

Safety of In Vivo Pharmacologic Vitreolysis with Recombinant Microplasmin in Rabbit Eyes

Toshiro Sakuma,¹ Minoru Tanaka,¹ Atsushi Mizota,¹ Junji Inoue,¹ and Steve Pakola²

PURPOSE. To investigate the safety of intravitreal microplasmin in rabbits and to confirm previous findings of posterior vitreous detachment (PVD).

METHODS. Different doses of microplasmin, from 12.5 μ g to 250 μ g, in 0.1 mL balanced salt solution (BSS) were injected into the vitreous cavity of rabbit eyes to induce PVD. Fellow eyes were injected with the same volume of BSS. Slit-lamp biomicroscopy, ophthalmoscopic fundus examinations, A- and B-mode ultrasonography, and electroretinography were performed to assess the retina. Electroretinograms (ERGs) were recorded up to 90 days after injection. Morphologic alterations were assessed by light microscopy, scanning electron microscopy (SEM), and transmission (TEM) electron microscopy.

RESULTS. A slight aqueous flare and cells were observed in the anterior chamber after microplasmin and BSS injection. A slight inflammatory reaction was also observed transiently in the vitreous cavity. In control eyes, B-mode ultrasonography and SEM examination demonstrated that PVD did not develop after BSS injection. Intravitreal injections of 125 μ g or greater of microplasmin induced complete PVD with an internal limiting membrane (ILM) devoid of vitreous collagen fibrils. Eyes injected with 12.5 μ g microplasmin had partial PVD, and SEM showed residual fibrils covering the ILM. In all eyes, there was a transient reduction in the a- and b-waves of the ERG on days 2 through 7. The ERGs showed less effect with <250 μ g microplasmin.

CONCLUSIONS. Intravitreal injection of recombinant microplasmin in the rabbit induces no ERG or retinal ultrastructural abnormalities. Pharmacologic vitreolysis with this agent may be a useful adjunct to vitreous surgery and could be used to induce PVD without vitreous surgery. (*Invest Ophthalmol Vis Sci.* 2005;46:3295–3299) DOI:10.1167/iovs.04-1517

The posterior vitreous cortex adheres to the inner retinal surface in the normal human eye, most prominently at the vitreous base, the optic disc, along the major retinal vessels, and in a fascial manner to the entire posterior pole.¹ Spontaneous posterior vitreous detachment (PVD) and vitreous liquefaction can develop, usually because of age-related changes in the human eye. Separation of the vitreous from the fovea can alleviate macular traction and, thus, may greatly reduce the risk for macular hole formation.^{2,3} Complete PVD may also prevent

retinal neovascularization in eyes with diabetic retinopathy and retinal vein occlusion.^{4,5}

Vitreous surgical procedures have been performed to relieve vitreoretinal tractions or adhesions to facilitate reattachment of a detached retina and to reduce retinal edema. The level of difficulty of vitreous surgery depends on the presence or absence of PVD and the degree of adhesion between the vitreous body and the retina.⁶ In particular, diseases such as proliferative diabetic retinopathy,⁷ macular hole,⁸ and proliferative vitreoretinopathy are associated with pathologic changes at the vitreoretinal interface induced by anomalous PVD.⁹

The techniques and instruments for vitreous surgery have greatly improved in recent years. However, the surgical removal of the vitreous cortex is still difficult in some patients and carries the risk for complications such as retinal breaks, retinal detachment, and retinal nerve fiber damage,^{10,11} especially in younger patients.¹²

Therefore, it would be helpful to have a biochemical agent that could cleave the vitreoretinal interface selectively without damaging the retina. If the vitreous gel can be liquefied or if enzymes, either alone or in combination with vitrectomy, can weaken adhesion of the vitreous to the retina, these changes would decrease the risk for surgical complications.

Several enzymes have been explored for this purpose.^{13–26} Plasmin, one of these enzymes, is a nonspecific protease that can be isolated from the patient's own serum. The efficacy of plasmin in inducing PVD has been demonstrated in several studies.^{14–21} In clinical studies, autologous plasmin has been used, but a considerable amount of time is required to isolate plasmin from patient plasma.

Microplasmin, a recombinant protein, is a truncated form of the human plasmin with retained protease activity. Microplasmin is in phase 2 development as the first neuroprotective agent with thrombolytic potential for the treatment of ischemic stroke.^{27,28}

The purpose of this study was primarily to evaluate the potential toxicity of intravitreal injection of microplasmin through histologic and electrophysiologic studies performed while replicating previous studies¹³ that demonstrated the efficacy of PVD induction with microplasmin.

