Posterior Vitreous Detachment Induced by Nattokinase (Subtilisin NAT): A Novel Enzyme for Pharmacologic Vitreolysis

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PURPOSE. To investigate the effects of intravitreal injection of nattokinase (subtilisin NAT), a serine protease that is produced by Bacillus subtilis (natto), for induction of posterior vitreous detachment (PVD).

METHODS. Different doses of nattokinase (1, 0.1, or 0.01 fibrin-degradation units [FU]) or physiologic saline as a control were injected into the vitreous cavity of rabbit eyes. Scanning electron microscopy was used to observe the retinal surfaces of four rabbit eyes per concentration. Histologic alterations were assessed by light microscopy, using four eyes from each group. Electoretinography (ERG) was performed to observe retinal function, ranging from 1 hour to 1 week after the nattokinase (1 or 0.1 FU) or saline solution administration, using four eyes from each group at each time point. Also, findings in all rabbits were monitored by slit lamp examination and by indirect ophthalmoscopy with a 20-D lens.

RESULTS. Scanning electron microscopy showed smooth retinal surfaces, indicating the occurrence of PVD at 30 minutes after intervention in all the experimental eyes injected with 0.1 or 1.0 FU nattokinase, but none of the control eyes. Light microscopy and ERG analysis showed no critical change even after the use of 0.1 FU nattokinase, an amount sufficient to induce PVD. However, toxicity in the forms of preretinal hemorrhage and ERG changes was noted with the higher dose (1 FU) of nattokinase.

CONCLUSIONS. The results suggested that nattokinase is a useful enzyme for pharmacologic vitreolysis because of its efficacy in inducing PVD. (Invest Ophthalmol Vis Sci. 2006;47:2075–2079) DOI:10.1167/iovs.05-0130

The vitreous is composed of a network of two major constituents, collagen, and hyaluronan. In patients with proliferative vitreoretinal disorders, the formation and recurrence of proliferative tissue at the vitreoretinal interface often cause a high incidence of vitreous hemorrhage, retinal detachment, and resultant visual loss after vitreous surgeries. The current treatment for vision-threatening proliferative vitreoretinal diseases consists of vitrectomy to remove the proliferative tissue and its scaffold of vitreous from the retinal surface surgically.

However, the surgical procedure for the creation of posterior vitreous detachment (PVD) is associated with a potential risk of the onset of retinal breaks and hemorrhage. In addition, even after vitreous surgeries, the occurrence and progression of cell proliferation on the residual vitreous gel sometimes cause serious postoperative complications, such as proliferative vitreoretinopathy (PVR). Therefore, the development of an enzymatic treatment for the complete removal of the vitreous (and associated proliferative tissue) is desirable, to reduce the incidence of complications during and after vitreous surgeries.

The vitreous is composed mainly of collagen and hyaluronan; therefore, hyaluronidase has been recommended for intravitreal injection to facilitate vitreous liquefaction and vitreous hemorrhage absorption. Plasmin has been also reported to be an effective adjunct for the induction of PVD. Previous reports on the clinical use of plasmin purified from autologous blood showed the usefulness of enzyme-assisted vitrectomy for vitreoretinal diseases.

Nattokinase (subtilisin NAT) is a serine protease composed of 275 amino acids that is produced by Bacillus subtilis (natto). It has potent fibrinolytic activity, enhances plasminogen activators, and inactivates a plasminogen activator inhibitor. It also has fibrinolytic activity when administered orally and is widely available in processed and health foods containing natto (fermented soybean) extracts. Therefore, we hypothesized that nattokinase might be an effective pharmacologic adjunct to surgery to induce PVD in patients with vitreoretinal disorders.

In the present study, we determined the efficacy and the safety of nattokinase for pharmacologic vitreolysis.

MATERIALS AND METHODS

Experimental Animals

Japanese adult albino rabbits (Kyudo, Kumamoto, Japan), 12 weeks old and weighing 2.0 kg, were used in the study. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Committee on Animal Research of Kumamoto University.

Nattokinase Preparation

Nattokinase was supplied by Japan Bio Science Laboratory Co., Ltd. (JBSL; Oita, Japan). It was purified from fermented soybean extract.

