Effect of Plasmin on Laminin and Fibronectin During Plasmin-Assisted Vitrectomy

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Objective: To determine whether plasmin will cleave the laminin and fibronectin located at the vitreoretinal junction during plasmin-assisted vitrectomy.

Methods: Western blot analyses were performed with antilaminin or anti-fibronectin antibodies on the internal limiting membranes collected from patients with macular holes or cystoid macular edema who underwent vitrectomy with or without plasmin. The results were compared with the results of in vitro experiments in which commercially available laminin and fibronectin were exposed to commercially available plasmin.

Results: In all eyes treated with plasmin, a posterior vitreous detachment was not present before surgery but was created easily with a minimal suction of less than 100 mm Hg during vitrectomy. Western blot analyses showed that the laminin and fibronectin in the internal limiting membrane exposed to plasmin during vitrectomy were degraded to several fragments of lower molecular weights, including a fragment of approximately 13000 Da for laminin and a fragment of approximately 30000 Da for fibronectin. These lower-molecular-weight fragments also appeared in the in vitro experiments.

Conclusion: The laminin and fibronectin at the vitreoretinal junction are degraded during plasmin-assisted vitrectomy.

Clinical Relevance: These findings provide evidence for the efficacy of using plasmin to create a posterior vitreous detachment during vitreoretinal surgery.


It is generally agreed that it is efficacious to create an artificial posterior vitreous detachment (PVD) during vitreoretinal surgery for diseases such as macular hole (MH) and cystoid macular edema (CME) in eyes in which a PVD has not developed spontaneously. However, it is not always easy to create a PVD, especially in the eyes of young patients. Enzymatic vitrectomy has recently been used to make the creation of a PVD easier and safer. The benefits of the use of enzymes during vitrectomy are less damage to the anterior retinal surface and the optic nerve head and the production of a clean separation between the internal limiting membrane (ILM) and the posterior hyaloid membrane. Therefore, the use of enzymes can reduce the surgical risk and time, lower the cost of surgery, and facilitate the transition to office-based vitreoretinal procedures.

Several enzymes, including dispase, chondroitinase, hyaluronidase, tissue plasminogen activator, and plasmin, have been tested as adjunctive therapy during vitreous surgery. Among these, plasmin is one of the most promising enzymes because it can be isolated from the patient’s serum, its activity decreases to an undetectable level within 24 hours without excessive enzymatic effects, and it is nontoxic to the retina. Plasmin is already being used during vitrectomy in eyes with MHs and CME.

The rationale for using plasmin during vitrectomy is that it can cleave the laminin and fibronectin that are located at the vitreoretinal junction and are believed to mediate the adhesion of the vitreous to the ILM. However, there have been no biochemical reports directly supporting the hypothesis that plasmin cleaves laminin and fibronectin during vitreoretinal surgery. The objective of this study was to test this hypothesis. To accomplish this, ILMs were collected from patients undergoing vitrectomy with and without plasmin for MHs or CME, and Western blot analysis was performed to determine whether the laminin and fibronectin were cleaved. The results were compared with the results of in vitro experiments in which commercially available laminin and fibronectin were exposed to commercially available plasmin.

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PLASMIN-ASSISTED VITRECTOMY

For patients who underwent plasmin-assisted vitrectomy, the plasmin was prepared from the patients' serum samples as previously reported. After confirming the sterility of the plasmin, 0.4 or 0.8 U of the plasmin in 0.06 to 0.14 mL was injected into the midvitreous through a pars plana incision with a 30-gauge needle. The concentration of plasmin in the vitreous was approximately 0.1 or 0.2 U/mL, assuming a vitreal volume of 30 to 90 minutes after the injection. For the in vitro experiments, human plasmin (Calbiochem, Darmstadt, Germany) was prepared to a concentration of 0.1 or 0.2 U/mL. Human laminin (10 µg; Sigma-Aldrich Inc, St Louis, Mo) and human fibronectin (5.0 µg; Biomedical Technologies, Stoughton, Mass) were incubated with 0.7 or 1.4 µg of human plasmin in 50 µL of phosphate-buffered saline for 15, 30, 60, and 120 minutes at 35°C. The digestion was stopped by the addition of 50 µL of sodium dodecyl sulfate sample buffer and subsequent boiling at 100°C for 5 minutes. The reaction products were placed on a polyacrylamide gel and electrophoresed, and the gel was stained with Coomassie blue.