MATERIALS AND METHODS

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Microplasmin Solution

Microplasmin (ThromboGenics Ltd., Dublin, Ireland) was stored at -20°C and was prepared under sterile conditions immediately before use by diluting the dry preparation with sterile balanced salt solution (BSS). The doses of microplasmin solution were 12.5, 25, 62.5, 125, and 250 μ g in 0.1 mL BSS.

Injection of Microplasmin

New Zealand albino rabbits weighing 2 to 3 kg each were anesthetized with intramuscular injection of 30 mg/kg ketamine hydrochloride and 6 mg/kg xylazine hydrochloride. In addition, topical proparacaine was applied to the eyes for all procedures. Additional ketamine hydrochloride

From the ¹Department of Ophthalmology, Juntendo University, Urayasu Hospital, Chiba, Japan; and ²ThromboGenics Inc., New York, New York.

Supported by a Grant-in Aid for scientific research from the Ministry of Education, Science, Sports, and Culture, Japan (16591767) and by grants from the Institute for Environmental and Gender-Specific Medicine, Juntendo University School of Medicine.

Submitted for publication December 24, 2004; revised March 28, May 13, and May 31, 2005; accepted July 25, 2005.

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Corresponding author: Minoru Tanaka, Department of Ophthalmology, Juntendo University, Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba Japan 279-0021; eyeofjun@mb.infoweb.ne.jp.

TABLE 1. Mean $\pm$ SD of a-Wave Amplitude of ERG (μ V)

		After Injection (days)			
		Before Injection	2	7	14
25 μ g	151.7 $\pm$ 20.0	116.0 $\pm$ 11.7*	134.4 $\pm$ 27.7	137.5 $\pm$ 19.6	182.3 $\pm$ 10.7
Fellow eye	153.8 $\pm$ 20.2	153.6 $\pm$ 26.7	157.2 $\pm$ 23.2	145.4 $\pm$ 19.4	184.2 $\pm$ 8.0
125 μ g	132.9 $\pm$ 17.1	92.2 $\pm$ 22.1*	104.9 $\pm$ 18.5*	115.3 $\pm$ 17.1	141.2 $\pm$ 28.9
Fellow eye	140.2 $\pm$ 8.0	163.8 $\pm$ 12.9	161.5 $\pm$ 15.2	154.5 $\pm$ 29.0	181.1 $\pm$ 20.4
250 μ g	155.7 $\pm$ 13.4	69.8 $\pm$ 22.4*	40.0 $\pm$ 18.5*	52.2 $\pm$ 15.4*	113.7 $\pm$ 3.2*
Fellow eye	170.3 $\pm$ 13.5	143.1 $\pm$ 27.1	152.0 $\pm$ 19.9	147.7 $\pm$ 22.1	154.0 $\pm$ 9.6

* $P < 0.05$.

ride was given as needed. To observe the fundus, the pupil was dilated with topical 0.25% scopolamine hydrobromide, 1% cyclopentolate, and 2.5% phenylephrine hydrochloride.

The microplasmin solution (0.1 mL) was injected into the vitreous cavity 2 mm posterior to the limbus while the eye was observed under a surgical microscope. Each fellow eye was injected with BSS (0.1 mL) as control. Care was taken to avoid damage to the lens and the retina.

Evaluation

This study was designed to determine the time course and dose dependency of microplasmin injections on the extent of PVD and its toxicity to the retina. Animals were randomly assigned to one of two groups. Group 1 consisted of 15 rabbits used to study the dose dependency of microplasmin. Microplasmin was injected into the vitreous cavity in doses of 12.5, 25, 62.5, 125, and 250 μ g/0.1 mL. Fifteen eyes were injected with different concentrations of microplasmin, and the fellow eyes were injected with the same volume of BSS as control. These eyes were also were examined for the incidence of PVD, histologic retinal changes, and electroretinographic (ERG) changes at 14 days after injection.

Group 2 consisted of nine rabbits, and microplasmin at concentrations of 25, 125, and 250 μ g/0.1 mL BSS was injected in the same way. Animals in group 2 were examined by electroretinography up to 90 days after injection to investigate the long-term toxicity of microplasmin.

