

Pharmacologic vitreolysis with microplasmin increases vitreous diffusion coefficients

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Received: 4 January 2006 / Revised: 7 June 2006 / Accepted: 10 June 2006 / Published online: 29 August 2006
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Abstract

Background Pharmacologic vitreolysis is a new approach to improve vitreo-retinal surgery and ultimately to liquefy and detach the vitreous from the retina to eliminate the contribution of the vitreous to retinopathy. The mechanism of action of the agents being developed for pharmacologic vitreolysis remains unclear. The effect of microplasmin on vitreous diffusion coefficients was investigated using the non-invasive technique of dynamic light scattering (DLS). **Methods** Vitreous diffusion coefficients in 18 intact porcine eyes were measured in vitro with dynamic light scattering (DLS). DLS was performed on all specimens at 37 °C 30 min after injections of human recombinant microplasmin at doses ranging from 0.125 to 0.8 mg, with 20-nm tracer nanospheres. **Results** DLS findings in untreated porcine vitreous were similar to the previously described findings in bovine and human vitreous. Microplasmin increased porcine vitreous diffusion coefficients in a dose-dependent manner (correlation coefficient, $r=0.93$), with an 85% increase after a 30-min exposure to the maximum dose.

This work is an abridgement of a thesis submitted in partial fulfillment of requirements for membership in the American Ophthalmological Society, May 2005.

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Conclusions Pharmacologic vitreolysis with human recombinant microplasmin increases vitreous diffusion coefficients in vitro. The results of these studies have implications for the dosing, route of administration, duration of action and methods of determining efficacy in future studies of pharmacologic vitreolysis to enhance vitreo-retinal surgery, as well as the design of clinical trials to induce prophylactic posterior vitreous detachment.

Keywords Vitreous · PVD · Anomalous PVD · Pharmacologic vitreolysis · Diffusion coefficients · Microplasmin · Dynamic light scattering

Introduction

Largely invisible by routine examination techniques [22, 30], the vitreous was previously not recognized as being important to health and as a cause of retinal pathology. In recent years, the importance of the vitreous in ocular physiology [9, 20, 25] and the pathogenesis of various retinopathies [18, 28, 29] has been increasingly appreciated, and the vitreous is being treated with ever-evolving therapeutic modalities, for the most part surgical [18, 20, 21, 25]. The future, however, will see an increase in the use of pharmacologic agents for therapy and prevention. Enabling such an approach is the present understanding that although seemingly clear, the adult human vitreous contains fine, parallel fibers coursing in an antero-posterior direction [13, 14]. Ultrastructural studies [17] have demonstrated that collagen fibrils are the only microscopic structures that correspond to these macroscopic fibers. Furthermore, the chemical interaction of these fibrils with other extracellular matrix components of the vitreous is now better understood [5, 23], making possible the

development of a pharmacologic approach to altering the molecular structure of the vitreous, known as pharmacologic vitreolysis [24].

The rationale of pharmacologic vitreolysis is based upon the fact that an innocuous posterior vitreous detachment (PVD) results from weakening of vitreo-retinal adhesion and concurrent liquefaction of gel vitreous [15, 16]. Although vitreo-retinal adhesion is known to weaken with age [19], the source(s) of adhesion and the exact mechanism(s) of weakening are not known. Similarly, the mechanism of endogenous gel liquefaction is not known. Vitreous liquefaction without vitreo-retinal dehiscence results in anomalous PVD with untoward effects ranging from retinal tears and detachments to macular holes or pucker, as well as the exacerbation of diabetic retinopathy [24]. To prevent anomalous PVD, it would be necessary to induce both dehiscence at the vitreo-retinal interface and liquefaction of the gel vitreous. Vitreous liquefaction would result in an increase in vitreous diffusion coefficients.

Previous studies [11, 35, 37] have suggested that plasmin from a variety of sources may be useful for pharmacologic vitreolysis. Autologous plasmin enzyme (APE) was reported to induce vitreo-retinal separation in rabbits [37], but there were no studies on the molecular effects of APE. These laboratory investigations were followed by pilot studies in small series of patients undergoing vitrectomy for macular holes (n=9) [34] and diabetic retinopathy (n=7) [38]. To date, no controlled clinical trials have been undertaken with APE. Recent studies found that microplasmin, a human recombinant molecule that is much smaller than plasmin, is able to detach the posterior vitreous cortex in pigs [36] as well as in cats and post-mortem human eyes [10]. However, as in the case of APE, there are no studies on the molecular or rheologic effects of microplasmin upon the gel vitreous.

