EFFICACY OF ANTIBODY DELIVERY TO THE RETINA AND OPTIC NERVE BY TOPICAL ADMINISTRATION

A thesis presented to the graduate faculty of
New England College of Optometry in partial fulfillment
of the requirements for the degree of Master of Science.

Stacy N. Hu

April 2015

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This manuscript has been read and accepted by the Thesis Examination Committee in satisfaction of the thesis requirement for the degree of Master of Science.

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ABSTRACT

Purpose

It has generally been assumed that potentially therapeutic, large, hydrophilic compounds such as proteins cannot reach the posterior segment following topical application; however, data from our and several other labs suggest that this assumption needs to be reassessed. The purpose of this study was to determine whether large antibody molecules of the isotype currently injected intravitreally in humans to treat wet macular degeneration can similarly accumulate in the rat retina following topical application. Furthermore, we attempted to develop a model system that could be used to demonstrate that antibodies that reach the posterior segment retain their pharmacological properties.

Methods

Ten microliter eye drops containing non-specific, polyclonal mouse IgG1 and the permeation enhancer saponin were topically applied to the eyes of two to three month old female Lewis rats. The concentrations of this antibody in the retina and optic nerve were determined 10 and 20 minutes later using an ELISA assay. We developed *in vivo* and *in vitro* models to assess the pharmacologic activity of topically-delivered antibodies in the retina. The *in vivo* model involved the induction of retinal leukostasis with lipopolysaccharide (LPS) followed by the topical treatment of the animals with mouse anti-rat intercellular adhesion molecule 1 (ICAM-1) antibody, which is known to interfere with this process of neutrophil

infiltration. Our indirect output measure of the density of neutrophils in the retina involved the use of a myeloperoxidase (MPO) ELISA. Our *in vitro* model was based on the fact that human umbilical vein endothelial cells (HUVECs) require vascular endothelial growth factor (VEGF) for growth and replication. In this model, we treated rat eyes with anti-VEGF antibody, harvested the retinas 20 minutes later, and then added these retinal homogenates into culture wells containing VEGF-containing media and HUVECs. If anti-VEGF antibody had accumulated in the retina after topical application, then the addition of these retinal supernatants should negatively impact the growth of the HUVEC cells.

Results

Statistically significant concentrations of IgG1 were detected in the optic nerve ($p < 0.001$) and retina ($p < 0.0001$) compared to controls. Anti-ICAM-1 antibody also accumulated in the retina after topical application, though levels were less than those seen with IgG1, probably owing to a lower starting concentration of antibody in our eye drop solution. Levels of MPO were significantly elevated 24 hours post-LPS injection ($p < 0.05$), and steadily declined to statistically insignificant levels 48 hours and 8 days later. Topical treatment of LPS-injected rats with two separate regimens of anti-ICAM-1 antibody, however, failed to suppress leukostasis. On the other hand, retinal homogenates from the eyes of rats treated with anti-VEGF antibody significantly suppressed HUVEC proliferation ($p < 0001$).

Conclusions

Our data support the contention that topically applied antibodies can accumulate in the posterior segment of the eye while retaining their pharmacological properties.

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INTRODUCTION

Due to the constraints of the blood-aqueous and blood-ocular barriers, the targeting of therapeutic drugs to the posterior segment of the eye has best been accomplished by their local delivery to target tissues by either transscleral or intravitreal injection (Kuppermann & Loewenstein, 2010; Rawas-Qalaji & Williams, 2012). Research demonstrated that the transscleral route could successfully deliver bioactive proteins to the posterior pole, and this method is currently used for the therapeutic delivery of the steroid triamcinolone acetonide (Retaane) for the treatment of wet age-related macular degeneration (wet AMD) (Ambati et al., 2000; Thrimawithana, Young, Bunt, Green, & Alany, 2011). The intravitreal route is used for the delivery of multiple pharmaceuticals such as steroids, antibiotics (e.g., vancomycin), and antivirals (e.g., foscarnet) (Peyman, Lad, & Moshfeghi, 2009). Perhaps the most exciting intravitreally-injected drugs to come on the market in the past several years have been those that target vascular endothelial growth factor (VEGF); these compounds will be discussed in more detail below.

The effectiveness of topical ocular delivery is limited by rapid drug elimination due to lacrimation and nasolacrimal drainage; drug binding to and metabolism by tear proteins; and target non-specificity as a result of systemic absorption through the nasal and lacrimal duct mucosa and conjunctival vasculature (Frangie, 1995; Koevary, 2003). Research showed that there are two general pathways that a drug can traverse from the ocular surface to the posterior segment: 1. corneal – through the anterior chamber, lens, pupil, or the iris or its root; and 2. conjunctival – directly across the sclera, choroid, choriocapillaris, and retinal pigment epithelium (RPE), or indirectly into the retrobulbar space and the optic nerve head

(Joshi, Maurice, & Paugh, 1996; Maurice, 2002). Once in the aqueous humor, drugs would have ready access to the posterior surface of the iris, which was estimated to have a permeability greater than the conjunctival and corneal epithelia but somewhat less than the RPE (Koyano, Araie, & Eguchi, 1993; Macdonald & Maurice, 1991). Mechanically blocking access to the corneal surface had little effect on drug penetration into the posterior segment, suggesting that the transscleral/conjunctival route is more important for posterior segment delivery (Kuppermann & Loewenstein, 2010). This finding is supported by data from our lab suggesting that insulin, applied topically, traversed the sclera before accumulating in the retina (Koevary, Lam, & Patsiopoulos, 2004).

In spite of the limitations alluded to earlier but in keeping with the description of potential routes of transport from the anterior to posterior segments described above, topically applied drugs have been reported to accumulate in the posterior segment, and several topical therapies for retinal and choroidal diseases are under active development (Eljarrat-Binstock, Pe'er, & Domb, 2010). TargeGen 801 (TG100801) is a VEGF receptor/src kinase inhibitor that is delivered as a prodrug with a low molecular weight. Animal studies showed that topical TG100801 significantly suppressed laser-induced choroidal neovascularization (CNV) in mice (Doukas et al., 2008). Based on promising phase I data, TargeGen is initiating a multi-center phase II clinical trial of this compound in patients with wet AMD. Topical treatment with pazopanib, a multitargeted tyrosine kinase inhibitor of VEGF receptors, resulted in improvement in best corrected visual acuity and macular edema in a subset of AMD patients (Danis et al., 2014). Efficient intraocular penetration of topical anti-TNF-alpha single-chain antibody (ESBA105) without the use of a penetration

enhancer was reported in rabbits (Ottiger, Thiel, Feige, Lichtlen, & Urech, 2009). Finally, low concentrations of the aldose reductase inhibitor imirestat and the steroid dexamethasone were detected in the vitreous following topical application, and these levels were suggested to have therapeutic potential (Kador et. al., 2007; Sigurdsson et. al., 2007). Data from our lab suggest that proteins of small to moderate molecular weight such as insulin (molecular weight [MW] 5.8 kDa), leptin (MW 16 kDa), and glial derived neurotrophic factor (GDNF; MW 20 kDa) can accumulate in the retina of rats following topical application (Koevary et al., 2004; Koevary, Nussey, & Lake, 2002; Koevary, 2002; Pardon & Koevary, 2012).

As outlined in more detail below, the overall objective of this project was to determine the efficacy of topical application for the delivery of therapeutic antibodies to the retina for the potential treatment of neovascular AMD, diabetic retinopathy, and retinal vein occlusions; these are the diseases most responsible for irreversible vision loss in the United States. Though these conditions have different presentations, their etiologies all stem from an increase in the level of VEGF in the eye (Falavarjani & Nguyen, 2013). When functioning normally, VEGF promotes the growth, maintenance and repair of tissues. When released in response to ischemia or tissue hypoxia, however, VEGF increases capillary permeability by stimulating endothelial cell production of proteases that break down basement membranes and tight junctions between cells. This leads to the proliferation and migration of endothelial cells, the latter of which is facilitated by expression of integrins such as intercellular adhesion molecule-1 (ICAM-1) (Miyamoto et al., 2000). This process results in the creation of new and structurally unsound blood vessels, and ultimately culminates in the breakdown of the

blood-retina barrier and the creation of choroidal neovascular membranes (CNVM) (Stewart, 2012; Velez-Montoya et al., 2014).

Several pharmaceuticals that specifically target VEGF have been developed since the early 2000s, initially for the treatment of cancer but more recently also for wet AMD. Bevacizumab (Avastin) was the first anti-VEGF agent to be FDA-approved in February 2004 for the treatment of colon cancer in combination with chemotherapy (Kim & D'Amore, 2012). Bevacizumab is a humanized anti-VEGF antibody that targets all five isoforms of VEGF. Just a few months later, pegaptanib (Macugen) was FDA-approved as the first anti-VEGF agent for the treatment of neovascular AMD (Kim & D'Amore, 2012). Pegaptanib is a ribonucleic acid aptamer that specifically targets VEGF165, the dominant isoform found in wet AMD. Patients that received intravitreal injections of pegaptanib every six weeks for one year lost seven letters of visual acuity compared to the loss of 15 letters in sham injected controls (Gragoudas, Adamis, Cunningham, Feinsod, & Guyer, 2004).

As VEGF's role in the development of neovascular AMD was further elucidated, researchers determined that off-label use of bevacizumab was even more successful for the treatment of wet AMD than pegaptanib. AMD is the most common cause of vision loss in patients 65 years of age and older in developed countries (Ferris, Fine, & Hyman, 1984). A small unmasked, single-center, uncontrolled study demonstrated that systemic, intravenous bevacizumab not only prevented further vision loss from wet AMD, but also improved visual acuity by an average of 12 letters after just 12 weeks of therapy (Michels, Rosenfeld, Puliafito, Marcus, & Venkatraman, 2005). Systemic administration is not without its extensive side effects, which will be elucidated later; further studies determined that

intravitreal injections also improved visual acuity in patients with wet AMD (Rosenfeld, Moshfeghi, & Puliafito, 2005), prompting its off-label use as an intravitreal injection for the treatment of the disease.

