High Subretinal Fluid Procoagulant Activity in Rhegmatogenous Retinal Detachment

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PURPOSE. An increased mRNA expression of genes related to blood coagulation has been demonstrated in an experimental retinal detachment model but has not yet been confirmed in human clinical specimens. Tissue factor (TF), the initiating factor of blood coagulation, may be a determinant of the extent of tissue injury after rhegmatogenous retinal detachment (RRD). This study was conducted to determine whether subretinal fluid and vitreous fluid collected from patients with RRD have a procoagulant effect.

METHODS. Calibrated thrombin generation (CAT) was used to investigate the thrombogenic properties of 28 subretinal fluids collected during scleral buckling surgery for RRD. Further, the thrombogenic properties of vitreous fluids from RRD (n = 12), macular pucker (n = 5), macular hole (n = 6), and proliferative diabetic retinopathy (n = 5) were compared with the properties of eye bank eyes (n = 11), which served as control specimens. The procoagulant activity of TF was determined with Western blot analysis.

RESULTS. The addition of subretinal fluid from all RRD patients (28/28, 100%) induced thrombin generation in normal and severely factor (F)XII-deficient plasma. Contrary to the subretinal fluid, the addition of vitreous fluids from various ocular disorders evoked very little thrombin generation in normal and severely FXII-deficient plasma (4/12, 33% RRD; 1/5, 20% macular pucker; 0/6, 0% macular hole; 0/5, 0% proliferative diabetic retinopathy; and 2/11, 18% eye bank eyes). The procoagulant activity in subretinal fluid was almost completely neutralized by antibodies against human TF. The presence of TF in subretinal fluid was confirmed by Western blot analysis.

CONCLUSIONS. Subretinal fluid of patients with RRD induces high procoagulant activity, determined by measuring the level of tissue factor. (Invest Ophthalmol Vis Sci. 2010;51: 5234–5239) DOI:10.1167/iovs.10-5354
High Subretinal Fluid Procoagulant Activity in RRD

Methods

Chemicals
Phospholipid vesicles consisted of 20 mol% phosphatidylserine (PS), 20 mol% phosphatidyl-ethanolamine (PE), and 60 mol% phosphatidylcholine (PC) and were obtained from Avanti (Alabaster, AL). Recombinant relipidated (r)TF not containing polybrene or Ca²⁺ was a kind gift from Dade Behring (Marburg, Germany). HEPES-buffered saline comprised 20 mM HEPES and 140 mM NaCl (pH 7.35). Fluorogenic substrate, Z-Gly-Gly-Arg-AMC, was obtained from Bachem (Bubendorf, Switzerland). On splitting by thrombin, Z-Gly-Gly-Arg-AMC releases the fluorescent AMC (7-amino-4-methylcoumarin), which is measured by a 390-nm excitation and a 460-nm emission filter set. A fresh mixture of fluorogenic substrate and CaCl₂ was prepared for each experiment as follows: To 25 mL of buffer (HEPES 20 mM, pH 7.35) containing 60 g/L BSA (A-7030; Sigma-Aldrich), 100 μL of 1 M CaCl₂ was added. At 37°C, 25 μL of a 100-mM DMSO solution of the fluorogenic substrate was squirted into the mixture and immediately vigorously stirred. The resulting clear solution, referred to as FluCa, thus is 2.5 mM in fluorogenic substrate and 100 mM in CaCl₂. A calibrator was prepared in-house and consisted of α₂-macroglobulin-thrombin (α₂-M-T) complex with an activity that is equal to that of 600 nM human thrombin in buffer. Severely factor (F)XII-deficient plasma was obtained from Siemens (Marburg, Germany). Antibodies against TF were purchased from American Diagnostica (Stamford, CT).