We hypothesize that microplasmin induces liquefaction with a decrease in bulk viscosity that can be detected by measuring diffusion coefficients. This hypothesis was tested in post-mortem pig eyes using non-invasive dynamic light scattering to measure vitreous diffusion coefficients repeatedly during the experiments.

Materials and methods

Microplasmin

Microplasmin (ThromboGenics, Ltd., Dublin, Ireland) is a direct-acting thrombolytic agent that lacks the five 'kringle' domains, but contains the protease domain of plasmin, making it a much smaller (molecular weight =29,000 kDa) molecule than plasmin [12]. The material used in these studies was produced with the use of the *Pichia Pastoris*

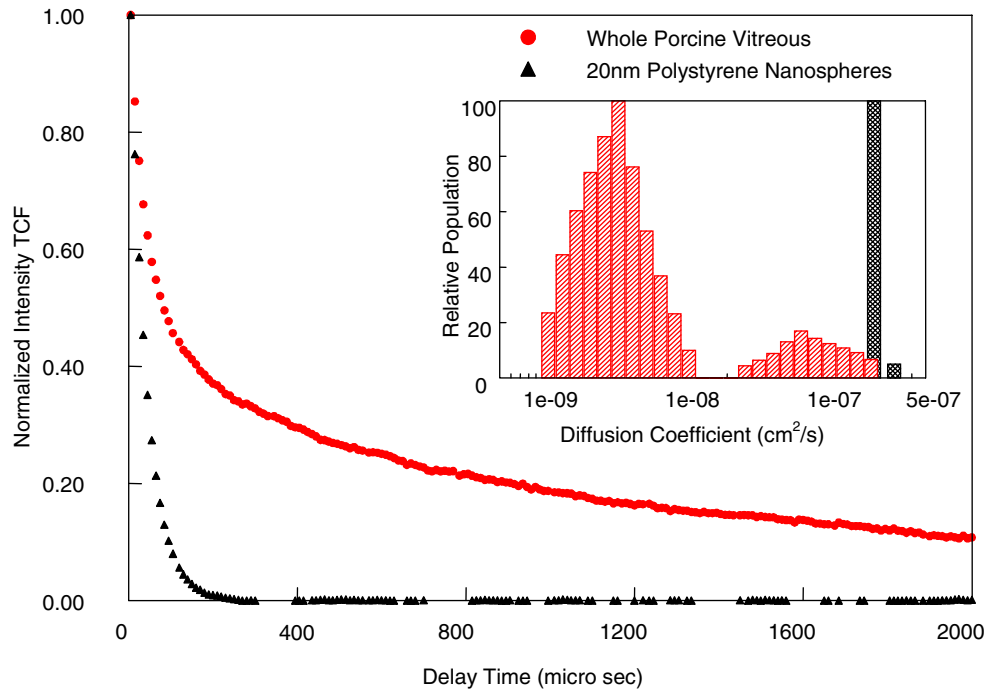
yeast expression system of human microplasminogen [12]. The product was purified, converted to microplasmin with staphylokinase, and equilibrated with 5 mM citrate. In these investigations, microplasmin was diluted with BSS Plus (ALCON, Fort Worth, TX) and added to the dissected porcine eyes at doses of 0.0125 mg, 0.08 mg, 0.125 mg, 0.4 mg and 0.6 mg, with one vehicle placebo specimen. In intact porcine eyes, microplasmin was injected at doses of 0.0125 mg, 0.125 mg, 0.25 mg, 0.5 mg and 0.8 mg. Controls were either untreated or placebos receiving vehicle injection without the microplasmin enzyme.

Dynamic light scattering

Dynamic light scattering (DLS) is an established laboratory technique to measure the average size or distribution of sizes of microscopic particles in solutions, ranging from 3 nm to 3 μ m in diameter [1]. DLS was originally developed to conduct fluid physics experiments and has been used in research aboard NASA's space shuttle and orbiting space station. In this system, light scattered by a laser beam passing through a dispersion of particles in a solution undergoing random Brownian motion will have intensity fluctuations in proportion to the Brownian motion of the particles. If the size of the particles is a constant, analysis of the scattered light intensity yields an index of the viscosity of the solution and can be quantitated in terms of diffusion coefficients. The theoretical basis for this methodology and all relevant formulas have been previously presented [6, 31].