Animal studies initially suggested that the full-sized bevacizumab antibody might not penetrate into the retina as well as antibody fragments, which was the rationale for the development of ranibizumab (Lucentis). Like bevacizumab, ranibizumab similarly targets all VEGF isoforms; however, ranibizumab is the 48 kDa Fab fragment of the full-sized antibody and was affinity-enhanced for VEGF (Kim & D'Amore, 2012; Stewart, 2012). Two groundbreaking, multi-center, randomized studies conducted on patients with wet AMD demonstrated that visual acuity improved with the use of ranibizumab. The Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration (MARINA) study showed that after one year, monthly ranibizumab injections resulted in an improvement of 7.2 letters, while monthly sham-injected patients lost 10.4 letters (Rosenfeld, Brown, Heier, Boyer, Kaiser, Chung, & Kim; MARINA Study Group, 2006). Additionally, the Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-Related Macular Degeneration (ANCHOR) study demonstrated that monthly intravitreal injections of ranibizumab resulted in better prognoses for patients after one year (gain of 11.3 letters) than those treated monthly with photodynamic therapy with verteporfin (loss of 9.5 letters), the long-standing treatment for CNVM prior to the development of anti-VEGF therapies (Brown, Kaiser, Michels, Soubrane, Heier, Sy, and Schneider; ANCHOR Study Group, 2006). Based on these studies, ranibizumab became the second anti-VEGF pharmaceutical FDA-approved

drug for the treatment of wet AMD. Thus, intravitreal injections of the anti-VEGF antibody ranibizumab edged out pegaptanib and photodynamic therapy as the standard of care for the treatment of wet AMD (Kim & D'Amore, 2012; Stewart, 2012). These drugs are not only the treatment mainstays for wet AMD, but also for retinal vascular occlusions, proliferative diabetic retinopathy (PDR), and diabetic macular edema (DME).

Though very effective in not only halting but actually reversing the course of neovascular diseases, the above antibody therapies must be administered by repeated intravitreal injections; specifically, they are directly injected into the vitreous cavity through the pars plana (Thrimawithana, Young, Bunt, Green, & Alany, 2011), thereby allowing high concentrations to reach the site of action at the retina. The requirement of repeated intravitreal injections in order to maintain treatment effectiveness has led to concerns regarding ocular and systemic side effects associated with this invasive therapy. Endophthalmitis is a potentially devastating complication of intravitreal injection that results in poor visual outcomes. In the MARINA, ANCHOR, and other trials, up to 1.3% of patients developed endophthalmitis, with a similar percentage developing uveitis (Tufail et al., 2010). Another ocular side effect that could present long-term problems involves changes in intraocular pressure (IOP). Immediate, transient elevations in IOP are a known side-effect of intravitreal injection, but cases in which IOP elevation were sustained over several visits have also been reported, which could increase the risk of developing glaucoma (Bakri et al., 2009; Hoang et al., 2012). RPE tears and retinal detachments have also been reported following intravitreal injections of anti-VEGF medications, with the incidence reported to be 1 per 7,188 injections in nearly 36,000 patients receiving anti-VEGF medications (Meyer et

al., 2011; Singh & Sears, 2006). A large review reported the incidence of retinal detachment to be as high as 0.9% per injection (Jager, Aiello, Patel, & Cunningham, 2004). No differences were reported in the rates of serious adverse effects between pegaptanib, ranibizumab, and bevacizumab (Falavarjani & Nguyen, 2013; van der Reis et al., 2011); however, incidence increased compared to controls. Patients injected with ranibizumab had a 2.1-2.9% incidence of grade 3-4 ocular inflammation, while no patients receiving photodynamic therapy and/or sham injection had any cases of grade 3-4 ocular inflammation (Brown et al., 2006; Rosenfeld et al., 2006; Tolentino, 2011). The risk was higher with anti-VEGF injections than with steroid injection because of the more-frequent treatment regimen in the former (Pielen et al., 2013). While the aforementioned ocular events were visually significant, other side effects of intravitreal injection include relatively benign but avoidable conditions such as intraocular and subconjunctival hemorrhages (Falavarjani & Nguyen, 2013; van der Reis et al., 2011).

Several trials evaluated whether less frequent or individualized dosing of ranibizumab or bevacizumab could reduce the risk for developing adverse events. In the PIER trial, 184 patients with wet AMD were randomly assigned to intravitreal injections of ranibizumab 0.3 mg, ranibizumab 0.5 mg, or sham monthly for three months, and then quarterly with follow-up at 12 months (Regillo et al., 2008). Patients in both treatment groups showed significantly less decline in visual acuity relative to those in the sham group (-1.6 and -0.2 letters in the 0.3 and 0.5 mg groups, respectively, versus -16.3 letters for control); however, these end points were worse than in previous studies, suggesting that patients benefit from more frequent injections (Tolentino, 2011). The IVAN study compared continuous monthly treatment with

either ranibizumab or bevacizumab for one year with discontinuous, as needed clinical treatment after the initial three monthly treatments (Chakravarthy et al., 2012). Patients treated on an as-needed basis demonstrated a clinically insignificant decrease of 0.36 letters of visual acuity compared to their continuously treated counterparts; however, patients who received continuous treatment had less subretinal fluid accumulation compared to those treated on an as-needed basis. Fewer patients also had fluorescein leakage in the continuous treatment group compared to the discontinuous group (24% vs 36%, respectively). The authors found no difference in the number of systemic and ocular adverse events between the treatment regimens after one year, but did find that those given discontinuous treatment had higher mortality rates after two years (Chakravarthy et al., 2013). When the results from multiple large-scale clinical studies were compared, the data showed that fewer treatments resulted in the loss of visual acuity, though the loss was clinically insignificant (Haller, 2013).

Thus, while anti-VEGF agents have been shown to be effective in the treatment of neovascular retinal diseases, the chronic need for intravitreal injections to prevent further pathology and maintain treatment effectiveness was associated with a variety of side effects. In light of these findings and because our previous findings suggested that topically applied proteins can accumulate in the retina, this thesis project was undertaken to provide pilot data regarding the efficacy of delivering antibodies to the posterior segment by topical application. A positive outcome would bode well for the future development of regimens for the treatment of the conditions described above. Furthermore, being noninvasive, such an

approach could lead to greater compliance and open the door to the direct treatment of patients with these conditions by optometrists or the patients themselves.

SPECIFIC AIMS

1. To determine whether topically applied mouse IgG1 antibodies can accumulate in the optic nerve and retina. This antibody isotype is the same as that which comprises the anti-VEGF antibody drug bevacizumab described above.
2. To develop a model system for determining whether topically applied antibodies can have a pharmacologic effect in the retina.
    a. The first system we developed was a leukostasis model in lipopolysaccharide (LPS)-treated rats. The bacterial product LPS triggers leukostasis i.e., the movement of leukocytes into tissues including the retina. Leukostasis is mediated by the interaction of ICAM-1, which is expressed on vascular endothelial cells, with LFA-1 on leukocytes. We determined whether topically applied anti-ICAM-1 blocking antibodies (IgG1 isotype) can reduce leukostasis in LPS-treated animals.
    b. The second system was an *in vitro* model that involved the use of human umbilical vein endothelial cells (HUVECs), the growth of which is dependent upon the presence of VEGF. Retinal homogenates, prepared from rat eyes previously topically treated with anti-VEGF antibodies, were added into the VEGF-containing culture media of HUVECs. The presence of anti-VEGF antibodies in the retinal homogenates would limit the growth of the HUVEC cells, which was determined using a cell proliferation assay.

EXPERIMENTAL DESIGN AND METHODS

Animals

Two to three month old female Lewis rats were used in this study. They were allowed access to food and water *ad libitum* in a climate-controlled room with a 12 hour light/dark cycle. All experiments were performed according to the guidelines set by the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the New England College of Optometry's Institutional Animal Care and Use Committee.

Studies involving the topical use of IgG1

In our first series of experiments, we used an ELISA assay to quantify IgG1 levels in the retina following topical application. Our first step was to validate the use of the ELISA with retinal homogenates, since the assay kit was designed for use with cell culture supernatants.

Validation of IgG1 ELISA

We initially spiked retinal homogenates with known concentrations of mouse IgG1 antibody (Sigma Aldrich Mouse IgG1, catalog #M7894) and read those samples off a standard curve generated using the same IgG1 antibody diluted in assay buffer according to the manufacturer's instructions. Our initial results suggested a loss of sensitivity with this approach. Next, we set up and compared the concentrations read from two standard curves: one created with the antibody diluted in the assay buffer provided by the kit, and one created

with the antibody diluted in retinal homogenates. If the values obtained from the standard curve generated with the retinal homogenates corresponded to the values obtained from the standard curve generated with assay buffer alone, then it could be concluded that the retinal homogenates did not contain any substances that interfered with IgG1 quantitation. On the other hand, should the values differ, then we would be obliged to use a standard curve generated using retinal homogenates rather than assay buffer alone in all future assays.

The ELISA kit (Enzo Life Sciences, catalog #ADI-900-109) uses a polyclonal antibody that is immobilized on a microtiter plate to bind to the mouse IgG1 in the standards or samples. Another polyclonal antibody conjugated to horseradish peroxidase targets the bound mouse IgG1 antibody for quantification; the reaction of the horseradish peroxidase with its substrate 3,3',5,5' tetramethylbenzidine (TMB) creates a color, and the optical density of that color is directly proportional to the concentration of mouse IgG1. Six serial dilutions of the IgG1 antibody were set up for each standard curve in a final volume of 100 µl per well, with concentrations ranging from 250 ng/ml down to 7.81 ng/ml. Each sample was placed into a Corning 96-well plate, and the assay performed according to the manufacturer's instructions. After an incubation of one hour with both antibodies on a shaking platform, the samples were washed to remove excess reagents. One hundred microliters of substrate solution were then added and left in the wells for 30 minutes, after which the enzyme reaction was stopped by the addition of acid. The color generated was read at 450 nm using a BioTek EL-808 plate reader. Results were expressed as ng of mouse IgG1 per ml solution.

Determination of optic nerve and retina IgG1 levels following topical application

Initially, when we topically treated rats with a solution containing 1 mg/ml of mouse IgG1 antibody, we were unable to detect any IgG1 in the retina with the ELISA; accordingly, we increased the concentration of our stock solution to 25 mg/ml.

To create the mouse IgG1 eye drop solution, 5 mg of IgG1 antibody (same as above) were reconstituted in 200 µl of sterile deionized water to create a final solution of 25 mg/ml IgG1. Serial dilutions of this reconstituted IgG antibody were also used to generate the standard curve used in the ELISA. Each 10 µl eye drop consisted of 9 µl of the 25 mg/ml IgG1 solution, and 1 µl of a 10% solution of the permeation enhancer saponin (Sigma Chemical Company, catalog # 8047-15-2) dissolved in sterile PBS. Our previous studies with other peptides suggested the need for this permeation enhancer to facilitate movement through the ocular surface (Koevary, Lam, Patsiopoulos, & Lake, 2003; Koevary et al., 2002). Thus, the final concentration of IgG1 per drop was 22.5 mg/ml.