Subjects
In our department, subretinal fluid and vitreous fluid samples are routinely collected during scleral buckling surgery and pars plana vitrectomy, respectively. From samples stored in our Biobank, we selected 28 subretinal fluid samples from patients with primary RRD. Eighteen patients had an uncomplicated postsurgical follow-up, and 10 patients had a redetachment due to development of PVR within 2 months. In the former group, two different collection techniques were applied. To investigate whether vitreous fluids of various ocular disorders would induce procoagulant activity, we selected 12 patients with primary RRD, 5 patients with macular pucker, 6 patients with macular hole, and 5 patients with PDR. Eleven human vitreous samples obtained from the Cornea Bank Amsterdam. All samples were core of the vitreous body. Vitreous samples from eye bank eyes were obtained from the Cornea Bank Amsterdam. All samples were collected in sterile tubes and immediately stored at −80°C until assayed. Sample volumes ranged between 50 and 250 μL for subretinal fluid and between 100 and 1500 μL for vitreous fluid.

Blood and Plasma
Blood was obtained through antecubital venipuncture (1 vol 0.13 M trisodium citrate to 9 vol blood) from healthy individuals who consented to participate in the study. Free flow or minimal suction was used: vacuum containers were avoided. The blood was centrifuged at 1000g for 10 minutes at room temperature, and the plasma was aspirated. This procedure was repeated once. The plasma was stored at −80°C in 1-mL aliquots.

The Automated CAT
The thrombograms were measured in a 96-well plate fluorometer (Ascent reader; Thermolab systems OY, Helsinki Finland) equipped with a 390/460 filter set (excitation/emission) and a dispenser. Flat-bottomed 96-well plates (Thermo Scientific, Waltham, MA) were used. Each experiment needs two sets of readings, one from a well in which thrombin generation takes place (TG well) and a second one from a well to which the calibrator has been added (CL well). Experiments were performed in duplicate (i.e., a set of two TG wells was compared to a set of two CL wells). A dedicated software program (Thrombinoscope; Synapse BV, Maastricht, The Netherlands) enables the identification of the (sets of) wells and determines the duration of the experiment and the sampling rate (usually 4/min). To each well, 80 μL of plasma (PPP) was added. The TG wells received 20 μL of buffer, containing the trigger (including 5 μL of the ocular fluid ≤5 μL of antibodies against TF) but no Ca²⁺, whereas the CL wells received 20 μL of the α₂-M-T solution. The trigger was the subretinal fluid or vitreous fluid obtained during ocular surgery, together with 24 μM PS/PC/PE vesicles in HEPES-buffered saline, in the absence of TF. The plate was placed in the fluorometer and allowed to warm to 37°C (minimum time, 5 minutes). The dispenser of the fluorometer was flushed with a warm 100-mM CaCl₂ solution, emptied, and flushed with warm FluCa. At the start of the experiment, the instrument dispenses 20 μL of FluCa to all the wells to be measured, registers this as time 0, shakes them for 10 seconds, and starts reading. During the measurement, the program compares the readings from the TG and the CL wells, calculates thrombin concentration and displays the thrombin concentration in time.

A typical course of a thrombogram is illustrated in Figure 1. The period in which no observable thrombin is formed reflects the lag time. After this phase, the concentration of thrombin increases rapidly until a peak is reached and then decreases again. The area under the curve is called the endogenous thrombin potential (ETP) and is obtained by adding an excess of thrombin substrate, so that the total amount of substrate converted is proportional to the area under the thrombogram. Other parameters of the thrombogram are the peak and the time it takes to reach the peak. Evident thrombin generation is defined as a peak of >50 nM.

In any experimental setting, the intrinsic pathway of the coagulation cascade is triggered through contact activation. To rule out the contribution of contact activation to the amount of thrombin generated, severely FXII-deficient plasma was used in addition to normal plasma in all the experiments. As a result, the use of severely FXII-deficient plasma prevented any thrombin from being generated in the absence of a trigger of the coagulation system.

Western Blot Analysis
Protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA). Ten micrometers of total protein was separated on a 4% to 12% Bis-Tris gel (Novex; Invitrogen), transferred to nitrocellulose and followed by immunodetection with rabbit polyclonal TF-
specific antibodies. Before immunodetection, the blot was stained with Ponceau S to assess equal loading.

**Statistical Analysis**

The nonparametric Mann-Whitney U test was used for the comparison of the parameters of the thrombogram between samples collected by the two different techniques and between samples from RRD patients, with and without postsurgical PVR. The nonparametric Wilcoxon signed-rank test was used to compare the procoagulant activity of the whole cell samples with the corresponding supernatants. The \( \chi^2 \) test was used to compare nominal variables such as diabetes mellitus. Differences at \( P < 0.05 \) were statistically significant.

