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Posterior Vitreous Detachment With Microplasmin Alters the Retinal Penetration of Intravitreal Bevacizumab (Avastin) in Rabbit Eyes

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Abstract

Purpose—Intravitreal bevacizumab (Avastin) is frequently used for the treatment of age-related macular degeneration. Previous studies have demonstrated full thickness retinal penetration. Intravitreal recombinant microplasmin (MP) has been shown to successfully induce a posterior vitreous detachment (PVD) and vitreous liquefaction in animals. It has been suggested that a PVD may alter the retinal penetration of molecules in the vitreous cavity. The aim of this study was to compare bevacizumab (BV) retinal penetration in rabbit eyes with and without a MP-induced PVD.

Methods—Twelve adult rabbits were injected with 0.1 ml (0.4 mg) of MP into the vitreous cavity of one eye. One week later, the rabbits were injected with 0.05 ml (1.25 mg) of BV into both eyes. Both eyes of three rabbits each were harvested at 6, 12, 24, and 72 hours after the BV injection. Frozen retinal cross sections were prepared, and BV retinal penetration was evaluated with immunohistochemistry using a fluorescence-labeled antibody against BV. Two eyes from one rabbit were not injected with either agent and used as controls to compare the background autofluorescence. Peripapillary retinal sections were recorded with a digital camera, and intra-retinal BV fluorescence-labeled antibody was measured by qualitative photographic interpretation. Two additional rabbits received an intravitreal injection of 0.1 ml of MP in one eye. One week later, both eyes from each rabbit were enucleated and frozen retinal sections were prepared and analyzed with light microscopy to evaluate for histologic damage.

Results—Full thickness BV retinal penetration was observed throughout the retina in both eyes of each rabbit. All of the MP-injected eyes exhibited increased antibody labeling in retinas evaluated 6, 12, and 24 hours after BV injection when compared with the contralateral non-MP-injected eyes. By three days after BV injection, all eyes demonstrated decreased antibody labeling compared to earlier time periods. At three days, one rabbit showed increased antibody labeling in the retina of the non-MP-injected eye compared with the contralateral MP-injected eye, and two

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rabbits exhibited similar antibody labeling in both eyes. When compared with control eyes, light microscopy demonstrated normal retinal histologic findings in eyes injected only with microplasmin.

Conclusions—Increased BV retinal penetration is observed initially in eyes with a MP-induced PVD, and the mechanism is likely multifactorial. By three days, retinal penetration is similar in eyes with and without a PVD. Although it is difficult to directly extrapolate to humans, our study suggests a PVD may alter the retinal penetration of BV.

Keywords

Bevacizumab; Avastin; Microplasmin; Posterior vitreous detachment; Retinal penetration

Age-related macular degeneration (AMD) is a leading cause of severe, irreversible vision loss in developed countries.^{1,2} Choroidal neovascular membranes (CNVMs) are responsible for nearly 90% of profound central visual loss (visual acuity 20/200 or worse) resulting from neovascular AMD.³ Vascular endothelial growth factor (VEGF) is thought to play a major role in the formation of CNVMs in neovascular AMD.⁴ Anti-VEGF pharmacotherapy, delivered by intravitreal injection, is considered a safe and effective treatment for neovascular AMD.⁵ Pegaptanib sodium (Macugen; Eyetech, New York, NY) and ranibizumab (Lucentis; Genentech, Inc., San Francisco, CA) are the only two anti-VEGF medications that are currently approved by the Food and Drug Administration (FDA) for intravitreal use to treat all subtypes of neovascular AMD.

Bevacizumab (Avastin; Genentech, Inc., San Francisco, CA) is a recombinant, humanized monoclonal IgG antibody that binds to and inhibits the biologic activity of human VEGF. It is approved by the FDA for intravenous use in the treatment of advanced colorectal, lung, and breast cancer. Numerous reports have supported the efficacy of off-label intravitreal bevacizumab (BV) in the treatment of neovascular AMD,⁶⁻⁹ and it is currently used by many retina specialists as a first-line therapy (Mittra RA and Pollack JS, eds. Preferences and trends survey. Poster presented at: 26th Annual ASRS meeting, October 11-15, 2008, Maui, Hawaii).

