Pharmacologic Vitreolysis with Plasmin: Basic Science Experiments

VI.D.1.

Thierry C. Verstraeten

Outline

Keywords
Vitreous • Vitreoretinal interface • Pharmacologic vitreolysis • Plasmin • Electron microscopy • Electrophysiology

Key Concepts

1. Vitreous and the vitreoretinal interface can be altered experimentally using a nonspecific serine protease derived from blood, called plasmin.
2. Plasmin effaces the retinal surface of all vitreous collagen in experimental models.
3. There are no untoward effects of plasmin pharmacologic vitreolysis as assessed ultrastructurally and by electrophysiology.

I. Introduction

Unmet needs in the treatment of vitreoretinal (VR) disorders provide the impetus to explore new pharmacologic treatments to complement the ever-improving quality and miniaturization of mechanical surgical tools [1, 2]. This is true today as it was 20 years ago when we undertook a novel exploration in biochemical manipulation of the VR interface. Plasmin, a nonspecific serine protease, was first used in 1990 in an experimental animal model to assess its potential to cleave the VR interface [1]. The physiologic adhesion between the inner retina and the posterior vitreous cortex is primarily attributed to glycoproteins, including laminin and fibronectin [see chapter II.E. Vitreoretinal interface and the ILM]. These molecules interact with the internal limiting membrane (ILM) of the retina and the posterior vitreous cortex collagens [3, 4], which over the posterior pole are mostly running parallel to the ILM, whereas perpendicular insertion of vitreous

T.C. Verstraeten, MD
Department of Ophthalmology,
Allegheny General Hospital,
Drexel University College of Medicine,
420 East North Ave, Pittsburgh, PA15212, USA
e-mail: verstraeten3@comcast.net

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collagen fibrils is seen at the vitreous base [5]. Laminin is primarily located in the basement membrane and attached to collagen type IV. Fibronectin is seen preferentially around blood vessels, in the basement membrane of most cells, and in the extracellular matrix. In the eye, it is present in the vitreous and in the ILM. These glycoproteins are also present in lens zonules, the lamina cribrosa, and the ciliary body. Variations in the distribution of laminin and fibronectin may underlie variability in the strength of the VR adhesion. Some pathologic conditions, like diabetes, can lead to a buildup of laminin and fibronectin at the VR interface [6, 7].

Clinical observations led to the choice of plasmin as a candidate for pharmacologic vitreolysis of the VR interface. In diabetic patients in particular, the presence of neovascularization with preretinal hemorrhage in the absence of a posterior vitreous detachment (PVD) often leads to localized areas of PVD, unless fibrosis is present with resulting increased VR adhesion. It was therefore hypothesized that a blood-derived molecule may alter the VR interface and contribute to the onset of a PVD. *In vitro* studies in the early 1980s were published in cancer research and demonstrated the degradation of basement membrane components, i.e., laminin and fibronectin, by plasmin [8]. Plasmin was thought to be a candidate worthy of further study [8].

II. Plasmin

A. Biochemistry

Plasmin is a two-chain serine endopeptidase (MW = 83 kDa) linked by a disulfide bond. It contains 1 heavy chain with 5 kringles and 1 light chain, which holds its active site. Plasmin is prepared from Glu-plasminogen using urokinase, streptokinase, or tissue plasminogen activator (tPA). Its main role in blood is lysis of fibrin clots. Plasmin has been shown to break down fibronectin and laminin into degradation products that can be chemoattractive to polymorphonuclear leukocytes [9]. These cells can, in turn, release elastase that degrades collagen type IV [10], even though plasmin does not cleave collagen type IV. Plasmin can cleave collagen type V, which is not thought to be present at the VR interface, but there is no experimental evidence to support that claim. Plasmin can also activate progelatinase A, a matrix metalloproteinase-2 (MMP-2) [11] which in turn can cleave collagen type IV. In planning our experiments 20 years ago, we hypothesized that plasmin may indirectly cause the release of various enzymes believed to contribute to the onset of PVD in addition to its direct effect on the breakdown of laminin and fibronectin [1].