**RESULTS**

To investigate whether subretinal fluid induces procoagulant activity, we added samples from 10 patients who underwent successful scleral buckling surgery for primary RRD to normal and severely XII-deficient plasma. All patients had an uncomplicated follow-up. The samples were collected by making a small incision into the sclera and choroid. In the absence of TF, the addition of subretinal fluid from patients with primary RRD induced thrombin generation to a different extent in all cases (Fig. 2), whereas no thrombin at all was generated in the absence of subretinal fluid. Antibodies against human TF almost completely neutralized the procoagulant activity of subretinal fluid (black). Only one example is shown.

**FIGURE 1.** Typical course of a thrombogram with its parameters. (A) Lag time, (B) endogenous thrombin potential (ETP), (C) peak, and (D) time to peak.

**FIGURE 2.** Thrombin generation in plasma; effect of the addition of subretinal fluid in the absence of TF. Ten subretinal fluid samples obtained during scleral buckling surgery for primary RRD provoked thrombin generation to a different extent in severely FXII-deficient plasma.

**FIGURE 3.** Thrombin generation in plasma, effect of antibodies against human TF after the addition of subretinal fluid. Thrombin was generated in the presence of subretinal fluid (red), whereas no thrombin at all was generated in the absence of subretinal fluid. Antibodies against human TF almost completely neutralized the procoagulant activity of subretinal fluid (black). Only one example is shown.

**FIGURE 4.** Western blot analysis of subretinal fluid samples using an antibody that specifically detects TF revealed a major band at \( \sim 50 \) kDa. Samples from six patients who underwent scleral buckling surgery for RRD were investigated. Samples from patients 1 to 3 are supernatants; samples from patients 4 to 6 are whole-cell specimens.
We also determined whether the method of fluid collection influenced our results. Therefore, we investigated eight subretinal fluid samples from patients with primary RRD that were collected by using a small needle with a pipette. All samples investigated clearly showed thrombin generation, although to a lesser extent than did samples collected with the incision technique (Table 2). Statistical analysis showed that only time to peak was significantly different between both groups (Mann–Whitney test; \( P = 0.033 \)). We also investigated the procoagulant activity of the supernatant of these samples. In comparison with the whole cell specimens, the supernatants showed similar procoagulant activity (Fig. 5), although differences were statistically significant for lag time (Wilcoxon signed-rank test; \( P = 0.042 \)), ETP (\( P = 0.012 \)), peak (\( P = 0.012 \)), and time to peak (\( P = 0.017 \)).

Further, we were interested in whether this procoagulant phenomenon could also be demonstrated in vitreous fluids of various other ocular conditions. Therefore, we compared the procoagulant activity of vitreous fluids collected during pars plana vitrectomy for primary RRD \( (n = 12) \), macular pucker \( (n = 5) \), macular hole \( (n = 6) \), and PDR \( (n = 5) \) with that of control vitreous fluid samples from eye bank eyes \( (n = 11) \). Of interest, the addition of vitreous fluids from the nonretinal detachment groups hardly affected the amount of thrombin generated in normal and severely FXII-deficient plasma. Thrombin generation was observed in one \( (20\%) \) of five samples from patients with macular pucker, zero \( (0\%) \) of six samples from patients with macular hole, and zero \( (0\%) \) of five samples from patients with PDR (Figs. 6A–C). Thrombin was generated in the retinal detachment group and in the eye bank group, in 4 \( (33\%) \) of 12 and 2 \( (18\%) \) of 11 vitreous samples, respectively (Figs. 6D, 6E).