Previous studies have demonstrated full thickness retinal penetration of intravitreal bevacizumab in rabbit¹⁰ and monkey¹¹ eyes. These studies, however, did not evaluate the presence or absence of a posterior vitreous detachment (PVD). It has been suggested that a PVD may alter the flow of molecules that cross the vitreoretinal (VR) junction.¹² Microplasmin (ThromboGenics NV, Leuven, Belgium) is a recombinant protein that is a truncated form of human plasmin.¹³ A single intravitreal injection of microplasmin (MP) has been shown to induce vitreous liquefaction^{14,15} and a complete PVD¹⁶⁻¹⁹ in animal and postmortem human eyes. Quiram et al.¹⁸ showed that a MP-induced PVD increases vitreous oxygen levels and increases the rate of oxygen exchange within the vitreous cavity in animal models. In addition, data from rats suggest a microplasmin-induced PVD alters the level of oxygen in the crystalline lens.¹⁹ These findings have led to the hypothesis that a PVD may also influence the retinal penetration of drugs that are injected into the vitreous cavity.¹² The aim of our study was to compare BV retinal penetration in rabbit eyes with and without a MP-induced PVD.

Methods

Animals

Fifteen adult albino rabbits (Kuiper Rabbit Ranch, Indianapolis, IN) weighing 2.0 to 2.5 kg were included in the study. Fourteen rabbits received intravitreal injections (outlined below),

and one rabbit received no injections (referred to as the control eyes). Both control eyes were fixed and processed in an identical manner as the non-control eyes in order to evaluate background fluorescence. The rabbits were allowed free access to food and water and were housed under 12 hour light/dark cycles. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Oakland University animal care and use committee. Prior to intravitreal injections, the rabbits were anesthetized with 40-44 mg/kg Ketamine and 7 mg/kg Rompun (xylazine). Topical anesthesia with proparacaine hydrochloride ophthalmic solution 0.5% was given for additional comfort. The pupils were dilated with topical phenylephrine 2.5% and tropicamide 1%.

Intravitreal injections

Twelve rabbits were injected with 0.1 ml (0.4 mg) of MP into the left eye. One week later, the rabbits were injected with 0.05 ml (1.25 mg) of BV into both eyes. The dose of MP employed was more than three times the minimum concentration needed to successfully induce a complete PVD with an internal limiting membrane devoid of vitreous collagen fibrils in rabbits.¹⁶ The chosen dose of BV is the same as that used to treat neovascular AMD in humans.^{6,7}

For all injections, a speculum was inserted to expose the limbus, and betadine 10% solution was applied to the injection site. A 30-gauge needle attached to a 1.0 ml tuberculin syringe was inserted approximately 1 mm posterior to the limbus under direct visualization using a Zeiss operating microscope. The drug was then slowly injected into the midvitreal cavity. Following the intravitreal injection, a single dose of topical moxifloxacin was administered.

Immunohistochemistry

All rabbits were sacrificed with an intracardiac injection of sodium pentobarbital 70mg/kg body weight after sedation with Ketamine/Rompun. Three rabbits were killed at the following intervals after BV injections: 6 hrs, 12 hrs, 24 hrs, and 3 days. Both eyes from each rabbit were fixed in 4% paraformaldehyde for 48 hrs. The eyes were washed in phosphate buffered saline (PBS) and infiltrated overnight with 20% sucrose in PBS. After overnight incubation, the tissue was embedded in OCT containing 20% sucrose (1:2), sectioned at 50µm, and mounted on poly-L lysine (Sigma) coated slides. Sections were blocked in 5% goat serum in PBS, and incubated with goat anti-human antibody (1:200 dilution) conjugated to AlexaFluor-488.

Whole eye sections through the optic nerve were evaluated. Because the rabbit retina does not contain a macula, we evaluated and compared the peripapillary retina to approximate a posterior location. Slides were viewed on a Nikon epifluorescence microscope with an attached SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Exposure times for photographs were kept constant for each animal. Retinal penetration was measured by photographic interpretation.

Light Microscopy and Electrophoresis

We used a relatively high concentration of MP in order to ensure the presence of a PVD since there is a direct correlation between increasing concentrations of MP and the degree of PVD.^{16,17} In order to evaluate for potential retinal toxicity, we injected 0.4 mg microplasmin into the left eye of two additional rabbits. The right eyes did not receive any injections (control eyes). One week after injection, all eyes from both rabbits were fixed and embedded as previously described above. Horizontal sections through the optic nerve were stained with hematoxylin and eosin and evaluated with light microscopy for evidence of structural damage.

