-Brn3a antibody 1:1000 (Sigma Aldrich) or rabbit anti-β-actin antibody 1:3000 (Cell signaling, Merck Millipore, Italy). The membrane was washed three times for 5 min in TBS, 0.05% Tween-20 before a 2 h incubation in a buffer (TBS, 0.05% Tween-20 and 2.5% nonfat milk) containing horseradish peroxidase-linked anti-mouse IgG and anti-rabbit IgG (Amersham, GE Healthcare Life Science, Milan, Italy) at 1:3000 dilution. The membrane was washed four times and specific protein bands were detected using the ECL Prime chemiluminescent agents (Amersham).

Western blot were analyzed using Imagej software (developed by Wayne Rasband, National Institutes of Health, USA) to determine optical density (OD) of the bands. The OD reading was normalized to β-actin to account for variations in loading. Four treatment groups (n=5 per group) were compared using One-way ANOVA with Bonferroni post-test.

4.6. Statistical analysis

Statistical analysis was performed using statistical software (GraphPad Prism Software, version 5.0). Values are presented as mean±SEM. Analysis of variance (One-way ANOVA) was used to compare all conditions and Bonferroni post-test was
used to compare mean values for all groups. Differences were considered statistically significant when the probability values were less than 0.05.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2012.08.014.

References