<table>
<thead>
<tr>
<th>Clinical Variable</th>
<th>Low TF Activity ( (n = 10) )</th>
<th>High TF Activity ( (n = 10) )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>Median (range) 62 (42–79)</td>
<td>62 (14–82)</td>
<td>0.940</td>
</tr>
<tr>
<td>Sex, %</td>
<td>Female 4 (40)</td>
<td>2 (20)</td>
<td>0.329</td>
</tr>
<tr>
<td></td>
<td>Male 6 (60)</td>
<td>8 (80)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>0</td>
<td>1 (10)</td>
<td>0.305</td>
</tr>
<tr>
<td>Pseudophakia, %</td>
<td>3 (30)</td>
<td>4 (40)</td>
<td>0.639</td>
</tr>
<tr>
<td>Duration of macular detachment, days</td>
<td>Median (range) 2 (1–5)</td>
<td>5 (4–17)</td>
<td>0.008</td>
</tr>
<tr>
<td>Size of retinal detachment, ( n ) quadrants</td>
<td>Median (range) 2 (1–5)</td>
<td>2 (2–3)</td>
<td>0.788</td>
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<tr>
<td>Retinal defects, ( n )</td>
<td>Median (range) 2 (1–2)</td>
<td>2 (0–6)</td>
<td>0.796</td>
</tr>
<tr>
<td>Preoperative visual acuity, logMAR</td>
<td>Median (range) 0.61 (0–2.52)</td>
<td>1.77 (0.60–2.52)</td>
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Again, these effects were neutralized by antibodies against human TF in all vitreous fluid samples in which procoagulant activity was shown (data not shown), indicating that it is the presence of TF that causes the generation of thrombin.

**DISCUSSION**

To our knowledge, this is the first report showing that subretinal fluid collected during scleral buckling surgery for primary RRD acts as a procoagulant. TF accounted for almost all procoagulant activity, since thrombin generation was almost completely reduced by neutralizing antibodies. We have hereby confirmed the results of the study by Hollborn et al.\(^5\) in human clinical specimens. Our results using CAT, a functional test that evaluates the biological activity of coagulation factors including TF, were further supported by Western blot analysis. Of interest, hardly any procoagulant activity was detected in vitreous fluids of patients with macular pucker, macular hole, or PDR, whereas vitreous fluids from RRD patients showed TF activity in one third of cases.

In our study, procoagulant activity was demonstrated in only a minority of vitreous fluid samples from patients with RRD, whereas all subretinal fluid samples from patients with the same ocular disorder were able to trigger the clotting cascade. This discrepancy may be explained by the site of sample collection. During vitrectomy the vitreous fluid is collected from the core of the vitreous body. Although we do not know the exact origin of TF in our samples, it may well be that there is less activity in this part of the eye than at the site very close to the retinal detachment. Procoagulant activity was detected in some control samples from eye bank eyes, perhaps because of lysis of cells in the postmortem tissues. However, analysis of data did not show a correlation between postmortem time and thrombin generation (data not shown). Finally, thrombin generation in subretinal fluid samples collected by pipette was lower than that obtained by the incision method. Possibly, the difference was caused by the small incision in the sclera itself whereby small amounts of TF may be released into the subretinal fluid.

TF is historically defined as the coagulation initiation factor, but is now recognized to have additional nonhemostatic functions on different cell populations. TF may induce a broad range of cellular responses, including inflammation and cell migration. This is illustrated by the upregulation of both interleukin (IL)-6 and IL-8 expression in macrophages and the stimulation of fibroblast migration, after complex formation of TF.

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**Table 1.** Comparison between Patients with Low TF Activity and Patients with High TF Activity Based on the Peak of the Thrombogram

<table>
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**Table 2.** Thrombin Generation in Subretinal Fluid Samples: Comparison of Two Collection Techniques

<table>
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<tr>
<th>Thrombogram Parameter</th>
<th>Incision Technique ( (n = 10) )</th>
<th>Pipette Technique ( (n = 8) )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time</td>
<td>Mean ± SD 4.6 ± 2.0</td>
<td>3.7 ± 1.4</td>
<td>NS</td>
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<tr>
<td>ETP</td>
<td>Mean ± SD 1249 ± 311</td>
<td>1485 ± 369</td>
<td>NS</td>
</tr>
<tr>
<td>Peak</td>
<td>Mean ± SD 241 ± 104</td>
<td>145 ± 131</td>
<td>NS</td>
</tr>
<tr>
<td>Time to peak</td>
<td>Mean ± SD 7.5 ± 2.6</td>
<td>11.5 ± 3.8</td>
<td>0.033</td>
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with factor VIIa. On the other hand, it was demonstrated that TF expression was upregulated by a variety of cell types after stimulation with inflammatory mediators. Among these cell types were monocytes that have been shown to be ubiquitous in the subretinal space after retinal detachment.

