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Bevacizumab clearance through the iridocorneal angle following intravitreal injection in a rat model

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ABSTRACT

Antivascular endothelial growth factor (Anti-VEGF) agents have been widely used for a variety of ocular disorders. The etiology of sustained ocular hypertension following intravitreal administration of anti-VEGF agents is yet to be unraveled.

Our study investigates and characterizes the presence of intravitreally injected bevacizumab in the aqueous outflow channels of a rat model. Choroidal neovascularization (CNV) was induced by diode laser photocoagulation to the right eye of twelve Brown Norway rats. Bevacizumab (25 mg/ml) was injected intravitreally after 3 days. Immediately after bevacizumab injection, and 3, 6, 24 and 48 h later, animals were euthanized for immunofluorescence staining. Donkey anti-human IgG labeled with Alexa Fluor® 488 was used for bevacizumab immunoreactivity detection. Anti-CD31 antibody was used as a marker for Schlemm's canal endothelial cells. Untreated eyes were used as negative controls. The intensity of the immunostaining was analyzed qualitatively. Bevacizumab immunoreactivity was found in the aqueous outflow channels including the trabecular meshwork and Schlemm's canal immediately after injection, and declined incrementally within the following hours. Forty-eight hours after the injection, no bevacizumab staining was detected in the aqueous outflow channel structures. Our manuscript demonstrates the presence of bevacizumab in the trabecular meshwork and Schlemm's canal structures after intravitreal injection in a CNV induced rat model. Bevacizumab molecules passed through the aqueous outflow channels within 48 h after intravitreal bevacizumab injection.

The use of intravitreal injections to inhibit vascular endothelial growth factor (VEGF) has grown tremendously in recent years. Anti-VEGF agents have been widely used for a variety of ocular disorders. It is currently the leading treatment for neovascular age-related macular degeneration (AMD), diabetic retinopathy, and retinal vein occlusions (Bressler, 2009; Iturralde et al., 2006; Spaide and Fisher, 2006). Anti-VEGF agents have also been used for patients with neovascular glaucoma (NVG) (Simha et al., 2009). Although these injections are relatively safe, one of the complications is elevated intraocular pressure (IOP) with acute, transient, delayed, persistent, or chronic presentation.

Bevacizumab is a full length recombinant humanized murine monoclonal antibody, that binds to and inhibits the biological activity of all five human VEGF-A isoforms who are responsible for vascular proliferation and can lead to a variety of ocular pathologies.

Over the last few years, several case reports and retrospective studies (Adelman et al., 2010; Choi et al., 2011; Hoang et al., 2013; Singh and Kim, 2012) have suggested that receiving multiple intravitreal anti-VEGF injections for AMD and other retinal vascular disease can cause sustained, elevated IOP in a subset of patients with no previous history of ocular hypertension.

Abbreviations: Choroidal neovascularization, CNV; vascular endothelial growth factor, VEGF; age-related macular degeneration, AMD; intraocular pressure, IOP; trabecular meshwork, TM; Schlemm's canal, SC; normal donkey serum, NDS; Immunoreactivity, IR.

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Sustained, elevated IOP was observed with both ranibizumab and bevacizumab. The etiology of persistently elevated IOP after anti-VEGF injections is still to be elucidated and requires insight into aqueous dynamics and outflow mechanisms. Several theories have been proposed (Bakri et al., 2008; Georgopoulos et al., 2009; Kahook et al., 2010; Kim et al., 2008; Liu et al., 2011) regarding the relationship between intravitreal anti-VEGF injections and sustained IOP elevation.

One proposed mechanism for the elevated IOP following intravitreal anti-VEGF injections includes a direct effect of the drug on the aqueous outflow channels, including the trabecular meshwork (TM) and Schlemm's canal (SC). It is known that glucocorticoids affect extracellular matrix deposition in the trabecular meshwork and alter aqueous dynamics and gene expression (Kubota et al., 2006; Shinzato et al., 2007). However, there are no similar studies on the effect of anti-VEGF agents. In this study, we investigated the hypothesis that bevacizumab may accumulate in the trabecular meshwork, thereby blocking aqueous outflow and leading to increased IOP.

Twelve male Brown Norway rats weighing 200–300 g each were used in this study (Harlan Laboratories, #3BN01). The animals were cared for in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Rabin Medical Center.

On day 0, choroidal neovascularization (CNV) was induced in the right eye of Brown Norway rats by indirect diode laser photocoagulation, as described previously (Antes et al., 2015). Rats were placed under general anesthesia using intramuscular administration of 50 mg/kg body weight ketamine hydrochloride and 5 mg/kg body weight xylazine. Pupils were dilated with tropicamide (0.5%) eye drops and supplemented with topical anesthesia (0.4% oxybuprocaine hydrochloride). Laser photocoagulation was performed on the right eye of each rat by indirect diode laser ophthalmoscope (Iris Medical Oculight SLX System©, Iridex, Mountain View, CA, USA) with the treatment beam set at 810 nm and the aiming beam at 650–670 nm, 450 mW power, 100 ms duration. Four to six laser spots were applied around the optic disc using a 90 Diopter condensing lens. This setting produced acute vapor bubbles, indicative of rupture of Bruch's membrane.

