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LABORATORY STUDY

Enzymatic vitreolysis with recombinant microplasminogen and tissue plasminogen activator

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Abstract

Purpose **To generate microplasmin (μPlm) using recombinant microplasminogen (μPlg) and recombinant tissue plasminogen activator (rt-PA) before intravitreous injection and to investigate the efficacy of μPlm in inducing posterior vitreous detachment (PVD).**
Methods **Forty-eight female or male New Zealand white rabbits were randomized into three groups. Recombinant human μPlg was incubated with rt-PA with a 200:1 molar ratio at 37°C for 40 min. The right eyes of groups 1, 2, and 3, were injected with 0.5, 1.0, and 1.5 U μPlm in 0.1 ml respectively, and 0.1 ml balanced salt solution (BSS) was injected into the left eye as controls. Scanning electron microscopy (SEM), gross specimen examination, B-ultrasonography and optical coherence tomography (OCT) were performed to detect vitreoretinal interface.**
Results **Over eighty percent of recombinant human μPlg could be activated to active μPlm by rt-PA after 40 min incubation. Complete PVD was found at vitreous posterior pole of μPlm-treated eyes without morphological change of retina. Complete PVD of 25, 75, and 87.5% rabbit eyes was induced by 0.5, 1.0 and 1.5 U recombinant μPlm respectively on day 1. The remnants of vitreous cortex at the posterior pole were dependent on the concentration of μPlm. Among the four approaches for detecting PVD, SEM, gross specimen examination, and B-ultrasonography were more effective methods than OCT.**
Conclusion **Intravitreous injection of 1.5 U μPlm can effectively induce complete PVD in rabbit eyes on day 1 without morphological change of retina.**
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Introduction

It is generally agreed that a complete posterior vitreous detachment (PVD) can greatly facilitate vitreoretinal surgery for macular hole (MH) repair and cystoid macular oedema (CME) in which a PVD has not developed spontaneously. The technique and instruments for vitreous surgery have developed greatly in recent years. However, surgical removal of the vitreous cortex carries the risk of retinal tear, retinal detachment, or retinal nerve fibre damage,[1,2] especially in young patients.[3] Moreover, residual vitreous may cause proliferative vitreoretinopathy.[4]

Enzymatic vitreolysis has potential as a simple and less invasive method than pars plana vitrectomy to relieve vitreoretinal traction by inducing PVD. Several enzymes, including chondroitinase,[5] hyaluronidase,[6,7] and dispase,[8,9] have been investigated for the induction of PVD. Among these, plasmin is one of the most promising enzymes because its activity decreases to an undetectable level within 24 h without excessive enzymatic effects,[10] and it is nontoxic to the retina.[11–13] However, isolation of autologous plasmin is a costly and time-consuming process. Furthermore, plasmin is produced with difficulty through gene recombinant technology because of its large molecular weight and is prone to degradation.

Recombinant microplasminogen (μPlg) is a truncated protein produced by protein engineering technology and consists of the catalytic domain of native plasminogen. Upon

activated to microplasmin (μPlm) by plasminogen activator, μPlg shares the same catalytic properties as human plasminogen.[14] μPlm is in phase 2 development as the first neuroprotective agent with thrombolytic potential for the treatment of ischaemic stroke.[15,16] Recently, μPlg has been expressed with high yield in *Pichia pastoris* (about 400 mg/l culture broth).[17] Thus, μPlg will be a commercially available product, which allows immediate availability and does not require long and difficult preparation time to isolate the plasmin from autologous blood.

The aim of the present study is to (i) generate μPlm by incubating recombinant human μPlg with the recombinant human tissue plasminogen (rt-PA) activator and (2) investigate the safety and efficacy of μPlm in inducing PVD in rabbit eyes.

Materials and methods

Animals

Forty-eight female or male New Zealand white rabbits weighing 2–3 kg were randomized into three groups; each group consisted of 16 rabbits. The animals were treated in accordance with the ARVO statement for the use of animals in vision and ophthalmic research.