It is unknown if MP is able to digest BV into smaller fragments, resulting in improved BV retinal penetration. Although it is unlikely that MP remains in the vitreous cavity one week after injection, the half-life of MP in the vitreous cavity is unknown. Therefore, we incubated 1.25 mg of BV with 0.4 mg of MP for 24 hours and then performed a SDS-PAGE analysis in order to determine whether or not MP digests BV.

Results

Bevacizumab antibody labeling was observed throughout the retina in both eyes of each rabbit. In all eyes, the highest concentration of antibody labeling was detected along the internal limiting membrane (ILM). In eyes that had been injected with BV 6 hrs prior to being harvested, all three of the MP-injected eyes demonstrated increased BV antibody labeling when compared with the non-MP-injected eyes (figure 1). Specifically, increased antibody labeling was present within the ganglion cell layer, inner and outer nuclear layers, in Muller cells extending across the retina, and in the photoreceptor outer segments. At higher magnification, increased antibody labeling is clearly visible in the MP-injected eye when photograph exposure time is held constant (figure 2).

BV antibody labeling is persistently increased in the MP-injected eye 12 hrs after injection (figure 3). In contrast, most of the antibody labeling in the non-MP-injected eyes is present along the ILM with less intense labeling in the inner and outer retina. There was mild variability in the amount of antibody labeling; however, similar findings were seen in all three rabbits sacrificed 12 hrs after BV injection.

At 24 hrs after BV injection, the antibody labeling remains increased in all of the MP-injected eyes in all layers of the retina (figure 4). Of note, many of the non-MP-injected eyes demonstrated an increased amount of vitreous just anterior to the retina, and antibody labeling was also detected in this vitreous. This finding was anticipated since these eyes were not expected to have vitreous liquefaction or a PVD.

By 3 days after BV injection, all eyes exhibited significantly less intense antibody labeling, compared with earlier time periods. When the brightness and contrast were increased, all eyes continued to show faint labeling above the background autofluorescence which was seen in the uninjected control eyes (figure 5). Using the same microscope and contrast settings, increased antibody labeling was detected in the non-MP-injected eye of one rabbit and only faint intraretinal labeling in the MP-injected eye (figure 5). Two rabbits demonstrated similar antibody labeling in both eyes (results for one animal shown in figure 6).

In the two rabbits injected with MP only, light microscopy demonstrated normal retinal histologic findings in both the microplasmin-injected and control eyes. Furthermore, no inflammatory cells were seen in the retinal specimens from any of the eyes. After incubating MP with BV, the SDS-PAGE analysis shows that the molecular weight of BV is unchanged in the absence or presence of MP (figure 7). These results indicate that MP is not able to digest BV, and the observed increased penetration of BV into the retina was not the result of BV degradation.

Discussion

We have confirmed findings from previous studies^{10,11} demonstrating full thickness retinal penetration of intravitreal BV in animals. We have also confirmed that MP does not digest BV into smaller fragments. Our results suggest the initial retinal penetration of BV is increased in rabbit eyes with a MP-induced PVD. Our findings support the theory that an attached posterior hyaloid (i.e. absence of a PVD) may create a semi-permeable barrier to

molecules that cross the VR junction, and a PVD may allow for increased diffusion across this junction. If the posterior cortical vitreous does, in fact, limit diffusion from the vitreous into the retina, then the rate of movement may be determined by specific characteristics of the diffusing molecule, such as its size and ability to bind other molecules.

When a molecule in the vitreous cavity diffuses into the retina in an eye without a PVD, it must first traverse through the posterior hyaloid. The posterior cortical vitreous consists of densely packed collagen fibrils arranged parallel to the retinal surface.²⁰ In cat eyes without a PVD, the cortical vitreous appears more densely compressed at the retinal surface as opposed to microplasmin-injected eyes which demonstrate a smooth retinal surface that is free of cortical vitreous.¹⁸ Perhaps these collagen fibrils act as a “filter” and prevent or slow the movement of large molecules from diffusing into the retina. Thus, it is possible that an attached posterior hyaloid presents a physical impediment to some molecules diffusing into the retina. BV is a relatively large molecule (molecular weight 149 kDa), and it is more than twice the size of albumin (molecular weight 68 kDa). Both molecules have been shown to achieve full thickness retinal penetration in animal studies that did not control for a PVD.^{10,11,21} However, our results show that a PVD increases the initial retinal penetration of BV, and perhaps this is due to the removal of the posterior hyaloid’s “filter.” It is possible that a PVD allows for improved diffusion of both small and large molecules across the VR junction; however, we are unable to substantiate this theory since we did not evaluate the retinal penetration of other molecules.