Ansari [3] recently authored a comprehensive review of ophthalmic applications of DLS demonstrating the sensitivity and accuracy of this technology in evaluating lens crystallins [7, 8] and the vitreous macromolecules hyaluronan (HA) and collagen [2, 26]. Water, which constitutes 98% of the vitreous, is trapped (non-free-draining) in the HA/collagen network that forms gel vitreous. As a consequence, the gel has two forms of viscosity: bulk viscosity and microviscosity. The former refers to the general state of the entire structure. The latter refers to viscosity on a molecular level in the spaces between the large molecules. The microviscosity of vitreous is, in effect, the viscosity of water. There is presently no accurate method to determine vitreous bulk viscosity. This is primarily due to the fact that vitreous gel is a non-Newtonian fluid, where the stress-strain relationship is non-linear, making all available methodologies inappropriate for vitreous applications. Nonetheless, by determining the ability of non-interacting tracer particles (e.g., 20 nm polystyrene beads) to enter the microenvironment of the vitreous gel, the measured diffusion coefficient of this tracer particle will reflect the bulk viscosity of the solution, in this case the vitreous. Alteration of the macromolecular

Fig. 1 Dynamic light scattering of whole porcine vitreous. The time correlation function (TCF) curve of whole porcine vitreous is compared to a solution of 20 nm polystyrene beads. The single exponential of the latter is clearly different from the double exponent configuration of the former

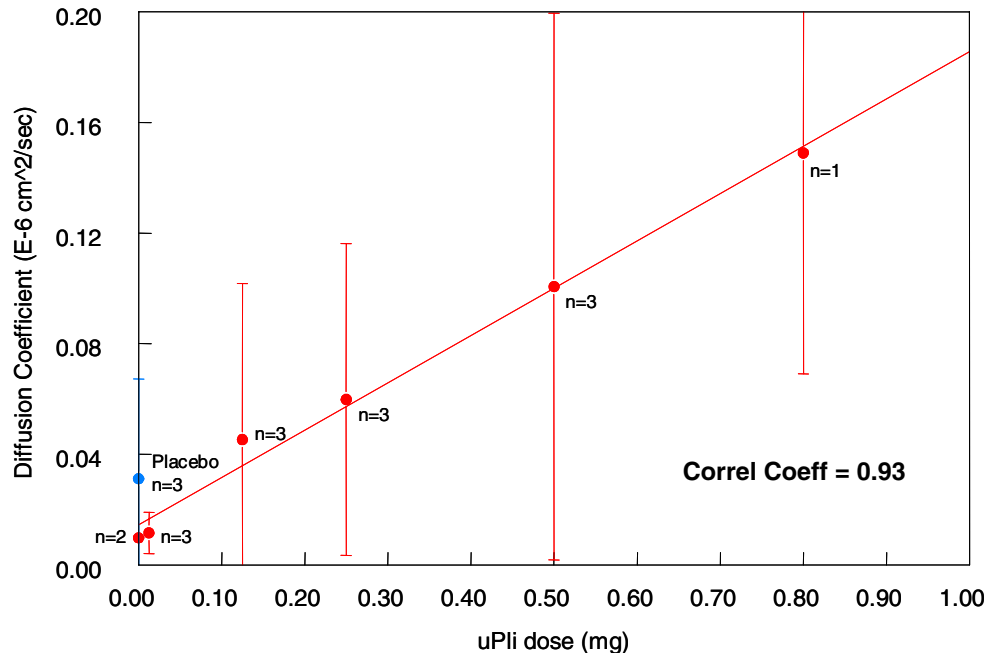


structure of the vitreous, as is hypothesized to occur in pharmacologic vitreolysis, will favor the entry of these tracer molecules and their movement through the vitreous, thereby assessing a drug's ability to alter vitreous bulk viscosity and induce liquefaction.

To perform DLS studies in the vitreous, the laser is focused into the specimen, and back-scattered light from a 30- μm^3 volume of the vitreous is captured by the probe.