Activation of µ*Plg*

Recombinant human μPlg was expressed in *P. pastoris* as described before.[17] rt-PA (Genentech, South San Francisco, CA, USA) was stored at –20°C until administration, when these powders were reconstituted in sterile balanced salt solution (BSS) to the required concentrations.

To determine optimal activation time, μPlg at 10 μM (290 μg/ml) was incubated with tissue plasminogen activator at a 200:1 molar ratio. The incubation mixture consisted of 10 μM μPlg in 0.99 ml of BSS. The activation reaction was initiated by the addition of 10 μl of 5.0 μM (280 μg/ml) rt-PA at 37°C. Samples of 50 μl were withdrawn at 10 min intervals, and the formation of μPlm was assayed by reduced sodium dodecyl sulphate gel electrophoretic (SDS-PAGE) analysis. Densitometric analysis was performed using the Image/J® 1.11 image processing and analysis program. The amidolytic activities of μPlm at different times were measured five times with the peptide substrate NH2-D-Val-Phe-Lys-pnitroanilide (S-2251, Sigma, South San Francisco, MO, USA) as described before.[14] Plasmin (Sigma) was used as calibration and the mean activity values and standard deviations of five assays were used as the test results.

Injection of enzymes

The animals were anaesthetized with an intravenous pentobarbital sodium 10 mg/kg. Pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin®-P, Santen, Osaka, Japan) or 1% atropine sulphate. Oxybuprocaine hydrochloride 0.4% (Benoxil®, Santen, Osaka, Japan) was applied for topical anaesthesia.

A prophylactic anterior chamber paracentesis was performed at the limbus to release approximately 0.1 ml of the aqueous to lower the intraocular pressure. Pars plana injection of 0.1 ml solution was performed 1 mm posterior to the limbus with a 30-gauge needle. The needle was maintained in place for 30 s to allow some pressure equilibration to prevent reflux of the solution. Groups 1, 2, and 3 were injected intravitreously with 0.5, 1.0, and 1.5 U of μPlm respectively. The same volume of BSS was injected into the left eye as control.

Clinical examination

Binocular indirect ophthalmoscopy and slit-lamp biomicroscopy with a $+90$ dioptre precorneal lens examinations were performed before and at periodic intervals after intravitreal injection of μPlm or BSS.

Eight rabbits were randomly assigned from each group on days 1 and 7 respectively for B-ultrasonography (BVI, Clermont-Ferrand, France) and optical coherence tomography (OCT). Then, rabbits were deeply anaesthetized and killed by intravenous injection of excessive 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 the fixative. After 24 h fixation with 2.5% glutaraldehyde and 2% paraformaldehyde (0.1 M phosphate buffer salt and pH 7.4) at 4°C, the eyes were bisected horizontally over the optic nerve into superior and inferior calottes in a circumferential manner. To avoid artificial mechanical PVD, the vitreous was dissected with a sharp razor. Care was taken to avoid damage to the adjacent retina. One of the calottes was used for gross specimen examination. The other was treated for histologic examination by scanning electron micrography.

Gross specimen examination

For gross specimen examination, the residual vitreous cortex on the surface of the posterior retina was assessed with the triamcinolone acetonide (TA) fine particle suspension (Bristol-Myers Squibb, Shanghai, China). Briefly, the calotte was rinsed three times with PBS and was laid horizontally opening upwards. TA fine particle

suspension (0.1 ml) was dropped uniformly onto the inner retinal surface. After incubation for 20 min at 25°C, the calotte was rinsed three times with PBS and photographed using a digital colour video camera (DXC-390, Sony Electronics Inc., Park Ridge, NJ, USA).