Forty six female Lewis rats were used in this study. Based on our previous data, we speculated that IgG1 would most likely reach the retina and optic nerve in less than 30 minutes. Furthermore, it was most likely that optic nerve accumulation would be maximal at 10 minutes post application, while retinal levels would peak 20 minutes post application (Koevary et al., 2004, 2002). As such, in the first series of experiments, accumulation of IgG1 in the optic nerve was determined 10 minutes after topical application. Specifically, the right eyes of 10 rats received one 10 µl drop prepared as above, while their left eyes served as untreated contralateral controls. Eight optic nerves from four untreated rats also served as negative controls. In the second series of experiments, accumulation of IgG1 in the retina

was determined 20 minutes after topical application. Specifically, the right eyes of 20 rats received one 10 µl drop prepared as above, while their left eyes served as untreated contralateral controls. Three additional rats received treatment in both their left and right eyes for a total of six treated retinas. Eighteen retinas from nine untreated rats served as negative controls.

All animals, including those left untreated, were anesthetized with 0.3 – 0.8 ml of a ketamine/xylazine solution (two parts 100 mg/ml ketamine and one part 20 mg/ml xylazine) prior to eye drop application. Ten µl of the aforementioned antibody solution were applied to the treated right eyes, after which they were closed and pressure was applied for five minutes to prevent lacrimal drainage. In the first series of experiments in which optic nerve IgG1 levels were measured, the animals were sacrificed 10 minutes after treatment as mentioned above. The optic nerves were harvested, weighed and placed into 100 µl of assay buffer in 0.6 ml polypropylene tubes. The tissues were then homogenized for at least 30 seconds on ice with a clean homogenizing pestle. Retinas were harvested in the same manner as the optic nerves, with the only difference being that animals were sacrificed 20 minutes after eye drop application. The tubes were spun down at 14,000 rpm for 3 minutes; supernatants were then harvested for use in the ELISA assay, which was performed as described above. Based on our results from the assay validation study, standard curves were generated using IgG1 antibody diluted in retinal homogenates from untreated rats.

BCA Protein Assay

Concentrations of antibody found in the retinas were adjusted per unit protein, whereas concentrations of antibody in the optic nerves were expressed per unit wet weight. Retinal samples invariably contained small amounts of vitreous, so using wet weights to express retinal antibody levels would have introduced error into our calculations; optic nerves, on the other hand, were harvested cleanly. Since the vitreous contains less than 1% protein (Angi et al., 2012), expressing retinal values per unit protein should not have resulted in the introduction of significant amounts of variation due to very small differences in the amount of vitreous between samples.

The Pierce BCA Protein Assay Kit (Life Technologies, catalog #23225) was used to quantify protein levels. The assay relies on the use of bicinchoninic acid (BCA) to colorometrically determine the quantity of protein in each unknown sample. Proteins reduce Cu^{+2} to Cu^{+1} in the presence of an alkaline medium; this reduced ion can then be detected using a reagent containing BCA. The acid-base reaction creates a color, and the measured optical density of that color is directly proportional to the amount of protein in each well. After the ELISA experiments were completed, any excess retinal homogenate supernatant was discarded from each sample, and 300 µl of 100 mM NaOH were added to the tubes and left overnight to solubilize the remaining homogenized tissues. The next morning, the samples were vortexed and 25 µl of each unknown sample were added to wells in a clean Corning 96-well plate. Six serial dilutions of bovine serum albumin (BSA) in 100 mM NaOH were set up to create the standard curve, with concentrations ranging from 2000 µg/ml down to 25 µg/ml. Each well then received 200 µl of working reagent containing cupric sulfate and

BCA. The reaction was allowed to incubate at 37°C for 30 minutes before the absorbances were read at 630 nm using the BioTek EL-808 plate reader. Results were expressed as μg of protein per ml solution; concentrations of antibody were then adjusted by the amount of protein per sample.

Development of a rat model to determine whether topically applied anti-ICAM-1 antibody can prevent LPS-induced leukostasis

In the next series of experiments, we sought to determine whether anti-ICAM-1 antibody (IgG1 isotype) can accumulate in the retina and exert a pharmacological effect i.e., prevent LPS-induced retinal leukostasis.

Determination of anti-ICAM-1 antibody levels in the retina following topical application

Monoclonal anti-rat ICAM-1 antibody (500 μg; anti-CD54, clone 141017; R&D Systems, catalog #MAB5832) was reconstituted in 50 μl of sterile PBS to a final concentration of 10 mg/ml. Since this antibody is a mouse IgG1 isotype, the assay manufacturer assured us, and we verified empirically, that the anti-ICAM-1 antibody could be sensitively and accurately measured in the IgG1 ELISA assay that we used above (data not shown).

Anti-ICAM-1 antibody eye drops were prepared as follows: 10 μl of the 10 mg/ml anti-ICAM-1 antibody solution were supplemented with one μl of 10% saponin dissolved in sterile PBS to create an 11 μl eye drop. The right eyes of 10 anesthetized Lewis female rats were treated with an anti-ICAM-1 eye drop in a manner similar to the protocol described

above for IgG1; the left eyes from the same animals served as untreated contralateral controls. Seven retinas from four untreated rats (one retina sample was lost) served as untreated negative controls. Twenty minutes after treatment, the retinas were harvested as described for the IgG1 experiments, and retinal supernatants were assayed for their levels of anti-ICAM-1 antibody using the IgG1 ELISA. Standard curves were generated using retinal homogenates spiked with anti-ICAM-1 antibody as before, with concentrations ranging from 250 ng/ml down to 3.91 ng/ml. The protein concentration in each sample was determined using the BCA protein assay as above, and the concentrations of anti-ICAM-1 antibody were adjusted accordingly.

Induction of leukostasis and measurement of its possible suppression after topical delivery of anti-ICAM-1 antibody

We developed a leukostasis model to determine whether topically applied anti-rat ICAM-1 antibody can have a pharmacologic effect in the retina. In this model, animals were injected with LPS to promote the movement of leukocytes in the retina. This movement is facilitated by the interaction of ICAM-1, which is expressed on vascular endothelial cells, with LFA-1 on leukocytes. Anti-ICAM-1 antibodies block this interaction and thus limit leukostasis; this is what we set out to measure.

To induce leukostasis, the hind footpads of anesthetized rats were injected with a 2 mg/ml solution of LPS (Sigma, catalog #L2630) in sterile PBS. Animals were anesthetized as described above, after which 50 µl of the LPS solution were injected into each hind footpad for a total of 200 µg per animal. Twelve female Lewis rats were used for the initial

determination of the time course for development of maximum leukostasis. Three animals injected with LPS and one animal injected with an equivalent volume of PBS were sacrificed at 24 hours, 48 hours, or 8 days after injection. Leukostasis was quantified using a rat myeloperoxidase (MPO) ELISA (Hycult Biotech, catalog #HK105). MPO is a glycoprotein expressed in all cells of the myeloid lineage, and is a marker for the presence of polymorphonuclear neutrophils. Retinas were harvested and placed into 100 µl lysis buffer containing protease inhibitors (Sigma, catalog #S8830), after which they were homogenized for at least 30 seconds on ice. As directed by the manufacturer's instructions, the retinas were then allowed to sit in the lysis buffer for 15 minutes, after which they were placed in a -80°C freezer for at least 20 minutes. These steps were taken to maximize cell disruption which was necessary to liberate MPO into the supernatants. All samples were allowed to thaw at 37°C for five minutes prior to being spun down in a microfuge at 14,000 rpm for three minutes. The MPO ELISA is a ready-to-use solid-phase assay. Samples and standards are captured by a solid bound specific antibody. Biotinylated tracer antibody binds to captured rat MPO, and then the streptavidin-peroxidase conjugate binds to the biotinylated tracer antibody. Finally, the streptavidin-peroxidase conjugate reacts with the substrate, tetramethylbenzidine (TMP), to produce a color. This reaction is stopped by the addition of citric acid, and the optical density of the reaction is measured at 450 nm with the BioTek EL-808 plate reader. No assay validation was required for this assay, as it was designed to measure MPO in tissue homogenates; accordingly, standard curves were created per the manufacturer's instructions.

The first series of experiments was conducted to determine whether the topical administration of anti-ICAM-1 antibody could reduce retinal MPO levels before the spike

seen 24 hours after LPS injection. Different eye drop treatment schedules were employed in attempts to uncover an effect. Since antibodies that accumulate in the retina likely do not linger there for long periods, we accepted the fact that we were unlikely to be able to completely suppress leukostasis, but might still be able to reduce it in measurable ways. Eight female Lewis rats were anesthetized and injected with LPS. Four hours after injection, all animals were anesthetized again, and four animals received 11 μl of the anti-ICAM solution in their right eyes; left eyes remained untreated. Eyes were held closed for 5 minutes to prevent lacrimal drainage. The remaining four animals were left untreated; one of these animals died. All eight animals, in addition to another two naïve, untreated animals, were sacrificed at the 24 hour point. The retinal homogenates were analyzed for their levels of MPO as described above. The protein concentration in each sample was determined using the BCA protein assay, and the concentrations of MPO were adjusted accordingly.

In addition to the experiment above, we sought to determine whether earlier treatment with anti-ICAM-1 could prevent the early rise of MPO levels four hours after LPS injection. Accordingly, we first verified that MPO levels at four hours after LPS treatment in four rats were elevated. We then treated separate cohorts of rats with anti-ICAM-1 antibody-containing eye drops 20 minutes after LPS injection and sacrificed the animals four hours later. Four rats received anti-ICAM-1 eye drops in their right eyes, with their contralateral eyes serving as untreated controls. Two rats served as LPS-treated positive controls, and two untreated rats served as negative controls. Again, the protein concentration in each sample was determined using the BCA protein assay as above, and the concentrations of MPO were adjusted accordingly.