B. Plasmin Procurement

Lyophilized human plasmin was obtained (Calbiochem, La Jolla, CA) and stored at −20 °C. It was then reconstituted in BSS at room temperature and used immediately at the concentration of 1 U per 0.1 ml for pars plana injection. Autologous plasminogen can be isolated from a patient's plasma by affinity chromatography. One can obtain plasmin by adding streptokinase [12], but this has been found to lead to increased proteolytic activity on fibronectin and laminin *in vitro*. A lower ratio of streptokinase-activated plasminogen (1:10) can generate increasing amounts of free plasmin [13]. The same authors have raised a valid caution when one compares the activity of a particular dose or unit of plasmin, as there exists a level of variability in calibrating the particular molecule in the absence of a uniform standard [13]. They concluded that one should test plasmin activity against a uniformed standard based on the 3rd International Reference Preparation (IRP). Finally tPA at a dose of 25 micrograms has been tried in combination with transscleral cryopexy to induce a breakdown of the blood–retinal barrier with presumed release of plasminogen [see chapter VI.C. Pharmacologic vitreolysis with tissue plasminogen activator]. In rabbit eyes, this technique has been shown to produce a PVD [14] as well as in diabetic human eyes [15]. Similar results have been achieved by pairing recombinant lysine-plasminogen with recombinant urokinase [16] or pairing recombinant microplasminogen with tPA [17]. Both techniques can induce PVD in the rabbit.

III. Experimental Models

A. Animals

1. Rabbit Model

a. Efficacy

The experimental model chosen in 1990 was the rabbit because of its availability and low maintenance as well as its known strong VR adhesion without any evidence of spontaneous PVD. Some shortcomings, however, include the presence of a constant ILM thickness as well as the absence of vitreous collagen fibrils inserting onto the lamina densa of the ILM, which makes this a standout difference from the human eye [6]. The rabbit, furthermore, has a particular feature that significantly differs from the human, the presence of a medullary ray with the emergence of blood vessels at 3 and 9 o'clock along the horizontal raphe. Here the nerve fiber layer is myelinated and exhibits extremely strong adhesions to the vitreous with perpendicular insertion of collagen fibrils very similar to that of the human vitreous base [6]. In the original experiments, 1 IU of lyophilized human plasmin

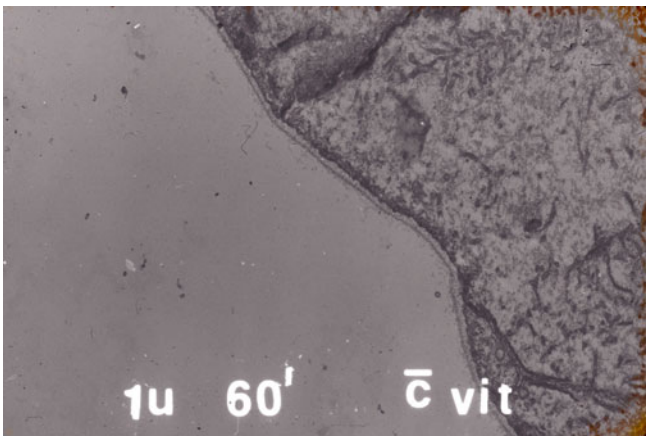

Figure VI.D.1-1 Transmission electron micrograph of the rabbit retina in an eye injected with plasmin followed by vitrectomy at 60 min. Note smooth ILM surface. Original magnification ×11,500

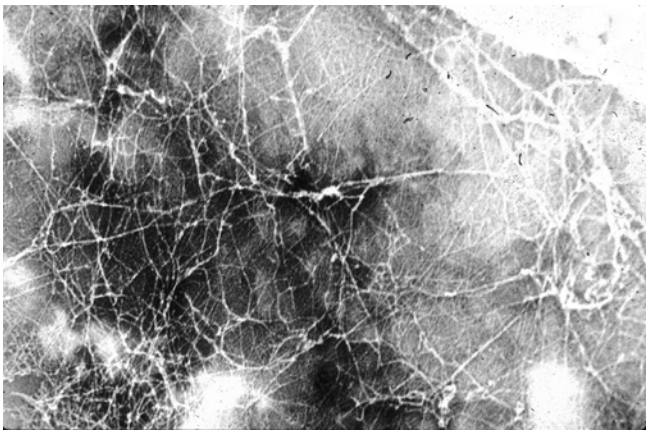

Figure VI.D.1-2 Scanning electron micrograph of the rabbit retina in an eye injected with plasmin without vitrectomy. Note sparse collagen fibrils over ILM. Original magnification ×2,500