Scanning electron microscopy

For scanning electron microscopy, a strip of tissue from the basement to the posterior pole was cut with a sharp razor. After 24 h of initial fixation, selected specimens were postfixed in osmium tetroxide 2% (Dalton's fixative), dehydrated in hexamethyldisilazane, dried to the critical point, sputter-coated in gold, and photographed using electron microscope (Hitachi S-520, 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 inner limiting membrane (ILM), whether single and sparse collagen fibrils were present on the ILM, or whether the ILM was devoid of any collagen fibrils.

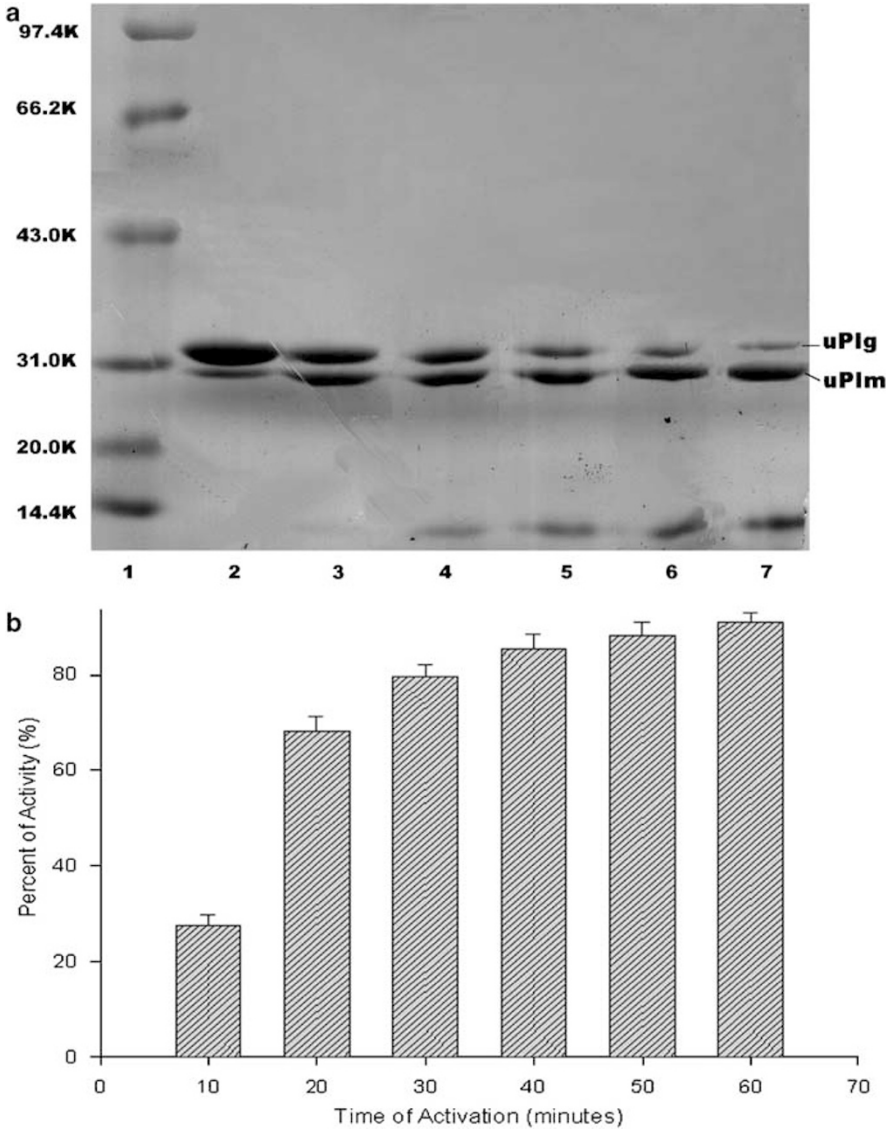

Figure 1 Time course of activation of recombinant human μPlg with rt-PA. (a) Reduced sodium dodecyl sulphate gel electrophoretic analysis showed that native μPlg was cleaved, and a major peptide of M_r 27 000 was observed and it increased with time. At 40 min, about 80% of the μPlg had been cleaved as shown by densitometric analysis. (b) Amidolytic activity of the incubation solution also increased during the time studied. At 40 min, the μPlm amidolytic activity was higher than 80% of the same molar plasmin. Results are given as mean $\pm$ SD ($n = 5$).