WESTERN BLOT ANALYSIS

Internal limiting membranes were collected during surgery, placed in 50 µL of phosphate-buffered saline, and stored at −80°C until use. For the analysis, they were thawed and added in 50 µL of sodium dodecyl sulfate sample buffer (0.125M Tris-hydrochloric acid, pH 6.0, 8.4% sodium dodecyl sulfate, 20% glycerol, and 10% dithiothreitol). The samples were sonicated for 30 seconds and boiled at 100°C for 5 minutes in preparation for 1-dimensional electrophoresis. The proteins were separated on a polyacrylamide gel (% stacking and % resolving gel) and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). A 60-V potential was applied across the membrane for 4 hours in the presence of Tris-glycine buffer (25mM Tris, 192mM glycine, and 20% [vol/vol] methanol, pH 8.3; Bio-Rad Laboratories, Hercules, Calif). The membrane was blocked with 1% bovine serum albumin and 0.1% Tween 20 in phosphate-buffered saline and incubated for 3 hours with a rabbit anti-human fibronectin antisera (1:5000 dilution; Biomedical Technologies, Stoughton, Mass). Other membranes were incubated with a polyclonal antibody to laminin (1:5000 dilution; Chemicon, Temecula, Calif), followed by horseradish peroxidase–linked anti-rabbit IgG (1:1000 dilution; Cell Signaling Technology Inc, Beverly, Mass). Finally, the membranes were incubated with enhanced chemiluminescence (Amersham Biosciences).

TIME COURSE OF PLASMIN DIGESTION OF LAMININ AND FIBRONECTIN

For the in vitro experiments, human plasmin (Calbiochem, Darmstadt, Germany) was prepared to a concentration of 0.1 or 0.2 U/mL. Human laminin (10 µg; Sigma-Aldrich Inc, St Louis, Mo) and human fibronectin (5.0 µg; Biomedical Technologies) were incubated with 0.7 or 1.4 µg of human plasmin in 50 µL of phosphate-buffered saline for 15, 30, 60, and 120 minutes at 35°C. The digestion was stopped by the addition of 50 µL of sodium dodecyl sulfate sample buffer and subsequent boiling at 100°C for 5 minutes. The reaction products were placed on a polyacrylamide gel and electrophoresed, and the gel was stained with Coomassie blue.

RESULTS

WESTERN BLOTTING OF ILMS

Laminin

A large-molecular-weight (MW) fragment was recognized by the anti-laminin antibody in an ILM from a patient with an MH who underwent vitrectomy without plasmin (Figure 1, lane C, arrow 1). The MW of the band was approximately 400,000 Da and corresponded to the α chain of laminin. This 400,000-Da band was not detected in the ILMs from 2 patients with MHs who underwent plasmin-assisted vitrectomy. However, a band with slightly lower MW was detected (Figure 1, lanes A-B, arrow 2), and several new bands with lower MWs were generated, including a fragment of 130,000 Da (Figure 1, lanes A-B, arrow 3).

These results were confirmed on 3 additional ILMs without plasmin exposure and 1 ILM with plasmin exposure from patients with MHs (data not shown). The ILMs from all patients with CME who underwent vitrectomy with or without plasmin demonstrated the same results (data not shown).

Fibronectin

A 400,000- to 450,000-Da band was identified in a control ILM (without plasmin) (Figure 2, lane C, arrow 1). This band corresponded with the MW of fibronectin...
dimer. In the ILMs from 2 patients with MHs who had plasmin-assisted vitrectomy, a new band with a MW of 30000 Da was identified. This band was not detected in the control (Figure 2, lanes A-B, arrow 2). The 400000- to 450000-Da fragment appeared to have been degraded to a slightly lower MW compared with the control (Figure 2, lanes A-B, arrow 3).