Clinical Examination

Each rabbit was examined clinically by indirect ophthalmoscopy and slit-lamp biomicroscopy with a +90 diopter precorneal lens before and at periodic intervals after intravitreal injection of microplasmin or BSS. A- and B-scan ultrasonography (UD-6000; Tomey, Nagoya, Japan) was performed to determine the condition of the posterior vitreous interface before and 30 minutes, 60 minutes, 1 day, and 7 days after injection in the group 1 animals. We watched the dynamic changes on the ultrasonograms of the vitreoretinal interface online.

Electrophysiologic Examination

ERGs were recorded from animals in group 1 before and on days 2, 7, and 14 after injection. In group 2, ERGs were recorded on the same

days and also at 90 days after injection. For ERG recordings, the rabbits were anesthetized with ketamine and xylazine, pupils were dilated, and a topical anesthetic was dropped on the cornea. After 1 hour of dark adaptation, ERGs were recorded from both eyes simultaneously with an ERG recording system (LE-2000; Tomey, Nagoya, Japan). ERGs were recorded with a contact lens electrode carrying light-emitting diodes as a stimulator and referred to an electrode on the forehead. The luminance of the stimulus was 20,000 cd/m² with a duration of 10 ms. The ground electrode was attached to the ear. Amplitudes and implicit times of a- and b-waves were evaluated.

Histologic Examination

Rabbits were deeply anesthetized and killed by intracardiac injection of sodium pentobarbital. After enucleation, the eyes were opened with a razor blade that penetrated the vitreous adjacent to the pars plana to ensure rapid penetration of fixative. Care was taken to avoid damage to the adjacent retina and lens. The eyes remained immersed in 2% paraformaldehyde plus 2.5% glutaraldehyde (0.1 M phosphate buffer and pH 7.4) for a minimum of 24 hours at 4°C.

For light microscopy, the globe was embedded in paraffin, and 10- μ m thick horizontal sections were cut through the optic nerve head and stained with hematoxylin and eosin. For scanning electron microscopy (SEM), a posterior calotte that included part of each quadrant of the eye was removed, dehydrated through a graded ethanol series, dried in carbon dioxide liquid to the critical point, sputter-coated in gold, and photographed using an electron microscope (S-800; Hitachi, Tokyo, Japan). For transmission electron microscopy (TEM), the tissue was dehydrated in an ethanol series, postfixed in 2% osmium tetroxide, and embedded in epoxy resin (Epok 812; Oken, Tokyo, Japan). Semi-thin sections were stained with 2% toluidine blue. Ultrathin sections were stained for contrast with tannic acid and lead citrate and were analyzed through an electron microscope (JEM-1200EX; JEOL, Tokyo, Japan).

Electron photomicrographs were evaluated for the degree of vitreoretinal separation by determining whether a continuous or a discontinuous network of collagen fibrils covered the ILM, whether single and sparse collagen fibrils were present on the ILM, or whether the ILM was devoid of any collagen fibrils.

TABLE 2. Mean $\pm$ SD of b-Wave Amplitude of ERG (μ V)

		After Injection (days)			
		Before Injection	2	7	14
25 μ g	299.4 $\pm$ 32.0	234.5 $\pm$ 38.5*	321.5 $\pm$ 40.0	326.1 $\pm$ 40.0	380.8 $\pm$ 20.5
Fellow eye	303.8 $\pm$ 38.9	282.5 $\pm$ 25.8	322.8 $\pm$ 41.0	312.5 $\pm$ 58.2	377.1 $\pm$ 36.1
125 μ g	291.4 $\pm$ 36.2	186.2 $\pm$ 52.3*	259.9 $\pm$ 111.5	303.9 $\pm$ 86.7	435.9 $\pm$ 112.7
Fellow eye	294.1 $\pm$ 36.4	309.7 $\pm$ 40.4	310.5 $\pm$ 48.92	301.5 $\pm$ 57.7	411.0 $\pm$ 72.8
250 μ g	281.4 $\pm$ 55.4	94.6 $\pm$ 25.4*	137.9 $\pm$ 52.5*	208.1 $\pm$ 95.1	417.3 $\pm$ 35.9
Fellow eye	285.9 $\pm$ 56.6	258.3 $\pm$ 76.8	291.0 $\pm$ 51.8	276.0 $\pm$ 48.6	382.5 $\pm$ 36

* $P < 0.05$. Implicated times of a- and b-waves did not show statistically significant changes through this experiment (Tables 3, 4).