(Calbiochem, La Jolla, CA) was injected in live rabbits' eyes and allowed to remain for varying lengths of time before sacrifice or performing vitrectomy. The first and almost immediate effect observed with indirect ophthalmoscopy at the time of plasmin injection was the appearance of a vitreous haze and a "schlieren" effect in the vitreous at the tip of the injecting needle. That observation formed this author's opinion that plasmin exerts an immediate biologic effect that includes a certain degree of liquefaction (synchysis) in addition to its activity on the VR interface. Following vitrectomy, the rabbits were sacrificed and the VR interface was studied histologically. Plasmin was found to have increasing efficacy with longer exposure time in the rabbit vitreous. Increasing the time interval between plasmin injection and performing vitrectomy from 5 to 60 min led to a smoother, cleaner retinal surface, constituting either vitreous separation or degradation of the posterior vitreous cortex. This was observed by light (LM), scanning electron (SEM), and transmission electron (TEM) microscopy, all showing bare ILM by 60 min of exposure. In the absence of vitrectomy, however, the PVD was partial, and it was only in combination with a vitrectomy that a complete PVD was observed on TEM (Figure VI.D.1-1) and SEM (Figure VI.D.1-2). In control eyes injected with balanced salt solution (BSS), vitrectomy alone resulted in a retinal surface still covered with dense collagen fibers (Figure VI.D.1-3).

b. Safety

To assess the health of the retina in these rabbits, dark-adapted electroretinography (ERG) was performed at intervals ranging from 1 h to 7 days after plasmin injection. Different surgical combinations were also tested. Vitrectomy was not found to affect the recorded amplitudes compared to controls. However, when ERGs were measured 1 h after plasmin injection, a transient 50 % decreased b wave amplitude was measured in 70 % of eyes and found to recover by day 3 (Figure VI.D.1-4). A possible explanation for this finding is the high osmolarity of the plasmin solution. Full recovery of normal ERG amplitude was always noted. Extensive histology studies by LM, TEM, and SEM have consistently shown absence of retinal toxicity not only in our rabbit model [1] but subsequently in the rat, pig [10], and monkey.

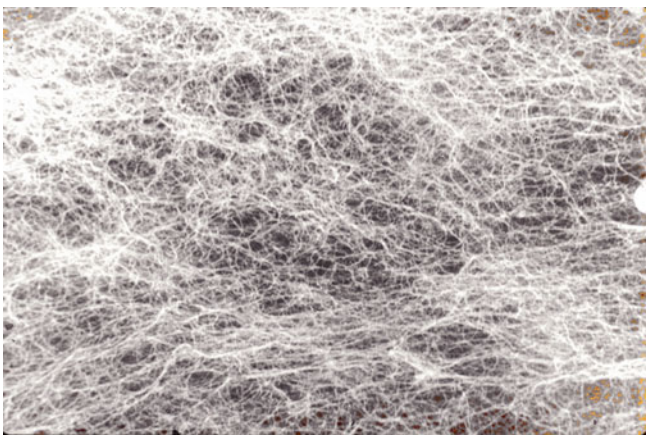

Figure VI.D.1-3 Scanning electron micrograph of the rabbit retina in an eye injected with BSS followed by vitrectomy. Note dense collagen of cortical vitreous. Original magnification ×4,200

2. Other Experimental Models and Considerations

In diabetic rats, it has been shown that it is harder to create a PVD than in normal rats, due most likely to the effects of diabetic vitreopathy [see chapter I.E. Diabetic vitreopathy]. In normal rats, plasmin alone was able to achieve a PVD whereas hyaluronidase was not. Diabetic rats required not only the use of plasmin but also hyaluronidase to achieve a complete PVD [18]. In other models, plasmin autologous to the species injected was used [19–21]. This may alleviate