Development of an in vitro HUVEC cell model to measure anti-VEGF activity

As an alternative to the leukostasis model, we also developed a system that was based on the fact that human umbilical vein endothelial cells (HUVECs) require VEGF for growth and replication. In this model, we treated rat eyes with anti-VEGF antibody and harvested the retinas 20 minutes later. We then added these retinal homogenates into culture wells containing VEGF-containing media and HUVEC cells. If anti-VEGF antibody had accumulated in the retina after topical application, then the addition of retinal supernatants containing these antibodies into the culture wells should negatively impact the growth of the HUVEC cells.

HUVEC Cells

HUVECs (ATCC, cell line CRL-1730) were passaged in F-12K Medium (ATCC catalog #30-2004). To make the complete growth medium, the following components were added: 0.1 mg/ml heparin; 0.03-0.05 mg/ml endothelial cell growth supplement (ECGS; Sigma, catalog #E-2759), which consists of acidic and basic fibroblast growth factors; and fetal bovine serum to make a final concentration of 10% (Life Technologies, catalog #10437-028).

VEGF Bioassay

Confluent HUVECs were trypsinized with 0.5% trypsin-EDTA (Life Technologies, catalog #15400-054) while being watched under a phase contrast microscope. Detached cells were washed in F-12K basic media without growth factors (Life Technologies, catalog

#21127-022), and resuspended to a density of 100,000 cells/ml. Ninety microliters of this cell suspension were added to the wells of a Corning 96 well tissue culture plate so that the final density of cells in each well was just below 10^4 cells/well. In a preliminary experiment, serial dilutions of recombinant human VEGF165 (R&D Systems, catalog #293-VE-010) ranging from 100 ng/ml down to 1.5 ng/ml were added to the wells (eight wells/dilution). This was carried out in order to provide us with information regarding the best concentration of VEGF to use in our subsequent assays. Cells cultured in the absence of VEGF served as negative controls.

In a separate assay, HUVECs were incubated as above in basic media, again in groups of eight wells per dilution, with the concentration of 100 ng/ml VEGF deemed ideal for growth as determined in the above assay. The cells were incubated either in the absence or presence of anti-VEGF antibody (R&D Systems, catalog #AF-293-NA) at concentrations of 10, 1, or 0.5 μg/ml. This assay was carried out in order to provide a frame of reference for the suppression of VEGF-mediated growth of HUVECs by anti-VEGF antibody.

In the final assay of this series, retinal homogenates from eyes previously treated with anti-VEGF antibody were tested for their ability to suppress VEGF-induced HUVEC growth, and results were compared to the antibody assay above. Ten female Lewis rats were used for this study; four right eyes from four rats served as untreated controls, while six right eyes from each of six rats were treated topically with anti-VEGF antibody. Three of these six rats were sacrificed and their eyes harvested after 10 minutes post-treatment, while the remaining three rats were sacrificed after 20 minutes post-treatment.

The anti-VEGF antibody solution was created as follows: a 200 μg stock bottle of anti-VEGF antibody (same as above) was diluted with 108 μL of sterile PBS and 12 μL of sterile 10% saponin (1% final solution) to achieve a final volume of 120 μL. All animals were anesthetized as above prior to eye drop administration. Ten microliters of this solution, which contained 166 μg of the antibody, were applied to the right eye of each treated animal, after which the eyes were closed and pressure applied for a full 10 minutes; pressure was applied for twice the amount of time as in the experiments above because of the particularly low concentration of our starting stock solution. As mentioned above, three rats were sacrificed 10 minutes after eye drop application and the remaining three were sacrificed 20 minutes after application. The retinas were harvested free of vitreous, weighed, and placed into 30 μL of sterile PBS in sterile 0.6 mL polypropylene microfuge tubes on ice, after which they were homogenized on ice with sterile homogenizing pestles, and the homogenates spun down in a microfuge for three minutes at 14,000 rpm. The homogenates were transferred to empty microfuge tubes and re-spun for an additional minute. Ten microliters of each of these homogenates were added into the assay wells in duplicate; as above, the assay wells contained just under 10^4 cells/well as well as 100 ng/ml of VEGF.

The plates in all of the above assays were incubated for 48 hours at 37°C in 5% CO2 in air, after which most of the media were aspirated from the wells and 100 μL of phenol red-free DMEM media (Life Technologies, catalog #A14430-01) were added back into the wells. This step was necessitated by the fact that phenol red interferes with the Vibrant MTT Cell Proliferation Assay Kit (Invitrogen, catalog #V13154) that we used to quantify cell proliferation. The MTT assay involves the conversion of the water soluble MTT (3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan that is then solubilized, and its concentration determined by optical density. Tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes located largely in the cytosolic compartment of the cell, so reduction of MTT depends on the cellular metabolic activity due to NAD(P)H flux – the greater the number of viable cells, the greater the optical density. The specifics of this assay were as follows: 10 µL of a 12 mM MTT stock solution were added to each well and the plates incubated for an additional four hours, after which all but 25 µL of media in each well were aspirated and 50 µL of DMSO (Life Technologies, catalog #20688) added to each well to dissolve the cells. The plates were then briefly shaken on a plate shaker and incubated for 10 minutes at 37°C, after which the plates were again briefly shaken and the absorbance in the wells determined at 540 nm using the BioTek EL-808 plate reader. Results were expressed as absorption units per mg tissue. Some cultures were photographed under phase microscopy prior to the initiation of the MTT assay.

Statistical analysis

All statistical analyses were conducted using JMP and Statistical Software from SAS, and graphs were created using Excel or SigmaPlot 12.0. Data in the graphs are expressed as the mean ± standard error.

To determine whether non-parametric statistical analyses were needed, all data were subjected to tests for normality. The data were analyzed using either student's *t*-tests or one-way ANOVAs with post-hoc comparisons when indicated through Tukey's HSD test assuming normal distributions. Non-normal data were analyzed via Kruskal-Wallis with pair-

wise post-hoc testing conducted using the Mann-Whitney test when indicated. All probability values were two-tailed. $p < 0.05$ was considered to be significant.

RESULTS

Validation of IgG1 ELISA

Recall that it was necessary to validate the use of the ELISA assay with retinal homogenates since the IgG1 ELISA kit was originally developed for the use with culture supernatants. We preliminarily spiked retinal homogenates with known concentrations of IgG1, and read those samples off the assay standard curve that was generated per the manufacturer's directions using IgG1 diluted in assay buffer. Our untreated controls in that assay registered a mean reading of 4.7 ng/ml of mouse IgG1, signifying a non-specific elevation in IgG1 concentration imparted by some unknown constituent(s) within the retinal homogenates harvested from untreated rats even though manufacturers reported only 0.9% cross-reactivity with rat IgG1. Furthermore, there was a paradoxical loss of sensitivity in retinal homogenates spiked with known concentrations of mouse IgG1 in that the read concentrations of antibody in the spiked retinal homogenates were lower than the known amounts that were added per well.

Consequently, we attempted to circumvent this problem by generating a standard curve using retinal homogenates spiked with known concentrations of IgG1. Our results confirmed that the retinal homogenate standard curves accurately determined the concentrations of IgG1; zero standards also accurately showed no readable levels of mouse IgG1 (**Figure 1**). A linear fit model with an intercept set at zero was the best fit for reading our unknown samples, and this was in keeping with the model recommended by the kits' manufacturer. On the other hand, a linear model was not the best fit for the standard curve generated using the assay buffer. Thus, for all of the reasons above, and as confirmed by the

non-overlapping standard curves as previously described, we read the values of our test samples off standard curves generated using retinal homogenates from untreated rats that were spiked with known concentrations of mouse IgG1.

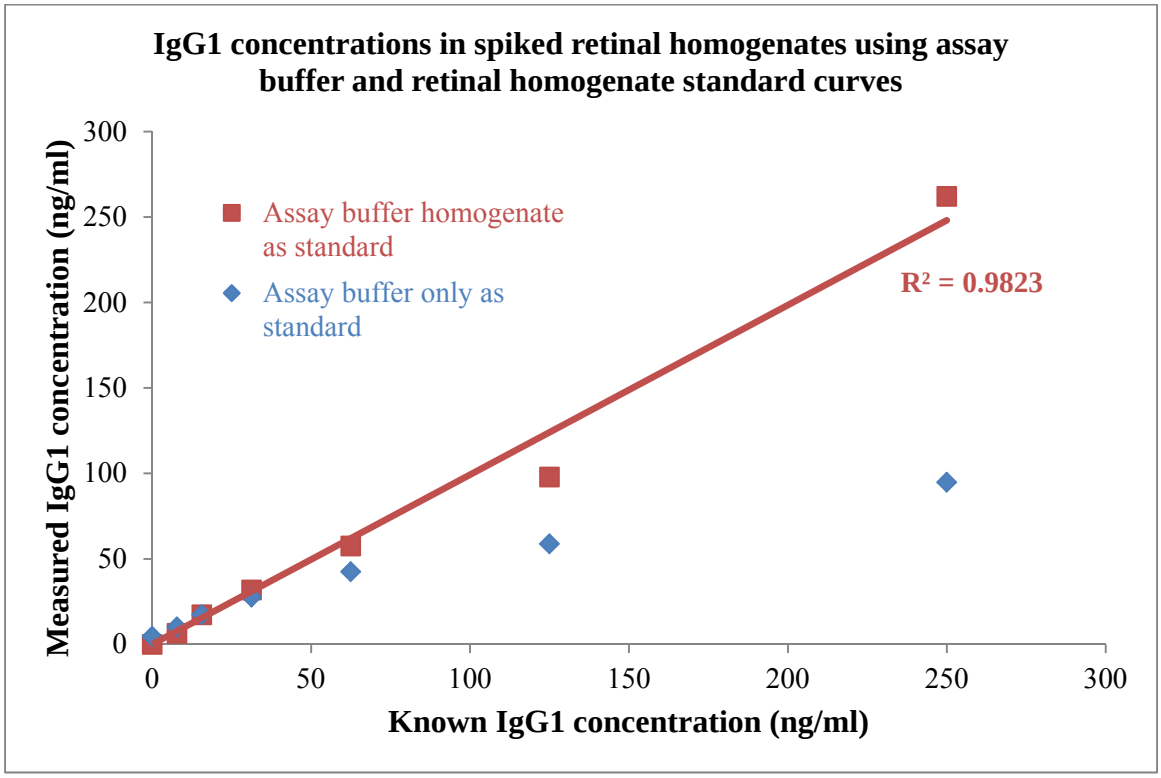


Figure 1. Standard curve generated per manufacturer instructions using assay buffer only registered artificially suppressed levels of IgG1 and did correspond to the known levels of mouse IgG1 added.