TABLE 3. a-Wave Implicit Times of ERG at Different Time Points after Injection (ms)

	Before Injection	After Injection (days)			
		2	7	14	90
25 μ g	6.5 $\pm$ 0.3	6.5 $\pm$ 0.2	6.9 $\pm$ 0.2	6.7 $\pm$ 0.4	6.7 $\pm$ 0.1
Fellow eye	6.6 $\pm$ 0.2	6.6 $\pm$ 0.2	6.8 $\pm$ 0.3	6.7 $\pm$ 0.3	6.8 $\pm$ 0.2
125 μ g	6.5 $\pm$ 0.2	6.7 $\pm$ 0.3	6.7 $\pm$ 0.4	7.1 $\pm$ 0.6	6.8 $\pm$ 0.3
Fellow eye	6.5 $\pm$ 0.2	6.5 $\pm$ 0.2	6.6 $\pm$ 0.2	6.6 $\pm$ 0.2	6.8 $\pm$ 0.2
250 μ g	6.5 $\pm$ 0.3	6.9 $\pm$ 0.4	6.9 $\pm$ 0.4	6.9 $\pm$ 0.4	7.0 $\pm$ 0.2
Fellow eye	6.7 $\pm$ 0.2	6.6 $\pm$ 0.3	6.5 $\pm$ 0.3	6.6 $\pm$ 0.2	6.9 $\pm$ 0.1

Statistical analysis

Results were compared using analysis of variance (ANOVA) and the Dunnett post hoc test. $P < 0.05$ was considered statistically significant. Amplitudes and implicated times of the a- and b-waves were analyzed by group mean comparisons. Amplitudes of all waves of the experimental and control eyes were measured.

RESULTS

Clinical Examination

Indirect ophthalmoscopy and slit-lamp biomicroscopy showed that the vitreous was slightly hazy and had some infiltrating cells on day 1 after injection of $>62.5 \mu$ g microplasmin. These findings decreased 3 days after injection and resolved completely approximately 5 days after injection.

Reactions in the vitreous of the eyes that received BSS were minimal. Inflammatory reactions were also observed in the anterior chamber after injection. These inflammatory changes decreased approximately 5 days after injection. In addition, no hemorrhagic complications and no cataracts were noted in any eyes.

The relationship between the dose and the incidence and degree of PVD was studied in group 1 animals based on the postoperative B-scan ultrasonographic findings. PVD was not created in control eyes or in eyes receiving 12.5μ g microplasmin. Partial PVD occurred in the eyes treated with 25 and 62.5μ g microplasmin, and total PVD developed in eyes receiving 125μ g or greater doses of microplasmin. There was a direct correlation between increasing concentrations of microplasmin and the degree of PVD. All PVDs occurred within 60 minutes of microplasmin injection, and no changes were found thereafter.

Electrophysiologic Examination

Representative ERGs from eyes injected with microplasmin are shown in Figure 1. Amplitudes of a- and b-waves were decreased 2 days after microplasmin injection at all concentrations. At 14 days after injection, a- and b-wave amplitudes recovered in the eyes receiving 25 and 125μ g microplasmin and did not differ significantly from those of the controls.

At 90 days, b-wave amplitude had recovered in the animals injected with 250μ g microplasmin, but the a-wave amplitude was still significantly smaller than in control eyes (Tables 1 and 2). The implicit times of a- and b-wave did not differ significantly from those of controls (Tables 3 and 4).

Histologic Examination

Light microscopy demonstrated normal retinal histologic findings in microplasmin-injected and control eyes. Histologic findings in the retinas were indistinguishable from those before the injections.

Postoperative SEM was performed on all eyes receiving microplasmin and BSS. If postoperative B-scan ultrasonography indicated vitreous separation, SEM demonstrated that the ILM had a fairly smooth surface and no apparent collagen fibrils. Where postoperative B-scan ultrasonography indicated that the vitreous was still partially attached, SEM showed persistent collagen fibrils on the retinal surface, especially over the retinal vessels and the medullary rays. These fibrils were consistent with attached cortical vitreous. SEM showed a smooth retinal surface, consistent with a bare ILM in the 125 and 250μ g microplasmin-treated eyes. These findings were found in both the superior and the inferior retina. At the vitreous base, all microplasmin-treated and control eyes had an attached cortical vitreous.