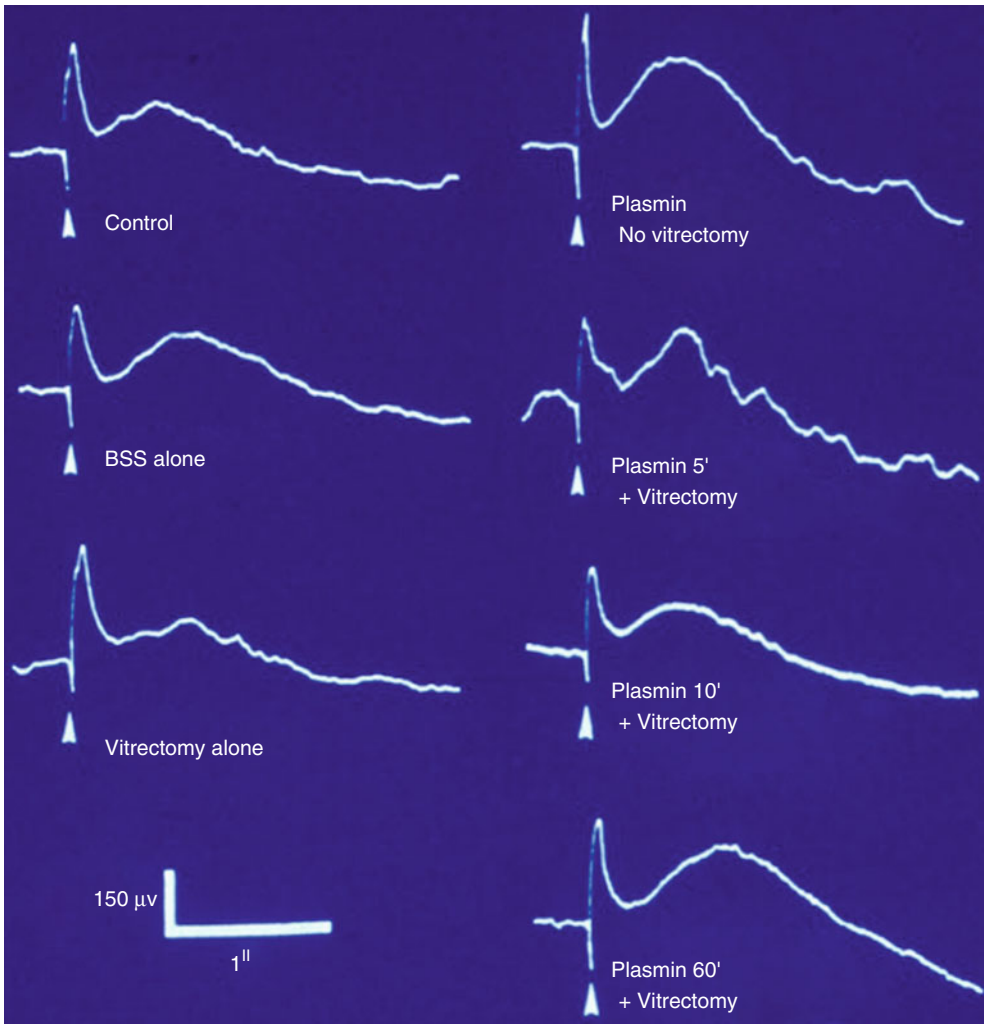

Figure VI.D.1-4
Electroretinograms taken on day 7 after different combinations of plasmin injection with or without vitrectomy. Note good b wave amplitude

some cross-species specificity concerns and potential lack of activity. In these models, plasmin has been shown to be able to produce a PVD by simply injecting it in the vitreous and not subjecting the eye to a surgical vitrectomy. Indeed, our own model used human plasmin in a rabbit model, and a smooth retinal surface was best achieved when plasmin was followed by a vitrectomy. More recently, in another rabbit model, plasmin was injected in combination with an SF6 gas injection [20, 21]. In these eyes, a total PVD was achieved whereas residual collagen fibrils and incomplete PVD was discovered in eyes injected with plasmin alone. In the pig eye model, porcine plasmin was able to achieve a clean retinal surface with total PVD within one hour of administration [19]. The investigators found a direct correlation between exposure time as well as concentration of plasmin and the degree of VR separation assessed by SEM and TEM. Postmortem human eyes were also used in this model, and a total PVD was observed after 30 min of incubation with plasmin at 37 ° C [22]. Histology revealed a smooth retinal surface in this model [23]. Interestingly, not incubating at this temperature failed to produce a PVD. Indeed a few experiments of pharmacologic vitreolysis are conducted at body temperature, an important consideration when dealing with enzymes whose activities can differ at different temperatures. This issue was raised in an experiment that found mid-vitreous and retinal temperatures dropped by about 9 ° C during vitrectomy [24]. Furthermore raising the vitreous temperature from 37 to 44 ° C for 1 min in ex vivo porcine eyes greatly facilitated the onset of an atraumatic PVD with no apparent retinal damage [25].