Topically applied mouse IgG1 antibodies accumulated in the optic nerve

Statistically significant levels of IgG1 were detected in the optic nerves in both the treated and contralateral, untreated eyes of rats 10 minutes post-eye drop application compared to controls ($p < 0.001$; **Figure 2**). IgG1 levels in optic nerve homogenates from treated eyes were 4.92 ± 2.08 ng per mg tissue, compared to 0.06 ± 0.06 ng IgG1 per mg tissue in control eyes ($p < 0.001$). Interestingly, and in concert with our previous studies (Koevary et al., 2002), untreated contralateral eyes within the same animal demonstrated increased levels of IgG1 compared to controls (0.84 ± 0.31 ng IgG1 per mg, $p < 0.01$), albeit at significantly lower values than compared to treated eyes ($p < 0.05$); this observation will be addressed in the Discussion.

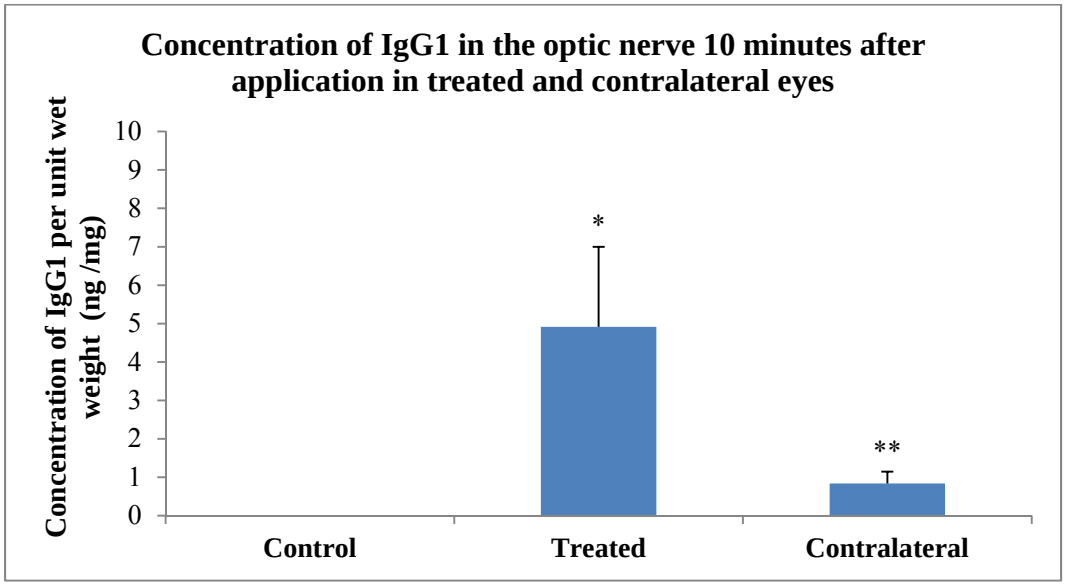


Figure 2. Levels of IgG1 in the optic nerves of treated and untreated, contralateral eyes were significantly higher than controls. IgG1 levels in treated and contralateral eyes were also significantly different from each other ($p < 0.05$). * $p < 0.001$ and ** $p < 0.01$ compared to untreated controls.

Topically applied mouse IgG1 antibodies accumulated in the retina

Similarly, statistically significant levels of IgG1 were detected in the retinas in both treated and contralateral, untreated eyes 20 minutes post-eye drop application ($p < 0.0001$, **Figure 3**) compared to controls. IgG1 levels in retinal homogenates from treated eyes were 8.07 ± 1.72 ng IgG1 per mg protein, compared to 0.17 ± 0.10 ng IgG1 per mg in untreated control eyes ($p < 0.0001$). Untreated contralateral eyes within the same animal also demonstrated increased levels of IgG1 compared to controls (1.83 ± 0.65 ng IgG1 per mg, $p < 0.001$). Again, levels of IgG1 in treated eyes were higher than in contralateral eyes ($p < 0.01$).

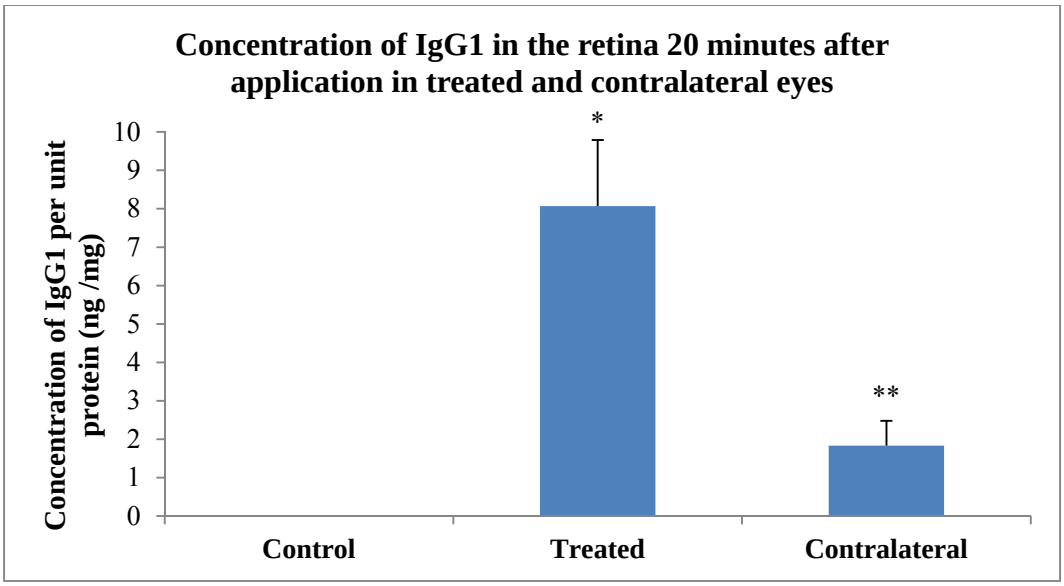


Figure 3. Levels of IgG1 in the retinas of treated and untreated, contralateral eyes were significantly higher than controls. IgG1 levels in treated and contralateral eyes were also significantly different from each other ($p < 0.01$). * $p < 0.0001$ and ** $p < 0.001$ compared to control.

Topically applied anti-ICAM-1 antibody accumulated in the retina

Anti-ICAM-1 antibody accumulated in the retina 20 minutes after topical application, though levels were less than those seen in our earlier experiments with IgG1. IgG1 levels in retinal homogenates from treated eyes were 1.90 ± 0.64 ng IgG1 per mg protein; no anti-ICAM-1 antibody was detectable in controls ($p < 0.05$, **Figure 4**). Unlike in our IgG1 experiments, there was no statistical significance in the concentrations of anti-ICAM-1 antibody levels between the untreated contralateral eyes and controls (contralateral: 0.89 ± 0.36 ng IgG1 per mg protein).

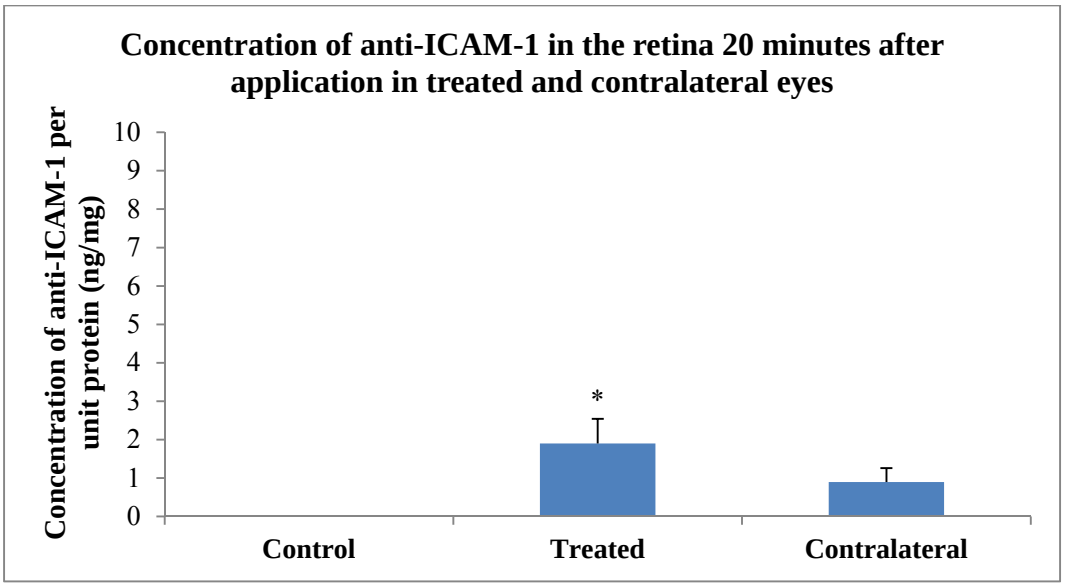


Figure 4. Levels of anti-ICAM-1 were significantly higher in the retinas of treated eyes compared to controls. There was no difference between antibody levels in contralateral and control eyes. * $p < 0.05$ compared to untreated controls.

Induction of retinal leukostasis

Recall that leukostasis was induced in rats by the injection of 100 µg of LPS, and retinas were harvested 24 hours, 48 hours, and 8 days after injection. Leukostasis was quantified by measuring the concentration of neutrophilic MPO. Levels of MPO were significantly elevated 24 hours post-injection (266.25 ± 53.88 ng MPO/mg protein; $p < 0.05$), and steadily declined to statistically insignificant levels 48 hours and 8 days later (148.54 ± 19.94 ng MPO/mg protein and 30.15 ± 18.25 ng /mg, respectively (**Figure 5**).