Histologic examination

For light microscopy, the tissue was transferred into phosphate buffer saline and kept at 4°C overnight. After dehydration through a graded ethanol series and propylene oxide, it was embedded in paraffin. Horizontal sections (5-μm-thick) were made and stained with hematoxylin and eosin.

Statistical analysis

Results were compared using analysis of cross-table and Pearson χ^2 test. $P<0.05$ was considered statistically significant.

Results

Activation of µ*Plg by rt-PA*

Figure 1a shows reduced SDS–PAGE analysis of the time course of activation of recombinant human μPlg with rt-PA in BSS. Native μPlg was cleaved, and a major peptide of M_r 27 000 was observed and it increased with time. At 40 min, about 80% of the μPlg was cleaved, as shown by densitometric analysis. There was also an increment of μPlm amidolytic activity during the time studied. At 40 min, the μPlm amidolytic activity was higher than 80% of the same molar plasmin (Figure 1b). Thus, we decided to incubate these two reagents for 40 min.

Clinical examination

No abnormalities were found in all pre-injection eyes and control eyes. A slight temporary haze in the posterior vitreous had been observed in the test eyes 6 h after injection, but fundus remained visible. Twelve hours later, the vitreous became clear again. The lenses were clear with no dislocation. Retinal oedema, bleeding, or detachment was not found in any of the eyes.

In most μPlm-treated eyes, typical acoustic properties of complete PVD were observed. The B-ultrasonograph showed a very fluid, continuous, and undulating echogenic band with moderate backward movements in front of the retina (Figure 2a). Eyes with partial PVD showed an echogenic band with focal attachment to the retina and were less mobile than complete PVD (Figure 2b). There was cavity formation between vitreous and retina.

Optical coherence tomography could also determine the incomplete and complete PVD, in which the posterior hyaloid face did not move very far anteriorly from the retina. But at 7 days after injection, the edge of the PVD was more peripheral than the OCT could detect in all directions in partial eyes with complete PVD (Figures 3a and b).

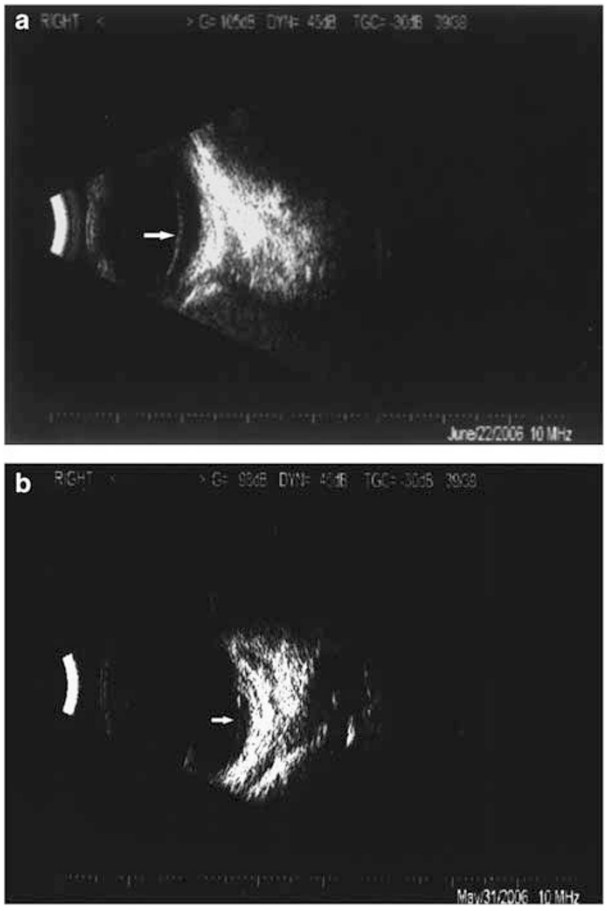

Figure 2 The B-ultrasonograph of vitreoretinal interface. (a) In complete PVD eyes, there was a continuous and undulating echogenic band with moderate backward movements in front of the retina, which was detached from the retina (arrow). (b) Eyes with partial PVD showed focal vitreous adhesions to the retina and less-mobile echogenic band than complete PVD (arrow). A cavity had been formed between vitreous and retina.