B. *Ex Vivo* Human Experimental Models

Postmortem human eyes were used to study the degradation of fibronectin and laminin after 60 min of exposure to plasmin [23]. Using an elegant technique with Western blot analysis of anti-laminin and anti-fibronectin antibodies, human ILM samples retrieved at the time of vitrectomy for macular hole or cystoid macular edema were tested. Some of these eyes were treated with pre-vitrectomy administration of plasmin. In these vitreous samples, fibronectin was degraded

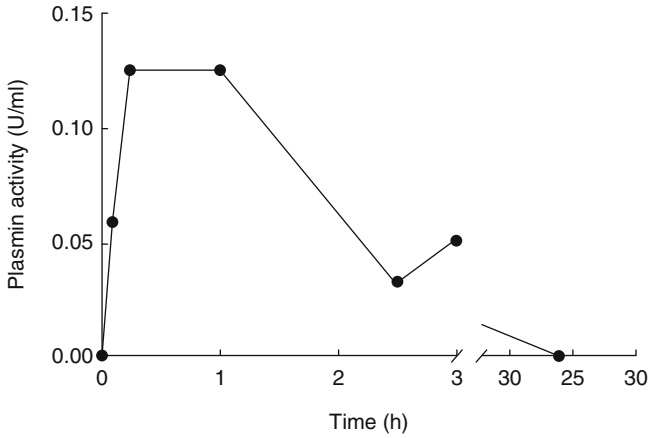

Figure VI.D.1-5 Plasmin activity in the rabbit vitreous over time after a single 1 U injection. Note total loss of activity by 24 h

in fragments of 30,000 Da, and laminin was degraded in fragments of 13,000 Da. This demonstrates the level of activity of plasmin and its capacity to break down fibronectin and laminin. Additionally, the investigators tested plasmin *in vitro* on commercially available laminin and showed no additional degradation beyond 2 h of exposure [23]. Against fibronectin, the activity of plasmin increased and peaked by 1 h of exposure. These results confirmed and validated our initial observations that seemed to indicate peak activity after 1 h of exposure to plasmin in the vitreous (Figure VI.D.1-5). Furthermore, we also found that plasmin displayed a rapid drop of activity beyond 3 h after injection in the rabbit vitreous. An in vitro caseinolytic chromogenic assay against D-Val-Leu-Lys-pNA (Sigma-Aldrich) allowed us to test undiluted vitreous samples retrieved at various time intervals following plasmin injection. It demonstrated the complete loss of activity of plasmin by 24 h (Figure VI.D.1-5). Another study looking at human donor eyes, which were injected with plasmin, showed reduced immunoreactivity for laminin and fibronectin at the vitreoretinal interface when studied histologically [26].

IV. Future Considerations

With increased understanding of the basic science factors governing a healthy vitreoretinal interface, we will be able to measure the variations present in different disease states. This will lead to the development of specific agents to target the abnormal molecules. It may turn out that as originally proposed by Sebag in 2002, the best treatment may be offered by a combination of more than one agent or enzyme acting on several key targets [27]. Likely the first step would involve the use of a "liquefying" agent followed by a second agent administered later. This second molecule would be injected right over the posterior pole with the patient in supine position, thereby avoiding loss of biologic activity caused by it being trapped in an intact vitreous gel some distance from the macula. This should lead to increasing efficacy, lower morbidity, and better adjunct therapy for diseases that still often require surgical treatment today.

Abbreviations

C	Centigrade
ERG	Electroretinogram
ILM	Inner limiting membrane
MW	Molecular weight
PVD	Posterior vitreous detachment
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
VR	Vitreoretinal

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