TEM of controls and 12.5μ g microplasmin showed persistent vitreous attachment with collagen fibers condensed over the retinal surface. In all eyes treated with 25μ g microplasmin and two of three eyes treated with 62.5μ g microplasmin, collagen fibrils could be seen adjacent to the ILM in some areas. In the eyes treated with 125 and 250μ g microplasmin, vitreous collagen fibrils were not seen on the retinal surface. The structure of the inner retina and of the ILM was unchanged. In particular, the ILM was not affected after microplasmin treatment.

DISCUSSION

Separating the vitreous from the retina is an important and critical step in vitreous surgery. Because the mechanical creation of a PVD often leads to complications, pharmacologic

TABLE 4. b-Wave Implicit Times of ERG at Different Time Points after Injection (ms)

	Before Injection	After Injection (days)			
		2	7	14	90
25 μ g	55.1 $\pm$ 5.3	53.8 $\pm$ 5.3	54.0 $\pm$ 2.6	51.3 $\pm$ 5.1	54.1 $\pm$ 2.8
Fellow eye	53.1 $\pm$ 6.0	53.6 $\pm$ 4.4	53.3 $\pm$ 2.9	54.6 $\pm$ 4.2	52.8 $\pm$ 3.1
125 μ g	56.1 $\pm$ 4.3	52.6 $\pm$ 4.6	53.8 $\pm$ 3.5	52.4 $\pm$ 3.5	57.8 $\pm$ 4.8
Fellow eye	56.5 $\pm$ 4.9	53.5 $\pm$ 4.0	53.0 $\pm$ 3.8	53.4 $\pm$ 3.9	57.6 $\pm$ 4.0
250 μ g	56.0 $\pm$ 4.0	55.4 $\pm$ 3.3	56.1 $\pm$ 3.4	53.5 $\pm$ 3.5	56.2 $\pm$ 2.3
Fellow eye	57.1 $\pm$ 3.1	56.1 $\pm$ 2.4	56.1 $\pm$ 2.8	55.4 $\pm$ 2.5	56.3 $\pm$ 3.3

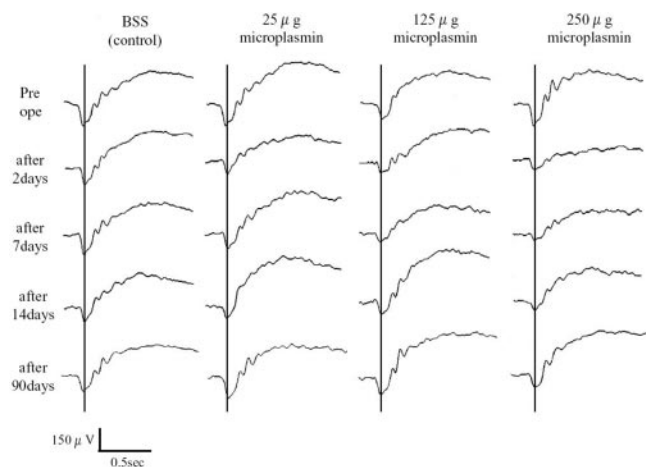


FIGURE 1. Electoretinograms recorded before and 2, 7, 14, and 90 days after the injection of different concentrations of microplasmin and BSS. Two days after all concentrations of microplasmin injection, the a- and b-wave amplitudes decreased. The form and amplitude in BSS-injected eyes and microplasmin-injected eyes were unchanged at 90 days after injection.

vitreolysis was investigated. Pharmacologic vitreolysis is a technique by which a chemical agent(s) is used to digest specific components of the extracellular matrix proteins in the vitreoretinal interface and in the gel to liquefy the vitreous, to form a PVD, or both. This technique was envisaged to increase or even replace standard mechanical vitrectomy because it had important advantages^{14,23,24}—fewer surgical complications, less surgical time, lower operation costs, greater patient access, and possible transition to office-based vitreoretinal procedures.^{14,23,24}

Earlier investigators have reported promising results dissolving the adhesion between the posterior vitreous cortex and the ILM enzymatically.^{14,23} The evaluation of various pharmacologic adjuncts, either alone or in conjunction with pars plana vitrectomy and intravitreal gas injections, to help with vitreoretinal separation has been the subject of many studies. Any attempt to induce PVD is fraught with hazard because inducing vitreous liquefaction without weakening the vitreoretinal adherence could cause serious problems related to vitreoretinal traction posteriorly and peripherally and can result in rhegmatogenous retinal detachment, vitreopapillopathy, vitreomacular traction syndrome, and macular hole.^{14,23}

Of the chemical agents examined, plasmin has received the most attention and appears to be the most useful. Plasmin is a nonspecific protease and has proteolytic activity against laminin and fibronectin, which are the components of the internal limiting lamina. Laminin and fibronectin are found between vitreous collagen fibrils of the posterior vitreous cortex and the internal limiting lamina. Thus, plasmin should exert its activity on the vitreoretinal interface.