It remains to be elucidated, however, what the exact meaning of TF in subretinal fluid is and whether its presence has any clinical implications. There are no reports indicating an increased incidence of thrombotic events in the retinal vasculature after retinal detachment. Nevertheless, retinal blood flow in eyes with RRD has been shown to be severely impaired, resulting in prolonged retinal circulation times. Activation of the blood coagulation cascade by TF may have caused this phenomenon. Another interesting possibility is that increased TF expression may reflect tissue injury after retinal detachment. It has been shown that TF gene expression is increased after experimental retinal detachment, and TF was detected in the normal human retina. We speculate that increased TF levels after RRD may be the result of the breakdown of the blood-retinal barrier, whereby retinal pigment epithelial cells and/or other retinal cells release TF into the subretinal space. This hypothesis is further supported by our findings showing that patients with high TF levels had a significantly longer duration of macular detachment than those with low TF levels. Moreover, there was a trend toward worse preoperative visual acuity in patients in the former group. A larger prospective study is needed to draw conclusions concerning the monitoring of tissue damage after RRD by the measurement of TF activity.

Since blood coagulation is the first phase in the wound-healing response, it may initiate the early changes that are responsible for the development of fibrotic membranes that are characteristic of PVR. Clinical studies have shown that intraocular hemorrhage during or after surgery is among the most important clinical risk factors for the development of

**FIG. 5.** Thrombin generation in plasma. Comparison of the effects of whole-cell subretinal fluid and its corresponding supernatant. Whole-cell specimens and their corresponding supernatants provoked thrombin generation to a similar extent, although differences regarding the various parameters of the thrombogram were statistically significant (Wilcoxon signed-rank test; $P < 0.05$ for lag time, ETP, peak, and time to peak). *Solid lines*: whole-cell specimens; *dashed lines*: supernatants. Only two examples (red and black) are shown.

**FIG. 6.** Thrombin generation in plasma. The effects of vitreous fluid from various ocular disorders in the absence of TF. Vitreous fluid samples obtained during pars plana vitrectomy for various ocular disorders hardly affected thrombin generation in severely FXI-deficient plasma. Thrombin generation was demonstrated in (A) 1 of 5 macular puckers, (B) 0 of 6 macular holes, (C) 0 of 5 PDR, (D) 4 of 12 primary RRD, and (E) 2 of 11 eye bank eyes.
In addition, several components of the coagulation cascade have been identified in PVR membranes, including fibrin. Fibrin clots may provide a scaffold for cellular attachment and for the proliferation of RPE cells and glial cells. Although many studies have shown the involvement of components of the coagulation cascade in the pathophysiology of PVR, we could not detect any significant differences in TF activity between patients with an uncomplicated RRD and patients who had postsurgical PVR.

Although some of our clinical findings suggest that tissue injury after RRD may have caused high TF activity, the origin of TF in the subretinal fluid samples may also be plasma-derived. Currently, several sources of blood-borne TF have been proposed. Most reports have focused on circulating TF-containing microparticles, which are cell fragments derived from cells undergoing activation or apoptosis. These are thought to derive mostly from platelets and activated circulating monocytes. Monocytes themselves have also been claimed as a source of circulating TF and are known to invade the subretinal space after RRD. The presence of soluble TF in plasma has also been suggested but is not generally accepted today. However, in our study the supernatants of subretinal fluid specimens elicited considerable procoagulant activity via the action of TF.

In conclusion, we have shown for the first time that subretinal fluid collected from patients with RRD induces high procoagulant activity, whereas procoagulant activity of vitreous fluids from various ocular conditions was negligible. Our findings indicate that TF levels may reflect tissue damage after RRD, although a large prospective study is needed to evaluate its clinical significance.

References

15. Hoffman M. Some things I thought I knew about tissue factor that turn out to be wrong. Thromb Res. 2008;122(suppl 1):S73–S77.