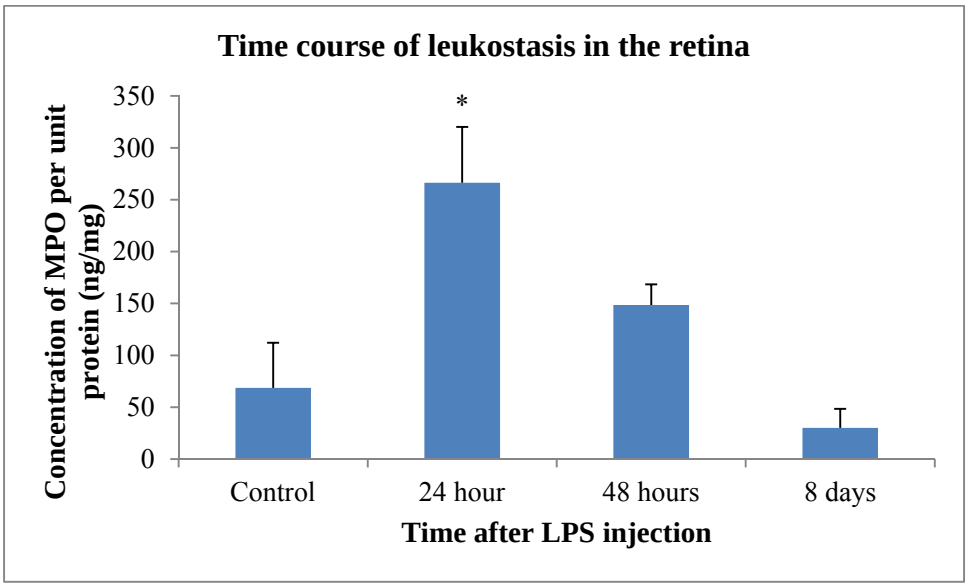


Figure 5. Levels of MPO in rat retinas peaked 24 hours after LPS injection and declined thereafter. * $p < 0.05$ compared to untreated controls.

Topically applied anti-ICAM-1 antibodies did not inhibit retinal leukostasis

Recall that in our first experiments, rats were topically treated with 10 mg/ml of anti-ICAM-1 antibody in their right eye four hours post-LPS injection; their left eyes were left untreated. Animals were sacrificed 24 hours post-LPS injection, and the retinas harvested.

As indicated in **Figure 6**, control animals did not have high levels of MPO; values were 62.81 ± 17.20 ng MPO per mg protein. Animals injected with LPS had higher levels of MPO in their retinas 24 hours post-induction (5103.98 ± 2139.98 ng MPO/mg protein). Treatment with anti-ICAM-1 antibody did not decrease the amount of leukostasis into the retina in either the treated or untreated contralateral retinas (treated: 6696.84 ± 2829.16 ng/mg; contralateral: 8523.20 ± 3003.55 ng/mg).

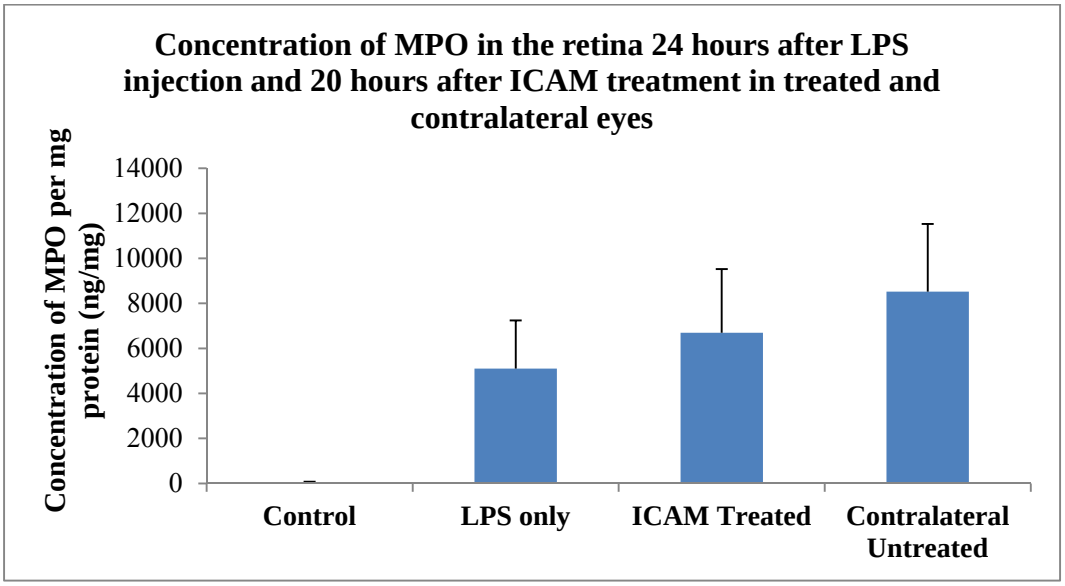


Figure 6. ICAM-1 antibody topically applied four hours post-LPS injection did not reduce the amount of leukostasis in either the treated or contralateral, untreated retinas.

In light of the above negative results, we compressed the treatment schedule down in the hopes of being able to suppress leukostasis. Accordingly, we administered antibody just 20 minutes after LPS injection and sacrificed the animals four hours later. Again, control animals had low levels of MPO (21.13 ± 1.92 ng MPO per mg protein) compared to animals injected with LPS (61.02 ± 17.43 ng/mg). Concentrations were likely lower in the LPS-treated animals here than in the animals sacrificed 24 hours post-induction because of the shortened incubation period. Statistical analysis revealed that animals injected with LPS tended to have higher levels of leukostasis than controls ($p > 0.0632$; **Figure 7**). Levels of MPO in eyes treated with anti-ICAM-1 were similar to those in the positive controls (treated: 62.40 ± 16.59 ng/mg protein), though there was a tendency for the contralateral eyes to have lower levels of leukostasis compared to the positive controls (contralateral: 23.56 ± 3.76 ng/mg; $p > 0.0803$).

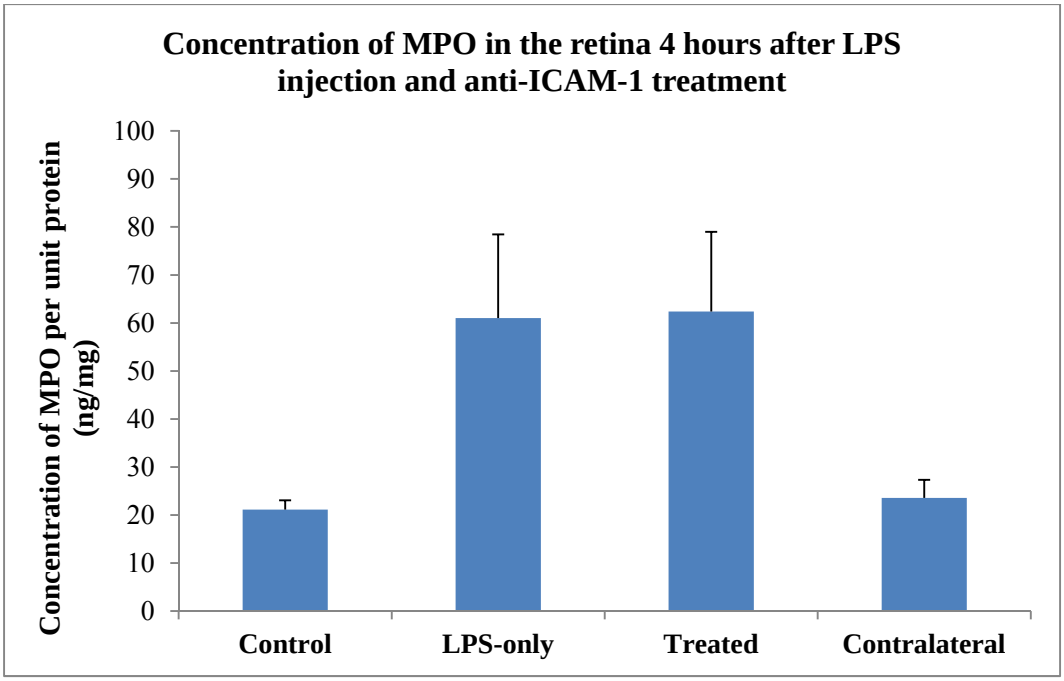


Figure 7. Topical treatment with anti-ICAM-1 antibody immediately following LPS injection did not lead to a reduction in leukostasis into the retina. Similarly, there was a statistically insignificant difference in the amount of leukostasis between the treated and contralateral eyes.

VEGF Bioassay

VEGF had a dose dependent effect on HUVEC cell growth (**Figure** 9**8**). Statistical analysis revealed a significant reduction in cell growth between cultures incubated with a dose of 100 ng/ml compared to all other doses below 25 ng/ml ($p < 0.001$). **Figure** 9**9** shows phase contrast microscope photographs of HUVECs cultured in high or low concentrations of VEGF. In the relative absence of VEGF, cells appeared clumped, detached and non-viable, while the cells cultured with VEGF appeared healthy and were attached. Based on the results of this assay, we used VEGF at a concentration of 100 ng/ml in all subsequent assays.

The addition of anti-VEGF antibody to cultured HUVECs significantly suppressed their growth in a dose dependent fashion (**Figure 10**). HUVECs cultured in the absence of VEGF served as negative controls, and their growth was also significantly reduced compared to cultures that contained VEGF. Retinal homogenates from the eyes of rats treated with anti-VEGF antibody 10 or 20 minutes earlier significantly suppressed HUVEC proliferation, as determined by the MTT assay (**Figure 11**; $p < 0.0001$). Though both time points were significantly different than controls, the nadir appeared to be at 10 minutes, since the 20 minute values were also significantly higher than those at the earlier time point ($p < 0.001$).