Connections between the vitreous and retina may provide a barrier to molecular diffusion due to specific binding properties. Laminin and fibronectin are two of the main glycoproteins that bind vitreous collagen fibers of the posterior vitreous cortex to the ILM.²² The failure of tissue plasminogen activator (tPA) to penetrate the retina has been attributed to its specific binding to fibronectin and other proteins at the VR juncture.²³ Fibronectin has been shown to interact with and bind all subclasses of IgG, including IgG1.²⁴⁻²⁶ BV is a monoclonal IgG1 antibody, and thus, it is reasonable to assume that BV is capable of binding fibronectin at the VR junction. Microplasmin’s ability to induce a PVD has been attributed to its active protease site which is capable of hydrolyzing several proteins, including fibronectin.^{27,28} Thus, fibronectin is degraded during a MP-induced PVD and is, therefore, unlikely to bind intravitreal BV at the VR junction which may facilitate its retinal penetration. Although this theory seems plausible, it is unclear if this same relationship exists in humans during a spontaneous PVD *in vivo*.

It is interesting that increased retinal penetration of BV in eyes with a PVD eventually slowed to an amount comparable to that of eyes without a PVD by three days. This observation may be due, in part, to microplasmin’s ability to liquefy the vitreous gel, and to the distribution of intraocular BV. Stefánsson has suggested the transport of all molecules to and from the retina is increased following a PVD due to the lower viscosity of liquefied vitreous.²⁹ Sebag showed that microplasmin leads to a decrease in vitreous bulk viscosity,¹⁴ and it also substantially increases vitreous diffusion coefficients in intact porcine eyes.¹⁵ Thus, vitreous liquefaction may enable a molecule in the vitreous cavity to acquire improved “mobility” or diffusion within the intraocular space. This may allow intravitreal BV to diffuse toward and reach the VR junction sooner, and it may also enable the molecule to leave the intraocular cavity via alternative exit routes. Intravitreal BV has been shown to penetrate into the iris, ciliary body, and anterior chamber angle shortly after injection, and its distribution in the anterior chamber angle is time-dependent.³⁰ Two exit pathways have been described to explain the distribution and the decline in ranibizumab levels in the vitreous cavity and aqueous humor.³¹ One pathway is directly through the retina to the choriocapillaris; the other is anterior through the aqueous drainage route. Therefore, it is possible that increased initial retinal penetration of BV is countered by increased exit

through the aqueous and trabecular meshwork. Furthermore, after injection of 1.25 mg intravitreal BV into a rabbit eye, the half-life in the vitreous was found to be 4.32 days.³² By three days after injection, it is possible that there are so few remaining BV molecules in the vitreous cavity that any advantage provided by a MP-induced PVD is now rendered obsolete.

Consequently, the treatment effect of BV may be diminished if vitreous liquefaction from microplasmin shortens the half-life of intravitreal BV. Thus with a microplasmin-induced PVD, it is possible that BV may have a shorter duration of effect resulting in lower overall efficacy even though the short-term penetration might be superior. This presumption suggests the vitreous may have a beneficial effect by serving as a drug reservoir during treatment. Even still, it is known that intravitreal BV results in high plasma concentrations and distribution into the intraocular tissues in fellow eyes via the systemic circulation.³³ Therefore, a microplasmin-induced PVD with vitreous liquefaction may also lead to elevated plasma and contralateral eye concentrations. Nevertheless, our study was not designed to specifically evaluate the ocular distribution of BV, and future studies are warranted to address these theories.

Our study has several limitations that should be addressed. Although, we cannot exclude the possibility that our dose of microplasmin was toxic to the retina and thereby promoted increased BV retinal penetration, it seems unlikely given the normal histologic appearance of the retina. Bevacizumab retinal penetration was measured by qualitative photographic interpretation. A quantitative analysis in future studies may provide a more objective measurement of retinal penetration. In addition, the retinal penetration of an intravitreal molecule may vary by region. Since neovascular AMD predominantly affects the macula, we only examined and compared the peripapillary retina in order to control for a consistent posterior location. Therefore, our findings are limited to this small sample of retina, and extrapolation of our results to include the entire retina should be used with caution. The rabbit eye is a well-established model used to study intravitreal pharmacokinetics of other drugs. Limitations of the rabbit model are that the retina is less vascular than in humans, the volume of the rabbit eye is much smaller than the human eye (1.5 ml and 4.5 ml, respectively), and the crystalline lens is larger than in humans.³² These factors may result in altered pharmacokinetics³² and retinal penetration as compared with the human eye.