Several clinical studies have shown the benefit of autologous plasmin in retinal disease.^{18–20} In our study, we chose recombinant microplasmin because it has several advantages over autologous plasmin. One advantage is the commercial availability of the product, allowing immediate availability and not requiring long and difficult preparation time to isolate the plasmin from autologous blood, which requires several days. Another advantage is its synthesis by genetic engineering, which reduces the safety problem of bacterial and viral contamination of blood derivatives. Even though microplasmin has not yet been administered into the vitreous of humans eyes, it has been demonstrated to be well tolerated when administered intravenously in healthy volunteers at doses approximately

2000 times those of the anticipated intravitreal dose for ophthalmic applications (Pakola S, unpublished data, 2004).

Our study demonstrated that microplasmin alone produced cleavage at the vitreoretinal interface of rabbit eyes, and no additional surgical procedures were required to induce PVD. Similar to the results of Gandorfer et al.,¹³ reports have shown the creation of PVD by autologous plasmin injection combined with other procedures in human eyes. Nevertheless, Gandorfer et al.¹⁶ demonstrated ultrastructurally that it is indeed possible to use intravitreal injections of plasmin alone to create a complete PVD. In controlled experiments on postmortem pig eyes, light microscopy and SEM verified that at sufficient concentrations and incubation times, plasmin-injected eyes developed PVD with the retinal surface smooth and free of cortical vitreous remnants. It is significant that enzymatic action alone was sufficient to induce PVD without the adjuvant gas bubble injection or cryotherapy necessitated in other studies.^{25,26}

In their study of microplasmin, Gandorfer et al.¹³ did not report any adverse effects. In our study, a transient decrease in a- and b-wave amplitudes was seen in eyes that received high doses of microplasmin. In addition, animals injected with the highest dose of 250 μ g showed ERG changes even 90 days after injection. Verstraeten et al.¹⁷ reported that an intravitreal injection of plasmin caused a transient decrease in ERG b-wave amplitude with excellent recovery. Their histologic observations showed that the retina was not damaged, and they suggested that the decrease in the ERG resulted from the high osmolarity of the plasmin solution. In our animals receiving 125 μ g or less microplasmin, there was a gradual recovery of the ERG amplitudes over several days; at 14 days after injection, a-wave and b-wave amplitudes were comparable to those of the control eyes injected with BSS. We believe that 125 μ g is the safe dosage to inject into the vitreous of rabbits, and our results showed that with this dose of microplasmin, PVD developed within 60 minutes. In terms of enzymatic action, 125 μ g microplasmin is equivalent to 2 U plasmin (Sigma-Aldrich, Poole, United Kingdom), which causes complete vitreous separation in porcine eyes and in human donor eyes.^{15,16}

The molecular weight of microplasmin is 28 kDa, which is lower than the molecular weight of human plasmin (88 kDa). This should enable microplasmin to penetrate the epiretinal tissue more effectively than plasmin.¹³ Histologic and ERG analyses, however, revealed no evidence of retinal damage in all rabbit eyes treated with 125 μ g or less microplasmin. Based on these results, we conclude that the 125- μ g dose of microplasmin was not toxic to the retina.

Microplasmin-assisted vitrectomy may not only facilitate the separation of the vitreous from the retina, it may also reduce the risk for subsequent cellular proliferation and fibrocellular contraction, as noted with autologous plasmin.^{14,23,24} Thus, we hypothesize that microplasmin may be useful as an adjunct to vitrectomy by creating PVD after a single injection. It should also be considered in an outpatient setting as an alternative treatment for eyes requiring vitrectomy. However, microplasmin must be evaluated for its activity and pharmacokinetics in human eyes.

Acknowledgments

The authors thank Duco I. Hamasaki (Bascom Palmer Eye Institute, Miami, FL) for his advice and editorial assistance.

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