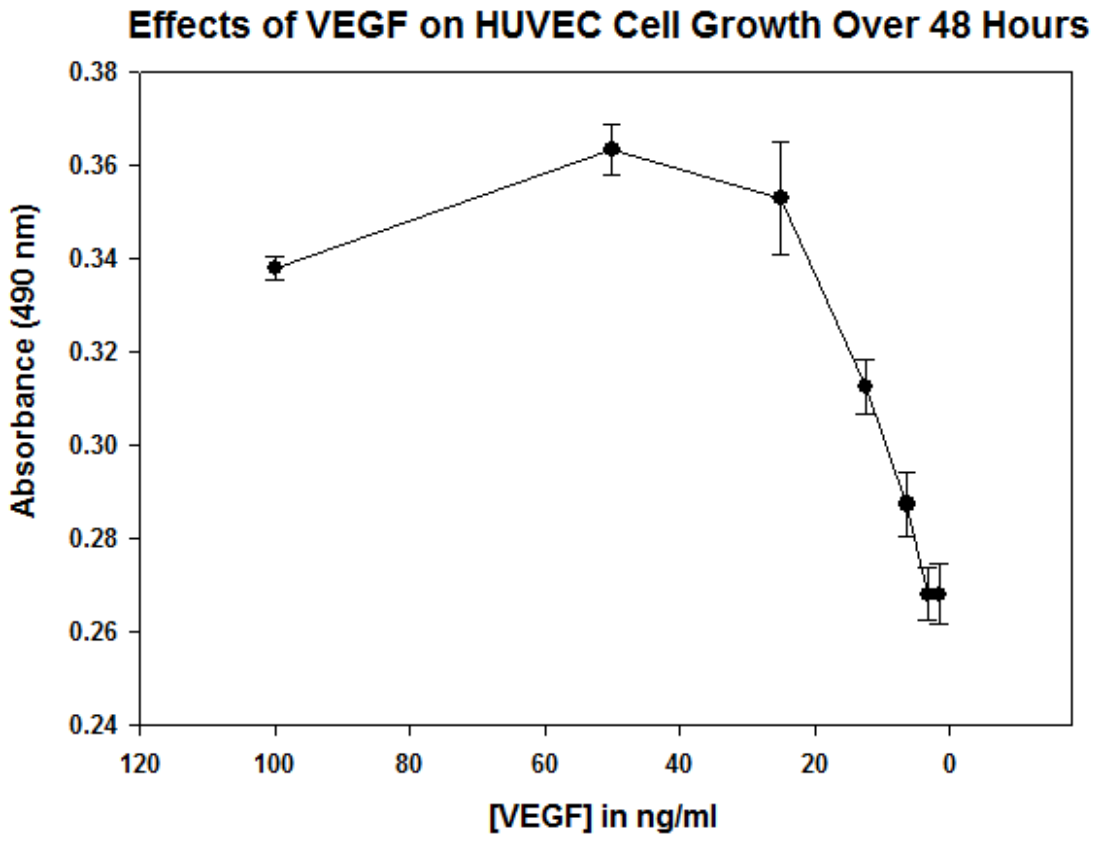


Figure 8. HUVEC cell growth was dependent on VEGF in a dose-dependent manner.

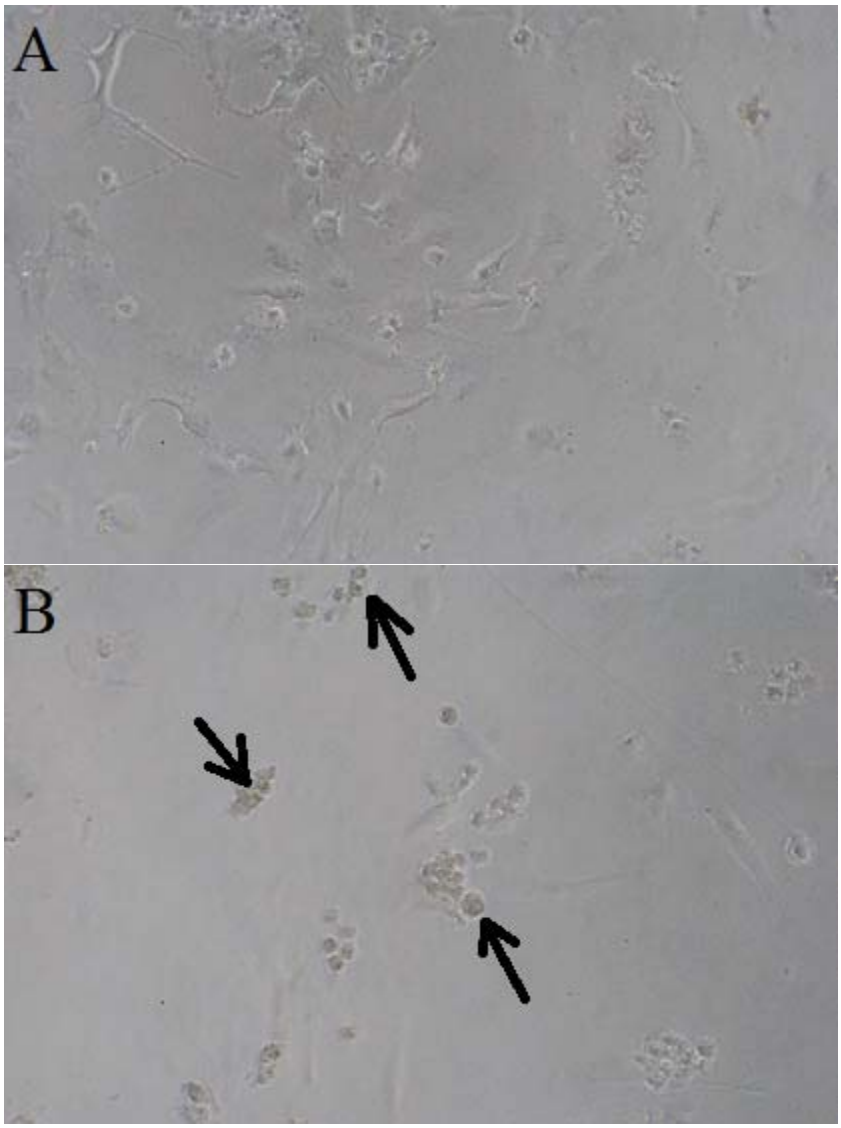


Figure 9. Phase contrast microscopy of HUVECs cultured in (A) high (100 ng/ml) and (B) low (1.25 ng/ml) concentrations of VEGF. Note the detached, clumped dead cells (B, arrows) compared to the healthy, attached cells in (A).

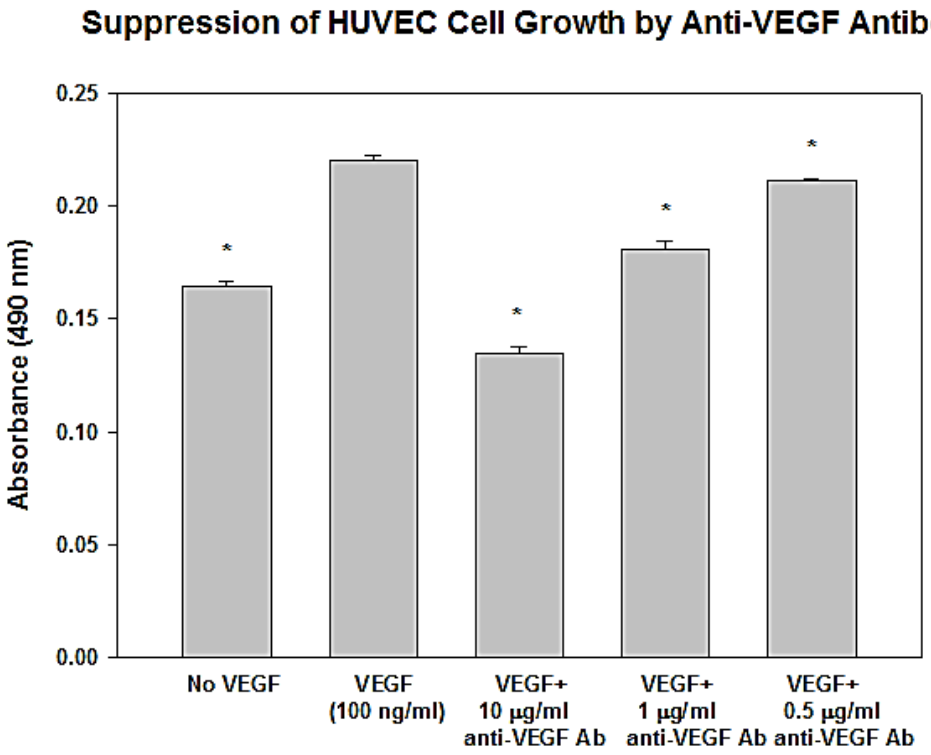


Figure 10. Anti-VEGF antibody added *in vitro* inhibited HUVEC growth in a dose-dependent manner. * $p < 0.01$ compared to VEGF alone.

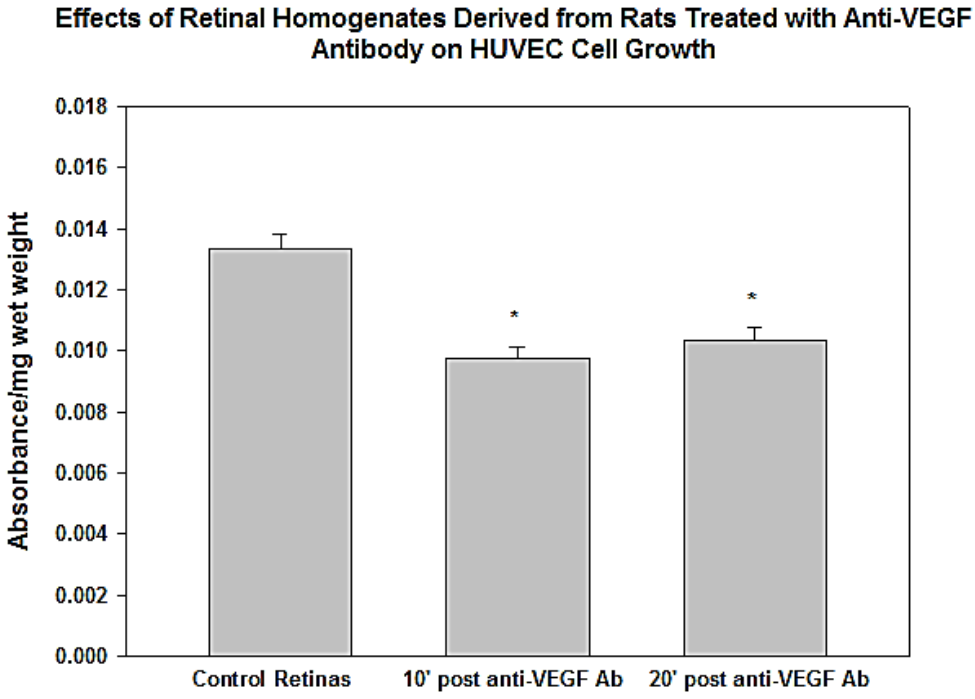


Figure 11. Retinal homogenates from eyes treated with anti-VEGF antibody inhibited proliferation of HUVECs *in vitro*. Animals were sacrificed either 10 or 20 minutes after topical anti-VEGF antibody application. * $p < 0.001$ compared to control.