Gross specimen examination

With TA fine particle suspension, the posterior hyaloid was clearly seen as a white-coloured gel on the whole inner retinal surface of all BSS-injected eyes (Figure 4a). Scattered islands of posterior vitreous and thin posterior hyaloid membrane were left on the retina of partial PVD eyes. There was a smooth light reflex from ILM where cortical vitreous had detached from the retinal surface (Figure 4b). In complete PVD eyes, smooth light reflex from ILM could be seen on the whole posterior retinal surface (Figure 4c).

Electron microscopy

Examination by SEM showed that retinal inner surface of BSS-treated eyes was completely covered with a dense meshwork of collagen fibres (Figure 5a). In SEM

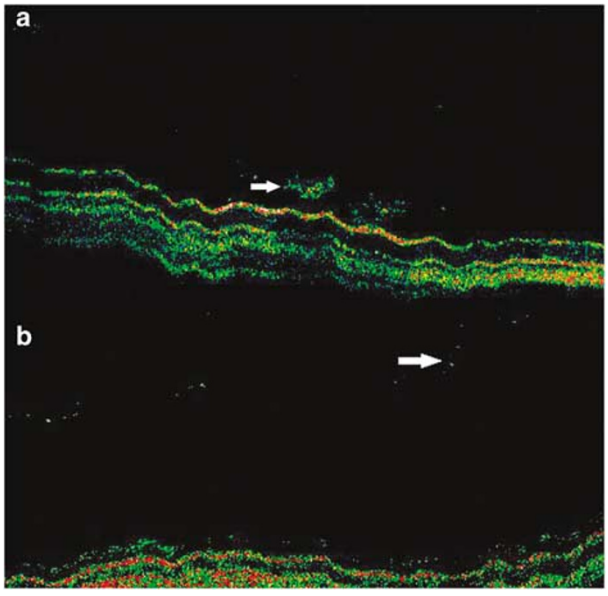

Figure 3 Optical coherence tomograph of vitreoretinal interface. (a) In an eye with complete PVD, the posterior hyaloid (arrow) had not moved very far anteriorly from retina and the ILM was smooth without remnants of posterior vitreous. (b) There were aggregated collagen fibre remnants (arrow) of posterior vitreous on the ILM in a partial PVD eye.

photographs of incomplete PVD eyes, the internal limiting membrane was covered by remnants of cortical vitreous at the equator (Figure 5b). There was a smooth and clean ILM in complete PVD eyes (Figure 5c).
A distinct correlation had been noted between the concentration of μPlm and degree of residual posterior vitreous cortical at 1 day after injection. Eyes that received 0.5 U μPlm had much more residual collagen fibrils than those that received 1.5 U μPlm (Table 1).

Histologic examination

In light micrographs, 0.5, 1.0, and 1.5 U μPlm-treated eyes on either 1 or 7 days after injection showed similar retinal structure to that of BSS-treated eyes. The cellular layers of the retina were clearly demarcated, and the ILM was presented as a continuous membranous structure. There was no inflammatory reaction observed in posterior vitreous or retina.