The same experiment was performed on the ILM from 1 patient with CME collected during plasmin-assisted vitrectomy and on the ILMs from 3 patients with MHs and 1 patient with CME who were not exposed to plasmin. The results for the control and plasmin-exposed specimens were the same as the results seen in Figure 2.

IN VITRO TIME COURSE OF PLASMIN DIGESTION OF LAMININ AND FIBRONECTIN

**Laminin**

The results of the analyses of commercially available human laminin exposed to commercially available human plasmin for 0, 15, 30, 60, and 120 minutes are shown in **Figure 3**. At 0 minutes, the gel showed several bands that were considered to be derived from laminin (Figure 3, lane A, arrowheads) because plasmin at the concentration used did not show any band (Figure 3, lane F).

At 15 minutes, 4 new bands were found, while the other bands were still present (Figure 3, lane B). Two of the new bands had MWs of about 60000 Da (Figure 3, lanes B-E, arrows 1-2), with another of about 30000 Da (Figure 3, lanes B-E, arrow 3) and another of about 13000 Da (Figure 3, lanes B-E, arrow 4). There was little change in the patterns between 15 and 120 minutes (Figure 3, lanes B-E, arrows 1-4). This experiment was repeated 3 times, and the results were reproducible.

**Fibronectin**

The results of the analyses of commercially available human fibronectin digested with commercially available human plasmin for 0, 15, 30, 60, and 120 minutes are shown in **Figure 4**. At 0 minutes, a band with a large MW was detected (Figure 4, lane A, arrow 1). It was believed to be about 450000 Da, corresponding to the MW of fibronectin dimer. Because there was no band in the lane for plasmin (Figure 4, lane F), all of the subsequent fragments were considered to be derived from fibronectin.

At 15 minutes, the 450000-Da fragment had been degraded to several new fragments (Figure 4, lane B). One of the new fragments had a MW slightly less than 450000 Da (Figure 4, lane B, arrow 2), and the other had a MW of 30000 Da (Figure 4, lane B, arrow 3). There was little difference in the appearance of the gels for exposure times of 15 and 30 minutes (Figure 4, lanes B-C). However, at 60 and 120 minutes, the 450000-Da fragment (Figure 4, arrow 1) was considerably weaker, and a new band appeared with a MW of about 200000 Da (Figure 4, lanes D-E, arrow 4). The 30000-Da fragment was more visible at 60 minutes.

After 60 minutes, all of the bands appeared to have reached equilibrium and showed no remarkable change thereafter. The results were confirmed by repeating the experiments 3 times.

**COMMENT**

Fibronectin exists on the surface of most cells and most extracellular matrices, and it is a component of basement membranes. It has a high affinity for extracellular matrix components such as collagen. Laminin, on the other hand, is located only in the basement membrane, where it binds to and promotes the adhesion of cells to collagen type IV. In the eye, fibronectin is present in the ILM and the vitreous, whereas laminin is located only in the...
As already noted, the effects of plasmin in the creation of a PVD and on the morphology of the vitreoretinal junction have been extensively studied; however, no one has reported on the biochemical effects of plasmin on laminin and fibronectin at the vitreoretinal junction, to our knowledge. Our results showed that the laminin and fibronectin located at the vitreoretinal junction were cleaved into several lower MW fragments. These results support our hypothesis and previous findings that plasmin is an efficient enzyme for separating the vitreoretinal interface.

The results of our in vitro experiments in which commercially available laminin and fibronectin were exposed to plasmin were compared with the results on ILMs obtained during vitrectomy. After exposure to plasmin, cleavage fragments with MWs of 13000 Da for laminin and 30000 Da for fibronectin resulted from the human ILM and the in vitro experiments. For fibronectin, the 30000-Da fragment has been detected in previous in vitro experiments and was reported to be flanked by a collagen-binding domain. These results suggest that the laminin and fibronectin at the vitreoretinal junction were cleaved during the plasmin-assisted vitrectomy.