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(product name NSK-SD; JBSL) including rich nattokinase produced by *B. subtilis* (natto), as follows. The fermented soybean extract (NSK-SD) was dissolved in 2 mM calcium acetate, dialyzed overnight in 10 mM phosphate buffer (pH 7.0), adsorbed on a pre-equilibrated Sepharose column (CM-Sepharose Fast Flow; GE Healthcare, Piscataway, NJ), and finally washed and eluted with a linear gradient of 10 mM phosphate buffer (pH 7.0) containing 500 mM sodium chloride. To obtain the active enzyme fraction, the sample was gel-filtrated in a Sephacryl column (5-100 HR; GE Healthcare) equilibrated with 10 mM phosphate buffer (pH 7.5) containing 150 mM of sodium chloride. The active enzyme fraction was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and confirmed as a single band.

**Nattokinase Activity**

Nattokinase activity was measured with a fibrin degradation assay developed by JBSL. First, 0.4 mL of 0.72% fibrinogen (fibrinogen fraction I, type I8, product number F8630; Sigma-Aldrich, St. Louis, MO) was placed in a test tube (15-mm inner diameter × 150-mm length) with 1.4 mL of 50 mM borate buffer (pH 8.5) containing 0.9% sodium chloride and incubated in a constant-temperature water bath at 37°C for exactly 10 minutes. Then, 0.1 mL of a 20-U/mL thrombin solution was added. The solution was incubated at 37°C ± 0.3°C for exactly 10 minutes. 0.1 mL of sample solution was added, and incubation continued at 37°C ± 0.3°C. This solution was again mixed after 20 and 40 minutes. At exactly 60 minutes, 2 mL of 200 mM trichloroacetic acid was added, mixed, and the solution was incubated at 37°C ± 0.3°C for 20 minutes. This solution was placed in a microcentrifuge tube and centrifuged at 15,000g for 5 minutes. Then, 1 mL of supernatant was collected and the absorbance at 275 nm was measured. In this assay, 1 unit (fibrin degradation unit, FU) of enzyme activity is defined as a 0.01-per-minute increase in absorbance at 275 nm of the reaction solution (excluding acid-insoluble material).

The substrate (fibrinogen) used in this assay is naturally derived, and so there can be considerable differences in quality among product lots. This makes it difficult to determine absolute values for enzyme activity. Therefore, JBSL also supplies a standard enzyme for use in the assay. The measured value divided by the labeled standard enzyme activity was used as a correction factor. All assay values were then multiplied by this correction factor. Measurement of human plasmin activity by this assay showed that 1 unit of human plasmin (Calbiochem, La Jolla, CA) had an activity equivalent to 1 FU. These activities were measured by JBSL, and the prepared nattokinase was used immediately after measurement of the activity.

**Anesthesia**

The rabbits were anesthetized for each procedure with pentobarbital (20 mg/kg injected intravenously) and ketamine hydrochloride (20 mg/kg injected intramuscularly).

**Administration of Nattokinase**

Rabbits were randomized into four groups. Group 1 received 1 FU nattokinase, group 2 received 0.1 FU, group 3 received 0.01 FU, and group 4 received physiologic saline (BSS Plus; Alcon Surgical, Tokyo, Japan) in one eye. The pupils were dilated with a mixture of 0.5% tropicamide and 0.5% phenylephrine hydrochloride. Nattokinase activity was adjusted by dilution with physiologic saline. After filter sterilization using a 0.22-µm membrane filter, 1-mL syringes of nattokinase dilutions were prepared (1 FU/0.1 mL, 0.1 FU/0.1 mL, and 0.01 FU/0.1 mL). A vitrectomy contact lens was placed on the cornea, and then under an ophthalmic microscope, a 30-gauge needle attached to a syringe was inserted at a point 2 mm from the corneal limbus. Then, 0.1 mL of nattokinase solution was carefully and slowly injected into the center of the vitreous cavity. Control eyes were injected in a similar manner with 0.1 mL physiologic saline. Because of increased intraocular pressure after injection, anterior chamber paracentesis was performed immediately with a 20-gauge ophthalmic knife to normalize the pressure and prevent the solution from coming out of the eye.