Discussion

Previous investigators have introduced several methods to achieve sufficient plasmin concentrations in the vitreous. Injection of purified plasmin into the vitreous cavity,[10,13,18–22] or stimulation of plasmin production in the vitreous by t-PA injection,[23] cryocoagulation followed by t-PA injection,[24] or injection of purified human

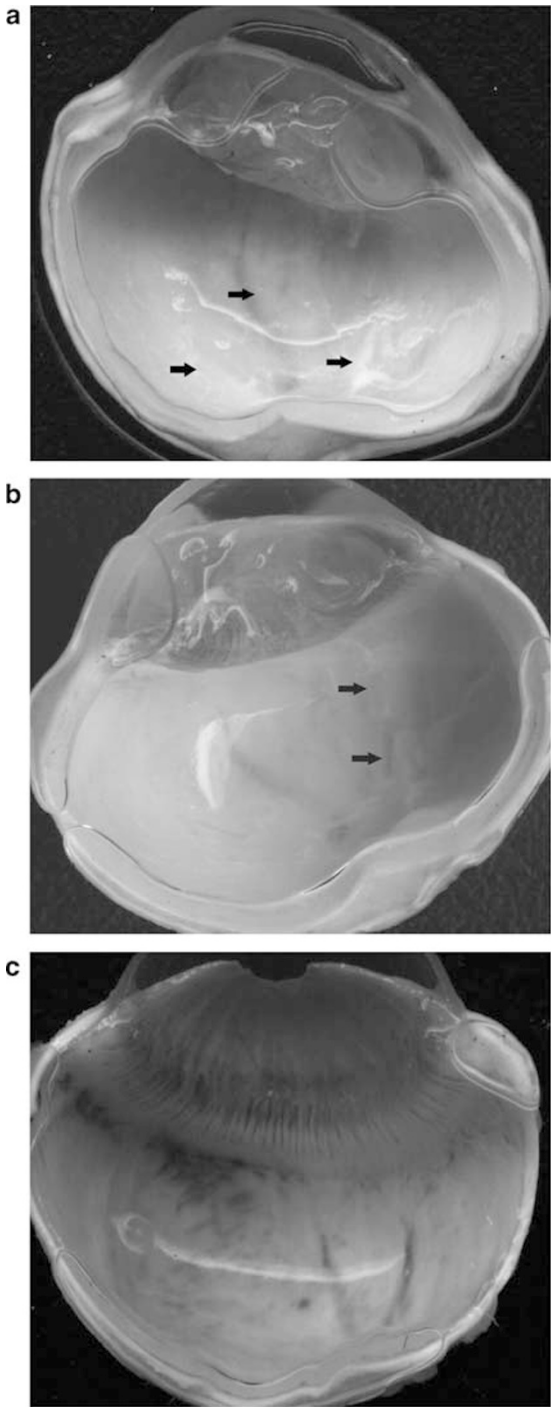

Figure 4 Photographs of gross specimen examination with triamcinolone acetonide (TA) fine particle suspension. (a) The posterior hyaloid was clearly seen as a white-coloured gel (arrow) on the whole inner retinal surface of a control eye. (b) The internal retinal surface of a partial PVD eye showed scattered islands of posterior vitreous (arrow). There was a smooth light reflex from ILM where cortical vitreous had detached from the retinal surface. (c) In a complete PVD eye, there were no remnants of vitreous on the retinal surface and smooth light reflex from ILM could be seen on the whole posterior retinal surface.