Despite our study's limitations, we have demonstrated a clear and consistent difference between initial retinal penetration of intravitreal BV in eyes with and without a PVD. Our findings add to the growing evidence that a PVD may have a significant influence on disease pathophysiology. A higher incidence of attached posterior vitreous has been reported to occur in patients with all types of AMD (exudative and nonexudative).³⁴ Additional studies have concluded that persistent attachment of the posterior vitreous cortex to the macula may be a risk factor for the development of exudative AMD.^{35,36} Our study is the first to show that intravitreal medications used to treat AMD may be influenced by a PVD. Because of artifactual retinal detachment during histologic specimen processing, we were unable to accurately evaluate the subretinal space. Therefore, it is unclear if the increased retinal penetration seen initially also leads to increased subretinal BV and improved clinical results. However, drug injection studies into the vitreous currently consider all vitreous cavities to be equal. In contrast, all vitreous cavities are not the same since vitreous liquefaction and PVD formation occurs to varying degrees in each eye. Researchers may need to adjust for the presence of a PVD in future drug trials and pharmacokinetic studies.

Summary Statement

We evaluated the retinal penetration of intravitreal bevacizumab in rabbit eyes with and without a microplasmin-induced posterior vitreous detachment (PVD). The initial retinal penetration is increased in eyes with a PVD. A microplasmin-induced PVD may alter the flow of molecules crossing the vitreoretinal junction.

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Figure 1.

Immunofluorescence of rabbit retinal tissue for eyes injected with microplasma and bevacizumab (A) or bevacizumab only (B) 6 hrs after bevacizumab injection. Increased antibody labeling is present in the retina of the microplasma-injected eye. An increased amount of vitreous gel is seen along the surface of the ILM in the non-microplasma injected eye (arrow). 10x magnification. ILM = Inner limiting membrane. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer. OS = photoreceptor outer segments.

Figure 2.

With increased magnification, retinal sections from the same eyes that are depicted in figure 1 clearly demonstrate increased antibody labeling in the eye injected with both microplasmin and bevacizumab (A) when compared with the eye injected with bevacizumab only (B). 20x magnification. ILM = Inner limiting membrane. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer. OS = photoreceptor outer segments.

Figure 3.

Twelve hours after bevacizumab injection, antibody labeling is still increased in the microplasmin-injected eye (A) versus the contralateral eye that did not receive intravitreal microplasmin (B). 10x magnification. ILM = Inner limiting membrane. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer. OS = photoreceptor outer segments.

Figure 4.

Antibody labeling remains increased in the microplasmin-injected eye (A) when compared to the fellow, non-microplasmin injected eye (B) 24 hrs after bevacizumab injection. 10x magnification. ILM = Inner limiting membrane. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer. OS = photoreceptor outer segments.

Figure 5.

Three days after bevacizumab injection, this rabbit demonstrates decreased antibody labeling in the microplasmin-injected eye (A) versus the fellow eye that did not receive intravitreal microplasmin (B). Brightness and contrast are increased but held constant for each eye. A control eye (C) that did not receive any intravitreal injections shows minimal background fluorescence. 10x magnification. ILM = Inner limiting membrane. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer. OS = photoreceptor outer segments.

Figure 6.

Three days after bevacizumab injection, this rabbit demonstrates similar antibody labeling in both the microplasma-injected eye (A) and the contralateral, non-microplasma injected eye (B). Brightness and contrast are increased but held constant for each eye. 10x magnification. ILM = Inner limiting membrane. GCL = Ganglion cell layer. INL = Inner nuclear layer. ONL = Outer nuclear layer. OS = photoreceptor outer segments.

Figure 7.

Twenty-four hours after incubating bevacizumab (1.25 mg) in the absence or presence of microplasmin (0.4 mg), aliquots (20 uL) from each treatment were subjected to SDS-PAGE. Results show that microplasmin does not degrade bevacizumab.