**Scanning Electron Microscopy**

Sixteen eyes were studied with scanning electron microscopy (SEM). Nattokinase (1, 0.1, or 0.01 FU) or saline was injected into the vitreous cavity of four rabbit eyes per concentration. At 30 minutes after injection, the eyes were enucleated and fixed with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer at 4°C. One hour later, the eyes were cut circumferentially at the limbus to make posterior cups and then placed in fixative solution (the same one as above) overnight. The posterior cup was cut across and vertically with a razor to prepare samples for SEM. The samples were placed in 2% tannic acid (Wako Pure Chemicals, Osaka, Japan) and allowed to stand overnight at 4°C. Then, the samples were washed six times with phosphate-buffered saline at 20-minute intervals, placed in 2% osmium on ice, and allowed to stand for 70 minutes. After recovery of the 2% osmium, the samples were washed three times with distilled water, dehydrated in an ethanol series (50% for 15 minutes, 70% for 15 minutes, 90% for 15 minutes, 95% for 15 minutes, and twice in 99.5% for 30 minutes), and immersed in t-butyl alcohol (20 minutes, three times). After freezing, the samples were freeze dried, mounted on an aluminum stage with double-sided carbon tape, coated with gold (JFC-1200; JEOL, Tokyo, Japan), and examined with a scanning electron microscope (JSM-5800 LV; JEOL).

**Light Microscopy and Electoretinography**

To evaluate the short-term effects of intravitreal injection of nattokinase (1, 0.1, or 0.01 FU) or saline solution as a control, four eyes from each group were used for a light microscopic study of the retina. The eyes were fixed with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer for 24 hours and embedded in paraffin, and horizontal sections were made and stained with hematoxylin and eosin.

Also, to investigate the adverse effects of nattokinase, we obtained scotopic full-field electoretinograms (ERGs) at times ranging from 1 hour to 1 week after the nattokinase (1 or 0.1 FU) or saline administration, using four eyes from each group at each time point. The pupils were dilated and dark adapted for 30 minutes before recordings. White-light-emitting diode built-in contact lens electrodes (LW-102; Tomey, Waltham, MA) were put on the cornea, and an electrode was attached to the forehead after the hair was shaved from the area. A ground electrode was attached to the ear. Stimuli (6000 cd/m², 0.5 ms) were delivered, and ERGs were recorded (LE-2000; Tomey). Statistical analyses were performed on a computer (StatView, ver. 5.0; SAS Institute, Inc., Cary, NC). In addition, a light microscopic study of the retina was performed 1 week after the administration of 1 or 0.1 FU nattokinase or saline solution in four eyes from each group.

**Slit Lamp Examination and Indirect Ophthalmoscopy**

Findings in all rabbits were monitored by slit lamp examination and by indirect ophthalmoscopy with a 20D lens.

**RESULTS**

**Scanning Electron Microscopy**

Figure 1 shows an example of the retinal surface observed by SEM. In all the saline-injected eyes, SEM showed very dense vitreous fibers covering the entire area of the retinal surface (Fig. 1, top left). All eyes treated with 0.01 FU nattokinase still showed numerous vitreous fibers covering the retinal surface (Fig. 1, top right). These fibers were attached to the retina, particularly in the area of the medullary rays. In contrast, all eyes in the groups treated with 0.1 or 1 FU nattokinase exhibit-
edited smooth retinal surfaces without any remnant of vitreous fibers, except in the regions of the medullary rays (Fig. 1, bottom).

**Light Microscopy and ERG**

Figure 2 shows an example of the retinal section 30 minutes after the administration of nattokinase. All eyes showed intact retinal surfaces and no abnormalities compared with control eyes.

Figure 3 shows the ERG findings for tested eyes. The ERG a- and b-waves in control eyes showed no significant reductions in the mean amplitudes from the preoperative examination ($P > 0.1$). In eyes treated with 0.1 FU nattokinase, the a-wave showed no significant reductions. Temporary reduction of the b-wave was observed 1 day after the administration of 0.1 FU nattokinase ($P = 0.021$); however, amplitude recovered 1 week after the intervention ($P = 0.170$). In eyes injected with 1 FU nattokinase, the b-wave amplitude 1 day and 1 week after injection showed significant reductions ($P = 0.012$ and 0.045, respectively). The a-wave 1 week after injection also showed significant reduction ($P = 0.006$). The histology of the retina 1 week after administration of saline solution or 0.1 FU nattokinase showed no adverse change. One week after 1 FU nattokinase, however, slight thinning of the inner plexiform layer was observed (Fig. 4).

**Slit Lamp Examination and Indirect Ophthalmoscopy**

Slit lamp examination and indirect ophthalmoscopy during follow-up showed no evidence of hemorrhage, retinal detachment. No retinal detachment was observed in any of the eyes treated with nattokinase. The histology 1 week after administration of saline solution or 0.1 FU nattokinase showed no adverse change. One week after 1 FU nattokinase, however, slight thinning of the inner plexiform layer was observed (Fig. 4).
ment, or any other complications after the use of 0.01 FU nattokinase in all the tested eyes. Also, all eyes injected with 0.1 FU nattokinase, which was sufficient to induce PVD, showed no abnormalities compared with control eyes. However, 1 day after injection of 1 FU nattokinase, we observed mild preretinal hemorrhages near the optic disc in all the eyes. One week after injection of 1 FU nattokinase, the mild hemorrhages were still observed, but the area gradually reduced.