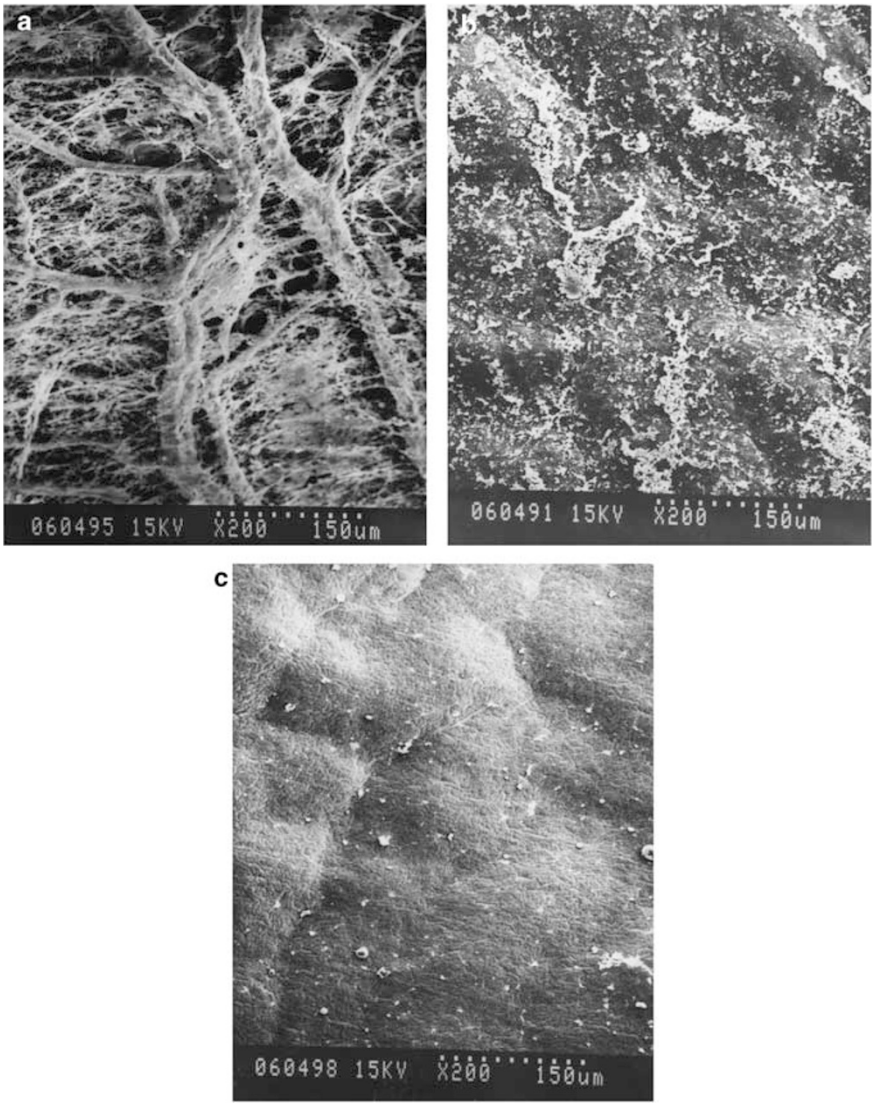

Figure 5 Scanning electron photomicrographs of vitreoretinal interface. (a) In a control eye, retinal inner surface was completely covered with a dense meshwork of collagen fibres. (b) The internal limiting membrane was covered by degraded remnants of cortical vitreous at the equator of eyes with partial PVD. (c) There was a smooth and clean ILM in complete PVD eyes. Magnification (a–c) $\times 200$.

Table 1 Dose, time, and degree of residual posterior vitreous cortical by SEM

Agents and dose	*Time (days)*	*Degree of residual posterior vitreous cortex*[a]			χ^2 *values*[b]	P-*values*[b] *(two-sided)*
		−	+	++		
BSS	1	—	—	24	—	—
	7	—	—	24	—	—
μPlm 0.5 U	1	2	2	4	13.71	0.002
	7	4	3	1	26.88	0.000
μPlm 1.0 U	1	6	2	—	32.00	0.000
	7	7	1	—	32.00	0.000
μPlm 1.5 U	1	7	1	—	32.00	0.000
	7	8	—	—	32.00	0.000

[a]++, continuous network of collagen fibrils, no PVD; +, sparse collagen fibrils, incomplete PVD; −, no collagen fibrils, complete PVD.
[b]Degree of residual cortical vitreous of test eyes in groups 1–3 compared with those of control eyes.

plasminogen and urokinase combination[25] were among the methods described.