DISCUSSION

Our data support the hypothesis that full-sized antibodies, applied topically together with a permeation enhancer, can accumulate in the rat optic nerve and retina. Specifically, polyclonal IgG1 and anti-ICAM-1 antibodies were detectable in the posterior pole less than 30 minutes after topical application. This time course is consistent with our previous results with topically applied insulin and glial derived neurotrophic factor (Koevary et al., 2004, 2002; Robinson, Mayo, Lee, & Koevary, 2006). Interestingly, the contralateral eyes also accumulated both IgG1 and anti-ICAM-1 following topical application, a phenomenon that we and others have seen in our previous studies with other peptides. Intravitreal and subconjunctival injection of antibody in rabbits both resulted in accumulation in the untreated, fellow eye; in those studies, the authors speculated that this might be due to uptake following systemic absorption (Nomoto et al., 2009). Our previous data, however, showed that contralateral uptake was even found in decapitated animals, indicating that systemic uptake was likely not the mechanism for this uptake in the contralateral eye (Koevary et al., 2002; Patsiopoulos, Lam, Lake, & Koevary, 2003). In light of our findings of high levels of topically applied peptides in the cerebrospinal fluid (CSF), we previously speculated that these peptides reached the posterior pole of the contralateral eye by diffusing through the CSF that surrounds the optic nerve and chiasm. Our previous data also suggested that while systemic uptake following topical application does occur, it did not influence the levels of peptide recovered in retinal homogenates, nor did it appear in under 30 minutes (Koevary et al., 2003); recall that our working time frame was 10 and 20 minutes post application. In our current study, we were not able to recover measurable amounts of IgG1 from serum at 10 and

20 minutes post application, though we were able to measure IgG1 in serum that was spiked with antibody (data not shown).

An important second aim of our study was to determine whether antibodies that accumulate in the retina following topical application retain their therapeutic potential. We developed both an *in vivo* and *in vitro* model to test this. The *in vivo* model was based on the known ability of anti-ICAM-1 antibodies to suppress leukostasis (Barouch et al., 2000; Miyamoto et al., 1999). While we were able to induce leukostasis four and 24 hours after LPS injection, we were unable to demonstrate a reduction in neutrophil concentration in animals treated with topical anti-ICAM-1 antibodies. This may have been due to the relatively low concentration of anti-ICAM-1 antibody present in our eye drop formulation. The shorter time course of treatment with immediate anti-ICAM-1 antibody and sacrifice 4 hours after LPS injection was selected in the hopes of halting leukostasis early in its development. Interestingly, ocular levels of MPO were lower in contralateral, untreated eyes harvested in this shorter time course compared to both positive controls and directly treated eyes. Though this difference was not statistically significant, this finding may have been due to the diffusion of peptide from the back of the treated eye, through the CSF, to the contralateral eye as described above; future studies using higher concentrations of anti-ICAM-1 antibody will need to examine this further. Since leukostasis is a dynamic process, and since it is as yet unclear as to how long these large antibodies are retained in the retina following topical application, it was likely that our single treatment was not sufficient to create a detectable inhibitory effect on neutrophil movement into the retina in the treated eye.

Due to cost limitations, we were unable to assess the effects of a higher concentration of anti-ICAM-1 antibody or multiple anti-ICAM-1 antibody treatments.

In light of these negative results, we developed an alternative, *in vitro* bioassay model to examine whether antibodies that accumulated in the retina following topical application retain their ability to effect pharmacological changes. Specifically, the model we developed was based on the need for VEGF for the growth of HUVECs. Anti-VEGF antibody was topically applied to rat eyes, and retinas were harvested and homogenized, and the homogenates were added into the VEGF-containing growth media for HUVECs. Our results showed that these anti-VEGF treated retinal homogenates suppressed cell growth and proliferation, supporting the notion that the topically delivered antibodies mediated this effect. It is important to note that this bioassay only indirectly suggested that anti-VEGF antibody was present in the retinal homogenates because of its *in vitro* nature, though this circumstantial evidence is strong. Further experiments would need to be carried out in order to confirm the presence and activity of this antibody *in vivo*.

Our data showing both directly and indirectly that topically applied antibodies accumulate in the retina and optic nerve, go against the conventional wisdom that suggests that molecules larger than 40 kDa are not able to reach the posterior pole in detectable levels because of limitations such as nasolacrimal drainage, tear protein metabolism, and systemic absorption (Kamei, Misono, & Lewis, 1999; Marmor, Negi, & Maurice, 1985; Nomoto et al., 2009; Peyman & Bok, 1972). That being said, there have been other reports of antibody movement to the posterior pole following topical application. Chen et al., showed that topically-delivered ranibizumab (Lucentis), the 48 kDa antibody fragment of the parent anti-

VEGF antibody bevacizumab (Avastin), is able to reach the retina in rabbits, though their sample sizes were small, and not all of their animals had detectable antibody levels in these tissues. Furthermore, the authors used confocal immunohistochemistry to localize the antibody and did not take quantitative measurements (Chen, Ebmeier, Sutherland, & Ghazi, 2011).

Nomoto et al., compared the pharmacokinetics of bevacizumab after topical delivery, subconjunctival injection, and intravitreal injection in rabbits. The authors found that intravitreal injections resulted in the highest concentrations of the antibody in the retina, but they also found detectable levels of bevacizumab in the retina/choroid in both treated and contralateral, untreated eyes following topical application at all time points tested. The authors did not state whether these levels were significantly different between the two eyes, or whether they were significantly higher than controls. As in the study by Chen et al., the authors did not evaluate antibody levels in the retina at the same time points as we did in our study. Furthermore, the data presented in the study were adjusted per retinal wet weight, which may be somewhat less reliable than expressing the data per unit protein for the reasons explained in the Materials and Methods. Finally, the authors did not address the issue as to whether retinal bevacizumab was able to inhibit the activity of VEGF. Our studies not only quantified the levels of antibody that accumulated in the retina following topical application, but also provided suggestive evidence that the antibodies that accumulate there may retain their pharmacological activity.

As the Baby Boomer generation ages, it is expected that the prevalence of patients with chronic retinal vascular disease requiring regular treatments will also increase, thereby

posing a challenge regarding their safe delivery. As mentioned, the development of anti-VEGF agents has certainly revolutionized the prognosis for patients with such vision-impairing diseases as wet AMD, proliferative diabetic retinopathy, and macular edema. Recall, however, that these agents need to be delivered to the posterior segment on a regular treatment regimen in order to retain their effectiveness and such intravitreal delivery is not without adverse local and systemic side effects.

While our data support the contention that topically applied antibodies can reach the retina, levels detected there were several orders of magnitude less than the concentration of the solution that was applied to the ocular surface. Similar reductions were reported following topical application of antibodies in the rabbit (Urtti et al., 1990). Thus, high concentrations would have to be applied to get potentially therapeutic levels into the retina; interestingly, our bioassay results suggest this can be achieved, since the concentrations of antibody that we applied to the surface of rat eyes were similar to the concentrations of stock solutions of bevacizumab and ranibizumab used for the treatment of humans (Martin et al., 2011). In one study it was reported that the minimum concentration of bevacizumab needed to inhibit VEGF *in vitro* was 975 ng/ml (Klettner & Roider, 2008), while another showed that complete inhibition of VEGF activity by bevacizumab could be achieved with significantly lower concentrations (Wang, Fei, Vanderlaan, & Song, 2004). Thus, inhibition of VEGF activity in the retina is likely possible after topical delivery provided that the initial drop concentration is sufficiently high; however, this would render such an approach fairly expensive, since topical application in humans would likely require multiple drop treatments;

the main advantage of the use of bevacizumab over ranibizumab was its decreased cost (Stein, Newman-Casey, Mrinalini, Lee, & Hutton, 2013).

Another potential advantage of topical administration of anti-VEGF is its decreased accumulation in the systemic circulation. Nomoto et al., showed that topical administration of bevacizumab six times a day for seven days resulted in detectable levels of the antibody in the plasma one week later, but others reported that systemic concentrations of a much smaller antibody fragment ESBA105 (a 26 kDa anti-TNF antibody) was 25,000 times lower after topical administration compared to systemic concentrations after intravitreal injection (Boddu, Gupta, & Patel, 2014). Intravitreal injection of anti-VEGF agents has a well-established association with systemic adverse events. The injected agents were quickly detected in the blood stream one day after the first dose, and resulted in a marked reduction in VEGF in the plasma that persisted for as long as 28 days (Avery et al., 2014; Carneiro et al., 2012). Likely as a result of their detectable inhibitory effect on plasma-free VEGF, administration of these agents is associated with an increased risk of systemic adverse events such as myocardial infarction, cerebrovascular accidents, transient ischemic attacks, deep vein thrombosis, pulmonary embolism, and death (Costagliola et al., 2012; Falavarjani & Nguyen, 2013; Tolentino, 2011; van der Reis et al., 2011). A Cochrane review found that 139 per 1000 patients treated with ranibizumab experienced at least one systemic adverse event after one year, compared with 177 per 1000 patients treated with bevacizumab; the difference in systemic safety profiles between the two drugs was not significant (Moja et al., 2014; Solomon, Lindsley, Vedula, Krzystolik, & Hawkins, 2014). Thulliez et al., concluded that intravitreal anti-VEGF antibodies did not result in statistically significant increases in major

cardiovascular or systemic hemorrhages, but agreed that "studies and meta-analyses were not powered enough to correctly assess these risks" (Thulliez et al., 2014). Thus, caution is still urged for patients receiving intravitreal injections of anti-VEGF antibodies who are especially at risk for systemic adverse events.

While data collected from all of our previous studies suggest only minimal systemic absorption following topical application, an extensive time course of antibody uptake in the bloodstream was not investigated. It is possible that over time, antibody levels would rise in the blood. This could result in systemic consequences that would need to be addressed if this approach is to be seriously investigated as an alternative to intravitreal injection.

Our study did not directly investigate whether topically applied anti-VEGF antibodies could prevent neovascular retinal diseases. Prior to assessing this, further research would need to be done using a permeation enhancer other than saponin; while saponin was approved for use in humans, it is not the preferred compound in light of the discomfort it causes. Further research is needed to assess how long the antibodies stay in the optic nerve and retina before elimination, and whether they can suppress VEGF activity in a primate model.

Thus, we have shown here that topically delivered full sized antibodies can accumulate in the retina and optic nerve, and provide suggestive evidence based on our *in vitro* model that they retain their pharmacological activity. There remain multiple hurdles that must be overcome in order to effectively treat posterior segment diseases with topical drops, notably the marked drop in the concentration of drug that reaches the retina and the potential for significant systemic uptake should multiple weekly treatment be required.

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