The MWs of several fragments from laminin and fibronectin in the ILMs obtained from patients (Figure 1 and Figure 2) differed from those in the in vitro experiments (Figure 3 and Figure 4). One possible reason for this difference is that the laminin and fibronectin for the 2 experiments were derived from different tissues; one was from human ILM and the other was from human plasma (fibronectin) or human placenta (laminin). Laminin is known to be composed of many distinct domains with different structure and function in each tissue. Another reason for the difference may be because different techniques were used. The total protein was stained with Coomassie blue in the in vitro experiments, whereas the fragments were made visible in the Western blot analyses using enhanced chemiluminescence in the ILM experiments.

Previous researchers have investigated the most effective exposure time for plasmin to create a PVD during vitrectomy. Verstraeten et al injected 1 U of plasmin into rabbit eyes, with reaction times of 5, 15, and 60 minutes, and reported that the longer reaction times were more effective. Gandorfer et al found that 2 U of plasmin was more effective than 1 U and that 60 minutes of exposure time was more effective than 30 minutes to produce a PVD in pig eyes. In human cadaver eyes, 1 or 2 U of plasmin reacted for 30 minutes at 37°C was effective in producing a PVD.

In patients, 0.4 to 0.8 U of plasmin has been used during vitrectomy, with an exposure time of 15 minutes. In an earlier study, exposure times of 30 to 90 minutes were used, and in the present in vitro experiments, laminin was completely degraded by 15 minutes, with no significant change seen for up to 120 minutes. On the other hand, fibronectin continued to be degraded for up to 60 minutes, with no significant change seen thereafter. Although these in vitro results may not completely reflect the time-dependent reactions in human eyes, they suggest that the exposure time of 15 to

Figure 4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of plasmin-digested fibronectin, stained with Coomassie blue. Lanes A through E, Results of incubating fibronectin with plasmin for 0, 15, 30, 60, and 120 minutes. Lane F, Results with plasmin alone.

ILM. Laminin and fibronectin are thought to play a role in stabilizing the vitreoretinal adhesion.

Plasmin is known to cleave laminin and fibronectin to specific products but not to cleave type IV collagen. The cleaving of laminin and fibronectin by plasmin is hypothesized to separate the vitreous cortex from the ILM to create a PVD. The effect of plasmin on the vitreoretinal junction has been studied morphologically in animals. Light and electron microscopic analyses in rabbit eyes showed a complete separation of the vitreous cortex from the retina, resulting in a smooth ILM surface. In pig eyes, a PVD was created by intravitreal injection of plasmin, and no morphological damage was detected in the retina.

Electron microscopic investigations of postmortem human eyes exposed to intravitreal plasmin revealed a smooth retinal surface with only sparse collagen fibrils, whereas the retinal surface showed a network of collagen fibrils in eyes not exposed to plasmin. The use of plasmin did not appear to cause any morphologic damage to the retina.

Based on these findings, enzymatic vitrectomy using plasmin was clinically tested. Margherio et al used plasmin-assisted vitrectomy in patients younger than 14 years with traumatic MHs. During the plasmin-assisted operation, the vitreous cortex was easily separated from the retinal surface, and all of the MHs were successfully closed, with significant visual improvement. Tresse et al reported that enzyme-assisted vitrectomy with 0.4 U of plasmin reduced the operation time in 9 eyes of 8 patients who had stage III MHs. Williams et al used 0.4 U of plasmin in 7 patients with both diabetes mellitus and macular tractional retinal detachments and 1 patient who had concurrent macular edema. They succeeded in creating a PVD or were able to remove the posterior hyaloid easily in all eyes. More recently, Asami et al used plasmin during vitrectomy for diabetic macular edema, and results showed that it was possible to induce a PVD spontaneously or with minimal suction. The surface of the peeled ILM was examined by electron microscopy, and it was found to be smooth, without collagen fibrils.
90 minutes may be effective for this concentration of plasmin during vitrectomy.

In conclusion, we have shown by Western blot analysis that laminin and fibronectin at the vitreoretinal junction were degraded during plasmin-assisted vitrectomy. These findings provide evidence for the efficacy of using plasmin to create a PVD during vitrectomy surgery.

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