Recombinant μPlg is the precursor of μPlm and is more easily preserved stably than μPlm and plasmin. For this reason, we considered it useful to incubate recombinant human μPlg with recombinant human rt-PA to generate μPlm before injection. Both reduced SDS-PAGE analysis and chromogenic substrate S-2251 confirmed more than 80% activation of μPlg by 0.5% molar ratio of rt-PA at 40 min. Our highest rt-PA dosage, 0.5μg/0.1 ml, was far below the values reported by Le Mer *et al*[23] and Hrach *et al*,[26] who observed clinical, electrophysiologic, and histological alterations in cat eyes receiving intravitreal rt-PA doses greater than or equal to 50 μg. Our histologic examinations confirmed the safety of very low dose of rt-PA. There was no difference in the retinal anatomy between μPlm-treated eyes and control eyes.

Similar to the results of Gandorfer *et al*,[27] our study demonstrated that μPlm alone produced cleavage at the vitreoretinal interface of rabbit eyes, and no additional surgical procedures were required to induce PVD. In controlled experiments on post mortem 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.[20,24]

To assess the cleaving effect of μPlm at the vitreoretinal interface *in vivo*, we administered three different doses into the vitreous cavity of adult rabbits and observed on 1and 7 days after injection. There was a distinct correlation between the concentration of μPlm and the degree of residual posterior vitreous cortical on 1 day after injection. Intravitreous injection into rabbit eyes of 0.5 U μPlm (about 50% of the vitreous volume of the human eye), which is equivalent to 1.0 U μPlm into the human eye, could only induce complete PVD of 25% rabbit eyes on day 1. An amount of 1.5 U μPlm induced complete PVD of 75% rabbit eyes during the same time. Comparison of three groups for days 1 and 7 did not show statistically significant deference in the degree of remnants of posterior vitreous hyaloid between days 1 and 7, suggesting consumption of the enzyme or an inhibitory mechanism against it within 24 h. This was consistent with the earlier studies on the plasmin.[19] The molecular weight of μPlm is 28 kDa, which is lower than the molecular weight of human plasmin (88 kDa). This should enable μPlm to penetrate the epiretinal tissue more effectively than plasmin.[27] On the other hand, this means a faster metabolism of μPlm than plasmin. As formal *in vivo* vitreous pharmacokinetic experiments have not been performed, the metabolism of μPlm in the vitreous cannot be determined at this time.

In addition, we used four approaches to detect complete PVD. According to the previous clinical finding, TA fine particle suspension improved the visibility of the hyaloid and the safety of the surgical procedures during pars plana vitrectomy and also inhibited the postoperative breakdown of the blood–ocular barrier.[28] Our results also showed that it succeeded in distinguishing complete PVD, incomplete PVD, and no PVD. The same efficacy was also seen in B-scan and SEM. Because the scanning depth of OCT was only 2 mm, OCT could not distinguish posterior hyaloid moving far from the retina in the complete PVD from posterior hyaloid attached to the retina in eyes with no PVD. Thus, according to our present study, SEM, B-Scan and gross examination were more effective approaches than OCT in detecting PVD. On the other hand, although SEM and gross examination are direct methods for PVD, there are also defects, because opening the eyecups, even with a sharp razor blade, can disrupt vitreous, particularly in rabbit eyes that have a very compliant scleral shell. In addition, crosslinking from fixation may alter the vitreous structure/vitreoretinal interface.

In conclusion, we described a new technique to produce μPlm by incubating two different recombinant compounds. μPlm is effective in inducing PVD and appears to be safe for the ocular ultrastructure. Compared with autologous plasmin, μPlm ensures the application of a pure substance at a defined dose. The rabbit vitreoretinal junction seems to be very strong, but is different from that in humans since it showed a constant ILM thickness and an absence of vitreal collagen fibrils inserting into the lamina densa of the ILM.[29] Further pharmacodynamic and toxicologic assessments in more appropriate animal models, such as the pig or monkey, are warranted before proceeding to clinical evaluation of this technique.

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