The vitreous VEGF concentration increased significantly from baseline in an experimental laser-induced CNV Brown Norway rat model, as described by Kim et al. (Kim et al., 2010). In this model, VEGF reached maximum concentration 5 days after CNV induction and remained significantly elevated for 14 days.

Intravitreal injection of bevacizumab was performed 3 days after laser photocoagulation. Under anesthesia, the pupils were dilated using tropicamide 0.5% eye drops. A 30-gauge Hamilton bevel-tip syringe needle was inserted into the vitreous, 1 mm posterior to the limbus, at about a 45° angle and 3 μl (0.075 mg) of bevacizumab (Avastin®, Genentech, South San Francisco, CA, USA) was injected. All experimental rats received injections in the right eye.

In order to evaluate the presence of bevacizumab, positive control, the left eyes of 8 rats (non-CNV induced) were intravitreally injected with bevacizumab, immediately prior to euthanization (time 0). As negative control, the left eyes of 4 rats (non-CNV induced) were left untreated as demonstrated in Fig. 1 (A1–2).

Immediately after bevacizumab injection and 3, 6, 24 and 48 h later, the animals were euthanized. The eyes were enucleated for immunofluorescence staining and histological analysis.

For immunodetection of bevacizumab in aqueous outflow channels, Alexa Fluor® 488 conjugated Donkey anti-Human IgG antibody was used. Mouse anti-rat CD31 was used as a marker for SC endothelial cells.

Enucleated eyes were fixed in 4% paraformaldehyde for 2 h and cryopreserved. 4 μm-thick cryosections were air-dried at room temperature for 2 h. Sections were blocked with 10% normal donkey serum (NDS) in tris buffered saline at room temperature for 1 h, and then incubated with primary antibody, mouse anti-rat CD31 (Millipore #MAB1393, 1:50 dilution) in 10% NDS overnight at 4 °C.

Donkey anti-mouse IgG labeled with Alexa Fluor® 568 (1:100; Molecular Probes #A10037) as a secondary antibody for anti-CD31 and Donkey anti-human IgG labeled with Alexa Fluor® 488 (#709-545-149, Jackson ImmunoResearch, West Grove, PA, USA, 1:200 dilution) as a secondary antibody for bevacizumab were added to the tissue at room temperature for 1 h. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI; Nucblue, Molecular Probes #r37606). The staining pattern of the tissue sections was observed under a standard fluorescence microscope and camera settings (Olympus Optical Co., Tokyo, Japan). For anatomic orientation, adjacent cryosections were then stained with hematoxylin and eosin (H&E; American MasterTech Scientific®, Lodi, CA, USA) and examined under a light microscope.

Immunoreactivity (IR) for bevacizumab was shown in the aqueous outflow channels, including the TM and SC of eyes that underwent intravitreal injection of bevacizumab. None of the untreated eyes which served as negative controls showed IR for bevacizumab (Fig. 1A2), confirming that anti-human IgG antibody was specific to the bevacizumab molecule.

The combined analysis of histology and immunostaining profiles provided insights into the localization of the bevacizumab molecule in the rat drainage tract, as depicted in Fig. 1(B1–3).

Iridocorneal angle sections prepared at different time points after bevacizumab injection were evaluated for their IR against the bevacizumab molecule (Fig. 2). IR for bevacizumab was shown in the TM and adjacent SC, of eyes that underwent intravitreal injection. Immediately and 3 h after the injection, positive bevacizumab IR was observed, primarily in the TM (Fig. 2A). Six hours after the injection, the TM still showed IR for bevacizumab (Fig. 2B), though less strongly, with faint staining present mainly on the outer surface of SC.

Twenty-four hours after the injection, the intensity of the staining of bevacizumab was decreased, but was detectable in different regions of the aqueous drainage tract, primarily around the episcleral veins and extending anteriorly to the corneal stroma (Fig. 2C). Forty-eight hours after injection, no specific IR for bevacizumab was noticeable in the TM and SC structures or in the corneal stroma (Fig. 2D).

Bevacizumab has become a common treatment for VEGF-mediated retinal diseases. Reports over the last few years suggest that sustained IOP elevation may occur in up to 10% of treated patients (Abedi et al., 2013; Bakri et al., 2008; Good et al., 2011; Tseng et al., 2012). Some suggested that repeated injections may increase the risk of sustained IOP elevation (Qv et al., 2012). However, no direct relationship between the number of anti-VEGF injections and sustained elevated IOP has been found (Good et al., 2011).

Although the mechanism by which sustained IOP elevation occurs is still unclear, several theories have been proposed. Kahook et al., 2009 indicated that protein aggregates and a high number of large molecules in some syringes could potentially lead to obstruction of the outflow pathways (Kahook et al., 2010). Another possibility is that contaminants, such as silicone oil from the syringe barrel or rubber stopper, could lead to obstruction of the outflow system (Bakri and Ekdawi, 2008).

Previous reports suggested that a likely mechanism for the