The detected signal is processed via a digital correlator to yield a time correlation function (TCF), which is used to determine the diffusion coefficient. When solutions (0.1 ml) of 20 nm polystyrene beads (10 mg/100 ml or 10%; Bangs Laboratories, Fishers, IN) were included in all injections except one control specimen, the measured diffusion coefficient results represented the bulk viscosity of the vitreous.

Fig. 2 Vitreous diffusion coefficients in intact pig eyes increase with increasing doses of microplasmin (correlation coefficient, $r=0.93$)



Vitreous specimens

In 18 intact pig eyes, experimental and control solutions were injected into the vitreous body with a 30-gauge needle inserted via the pars plana. The eyes were immersed in a water bath at 37°C, but shielded from direct contact with the water so as to prevent artifacts. After 30 min of incubation, the anterior segment was excised. With the eyes upright in the 'supine' position, the DLS probe was positioned over the anterior vitreous cortex and the air/vitreous interface was identified by light-scattering criteria. DLS measurements were obtained from multiple points (mean =18.6; SD=11.8) 0.5 mm apart, extending along the optical axis from the air/vitreous interface to the vitreo-retinal interface. The micro-positioning control was also used to position the focal point of the DLS laser probe at a depth of 4 mm behind the air/vitreous interface, and DLS measurements were obtained at multiple points (mean=30.8 points; SD=18.0) 0.25 mm apart along the horizontal axis from one side of the eye to the other.

Statistical analyses

Pearson's correlation coefficient (r) was used to evaluate the dose-response relation in intact porcine eyes injected with various doses of microplasmin.

Results

The DLS findings in untreated whole porcine vitreous compared to a solution of 20 nm polystyrene beads are shown in Fig. 1. These findings are consistent with previous DLS findings in the bovine [33] and human [34] vitreous.

Dose-response studies were performed in intact pig eyes. Different doses of microplasmin (0.0125 mg, 0.125 mg, 0.25 mg, 0.5 mg and 0.8 mg) and placebo solutions were injected via the pars plana into the anterior vitreous (approximately 3–4 mm behind the lens) in whole pig eyes ($n=18$) that were then incubated for 30 min at 37°C. Figure 2 demonstrates the results obtained across the entire range of microplasmin doses. As compared to untreated and vehicle controls ($n=5$), eyes with microplasmin injection had a significant increase in vitreous diffusion coefficients. This effect was strongly dose-dependent, as the correlation coefficient across the range of microplasmin doses was significant ($r=0.93$).

Discussion

Pharmacologic vitreolysis has emerged in recent years as a new treatment modality to enhance vitreo-retinal surgery,

thereby improving therapy, and potentially to eliminate untoward effects of the vitreous upon the retina, thereby enabling prevention. Although several agents have been investigated [4, 24, 27, 32, 33], the mechanism of action of these agents is not presently known.

In the investigations reported herein, microplasmin induced a dose-dependent increase of vitreous diffusion coefficients in intact pig eyes, with a correlation coefficient of 0.93. At the highest dose there was an overall increase of about 85% after 30 min. This duration seems acceptable in terms of the amount of time that would be desirable for pharmacologic vitreolysis to have a clinical effect, especially as an adjunct to surgery in the operating room. The doses employed in this study are within the range of those used in previous studies where vitreo-retinal separation was induced in pigs, cats and post-mortem human eyes [10, 36]. Those findings in conjunction with the findings reported herein, showing that microplasmin substantially increases vitreous diffusion coefficients, suggest that microplasmin can induce both components of PVD, i.e., vitreo-retinal dehiscence and gel liquefaction. The increase in diffusion coefficients and a decrease in vitreous bulk viscosity should be useful in 23- or 25-gauge vitrectomy surgery, since a decrease in vitreous bulk viscosity should enable more complete and faster surgical removal, facilitating this surgery in outpatient and perhaps even office settings. Vitreous liquefaction is also important in the induction of prophylactic PVD. In this clinical application it may be advisable to lyse vitreo-retinal adhesion prior to the induction of liquefaction so as to avoid creating an anomalous PVD [27]. Prophylactic PVD should be advantageous in diabetic patients at risk of developing neovascularization from the optic disc or retina, in high myopia, and in fellow eyes of patients with macular holes and rhegmatogenous retinal detachments, as well as other clinical circumstances. Indeed, successful development of pharmacologic vitreolysis may one day eradicate all diseases that result from anomalous PVD and markedly decrease the need for vitrectomy surgery.

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