**Discussion**

Safer surgical procedures for removing the vitreous in the treatment of vitreoretinal diseases such as proliferative diabetic retinopathy (PVR) and numerous macular diseases are constantly being sought. Among them, enzyme-assisted vitrectomy has been advocated as a useful surgical modality for the treatment of vitreoretinal diseases. Hyaluronidase, which breaks down hyaluronan, facilitates vitreous liquefaction and vitreous hemorrhage absorption; however, it is known that hyaluronidase does not induce PVD.\(^{23,24}\) In contrast, it has been reported that autologous plasmin is useful in inducing PVD and in facilitating vitrectorial surgery in clinical cases.\(^{15–18}\) However, the purification of autologous plasmin requires sterile facilities and trained personnel to perform. Thus, the development of another form has been regarded as important in the treatment of many proliferative vitreoretinal diseases and macular diseases.

In this study, ultrastructural observation with SEM revealed a loss of vitreous fibers on the retinal surface, suggesting the occurrence of PVD induced by nattokinase. Also, concerning the safety of the nattokinase injection, the histology of the retina 30 minutes after the administration of several doses of nattokinase showed no significant damage. In addition, ERGs after the injection of amounts of nattokinase sufficient to induce PVD revealed no critical reductions in the a- and b-wave amplitudes. These results suggest that using an adequate dose of nattokinase as a pharmacologic adjunct to pharmacologic vitreolysis and/or enzyme-assisted vitrectomy in patients with vitreoretinal disorders is very promising.

The mass purification of this *B. subtilis* (natto)-derived enzyme is achieved with an easy and convenient protocol in comparison with plasmin. The clinical application of this enzyme will enable us to perform pharmacologic vitreolysis and/or enzyme-assisted vitrectomy even in medical centers without the facilities or trained personnel to purify autologous plasmin, and in patients requiring urgent surgery. In addition, unlike with autologous plasmin, we can obtain uniform enzymatic activity by the use of purified nattokinase among individual cases. These advantages make nattokinase a reasonable alternative in all medical centers for performing safer and less invasive treatment for vitreoretinal disorders.

Nattokinase hydrolizes collagen fiber, which is a matrix component of the vitreous, indicating effectiveness in liquefying the vitreous gel when injected into the vitreous cavity. In the present study, SEM showed smooth retinal surfaces without vitreous fibers, suggesting strong and broad cleavage of some materials in the interface between the retina and vitreous by nattokinase. Regarding the mechanisms of PVD induction, our previous study suggested that the activation of endogenous matrix metalloproteinase-2 by exogenous plasmin is associated with the induction of PVD.\(^{25}\) Despite having considerably different amino acid sequences, nattokinase and plasmin share some common features: both are serine proteases and both have a high affinity for fibrin. Nattokinase not only has potent fibrinolytic activity but also enhances plasminogen activators and inactivates a plasminogen activator inhibitor.\(^{20–22}\) Therefore, regarding the PVD-inducing mechanism, we think that nattokinase may have two major effects: one is the direct effect of liquefying the vitreous gel by its proteolytic activity and the other is the indirect effect of increasing the plasmin activity that induces the vitreoretinal dehiscence. In further studies, the details of the mechanisms related to nattokinase’s PVD-inducing effects should be revealed. Concerning the therapeutic dose of nattokinase from this study, we think that 0.1 FU is adequate for pharmacologic vitreolysis. After administration of 1 FU nattokinase, the experimental eyes showed preretinal hemorrhage and histologic alteration. Although we did not determine the relationship between these findings, it may not be a direct toxic effect on ocular tissues, but rather the result of sudden separation of the posterior vitreous cortex from the retina that was induced by the higher dose. Further study of the optimal concentration of nattokinase and the mechanism of its adverse effects should be conducted. Further study is also necessary to determine an adequate exposure time to this enzyme and any adverse effects after an injection of nattokinase combined with a mechanical vitrectomy.

In conclusion, our findings suggest that nattokinase may be a useful pharmacologic adjunct in vitreous surgery.

**References**


