CONCISE REPORT

Thrombin-Mediated Release of Factor VIII Antigen From Human Umbilical Vein Endothelial Cells in Culture

By James D. Levine, John M. Harlan, Laurence A. Harker, Michael L. Joseph, and Richard B. Counts

We have examined the effects of purified human α-thrombin on factor VIII antigen (FVIII-Ag) release by human umbilical vein endothelial cells in culture. α-Thrombin induced a time and dose-dependent release of FVIII-Ag into supernatant medium. α-Thrombin-mediated FVIII-Ag release was not dependent on protein synthesis and was observed in both serum-free and serum-containing media. FVIII-Ag release, however, was prevented when the serine esterase activity of thrombin was inhibited. Pretreatment of human endothelial cells with α-thrombin, but not diisopropylfluorophosphate-thrombin, prevented subsequent FVIII-Ag release by α-thrombin. Thrombin-mediated FVIII-Ag release was not associated with significant 51Cr release from prelabeled endothelial monolayers. We conclude that α-thrombin induces release of preformed FVIII-Ag from human umbilical vein endothelial cells by a receptor-independent, nonlytic mechanism requiring serine esterase activity.

ELEVATED BLOOD LEVELS of factor VIII antigen (FVIII-Ag) have been reported in patients with a variety of acute and chronic vascular disease states, including deep venous thrombosis, adult respiratory distress syndrome, burns, trauma, and diabetes mellitus.1,3 Since endothelial cells synthesize and release FVIII-Ag, flat it has been postulated that the elevated plasma levels of FVIII-Ag may reflect endothelial “injury.” However, a number of the clinical conditions associated with increased FVIII-Ag plasma levels are also associated with activation of the coagulation cascade and consequent generation of thrombin. Since thrombin binds to high affinity endothelial cell receptors,5 induces the synthesis and release of prostacyclin,6 decreases intracellular plasminogen activator activity,7 and cleaves endothelial extracellular proteins,9,10 we have examined the possibility that thrombin might also mediate FVIII-Ag release from endothelium. In these studies we have determined the effect of purified human α-thrombin on FVIII-Ag synthesis and release by human umbilical vein endothelial cells in culture.

MATERIALS AND METHODS

Human Umbilical Vein Endothelial Cells (HEC)

HEC were prepared by collagenase treatment of human umbilical cord veins as previously described.11 Cells were maintained in 20% newborn calf serum (NBCS) (GIBCO, Grand Island, N.Y.) in Waymouth’s 752/1 medium (Irvine Scientific, Santa Ana, Calif.) over a 1-hr incubation. This procedure was shown not to affect residual FVIII-Ag; these values were consistently <1 ng/well. The remaining medium was gently aspirated, and 1 ml of Waymouth’s medium (in a 4-hr incubation) or 5% NBCS/Waymouth’s medium (in a 24-hr incubation) with or without thrombin or inhibited thrombin was added and the wells were incubated at 37°C. Two-hundred microliters of medium were removed at sequential intervals for determination of FVIII-Ag.

Total cellular FVIII-Ag was determined by solubilizing the cell layer in 0.5% Triton X-100 (New England Nuclear, Boston, Mass.) over a 1-hr incubation. This procedure was shown not to affect FVIII-Ag measurement by RIA (data not shown).

Results were obtained from a minimum of 3 replicate wells per experiment and expressed as ng FVIII-Ag/106 cells ± SEM.

51Cr-Release Assay

51Cr release assay was determined as previously described.11

FVIII-Ag Radioimmunoassay (RIA)

FVIII-Ag was measured by radioimmunoassay as previously described.11 The lower limit of detection in this assay was 0.5 ng/ml.

Reagents

Diisopropylfluorophosphate-thrombin (DIP-thrombin), α-thrombin (3.2 U/ag), and N-tosyl-L-lysine-chloromethyl ketone (TLCK)-thrombin were generously provided by Dr. John Fenton III (Buffalo, NY).
N.Y.). Hirudin (grade IV) and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Mo.

Statistics

Significance was determined by two-tailed unpaired t statistics.

RESULTS

Effect of α-Thrombin on FVIII-Ag Determination by RIA

To test the possible effects of α-thrombin on FVIII-Ag immunoreactivity in the RIA, purified FVIII-Ag was incubated with α-thrombin for 4 hr at 37°C in the absence of endothelial cells. Control values of FVIII-Ag by RIA were 0.93 μg/ml ± 0.28 compared to 0.87 μg/ml ± 0.24 following treatment with α-thrombin (8.8 μg/ml) (p > 0.05).

Time and Dose-Response of α-Thrombin

Unstimulated HEC released small amounts of FVIII-Ag into supernatant medium (Fig. 1). With α-thrombin, however, release of FVIII-Ag into the supernatant medium increased significantly in a time and dose-dependent manner (Fig. 1). This effect was also observed in the presence of 5% NBCS [66 μg/ml ± 6.5 at 4 hr with α-thrombin (8.8 μg/ml) compared to 27 μg/ml ± 1.1 in control (p < 0.001)]. Table 1 demonstrates that α-thrombin released 50% ± 6% of the initial cellular content of FVIII-Ag into the supernatant medium at 4 hr compared to 18% ± 2% in untreated control studies (p < 0.001). Alpha-thrombin also appeared to stimulate synthesis of FVIII-Ag, since total FVIII-Ag (cell-associated plus medium) was significantly increased after a 24-hr incubation.

Table 1. Effects of α-Thrombin on FVIII-Ag Synthesis and Release

<table>
<thead>
<tr>
<th>Time</th>
<th>Medium</th>
<th>Cell Associated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (time 0)</td>
<td>10.1 ± 2.1</td>
<td>168.0 ± 21.3</td>
<td>178.1 ± 19.4</td>
</tr>
<tr>
<td>4-hr Incubation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32.1 ± 1.6</td>
<td>(p &lt; 0.01)</td>
<td>—</td>
</tr>
<tr>
<td>α-Thrombin</td>
<td>89.3 ± 4.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>24-hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>102.0 ± 8.7</td>
<td>(p &lt; 0.01)</td>
<td>98.7 ± 10.9</td>
</tr>
<tr>
<td>α-Thrombin</td>
<td>166.8 ± 12.7</td>
<td>(p &lt; 0.01)</td>
<td>106.7 ± 9.8</td>
</tr>
</tbody>
</table>

FVIII-Ag values were determined in first passage HEC maintained in serum-free Waymouth's medium with and without exposure to α-thrombin (8.8 μg/ml). Medium values were determined from aliquots of supernatant culture medium. Cellular FVIII-Ag values were determined from Triton-solubilized cell layers. Total FVIII-Ag represents the sum of cellular and medium determinations. Values represent the means of 3 replicates ± 1 SEM.
Fig. 3. Alpha-thrombin-treated HEC are refractory to subsequent restimulation of FVIII-Ag release by α-thrombin. FVIII-Ag release was first determined in HEC incubated for 6 hr in 5% NBCS/Waymouth’s medium alone (open circle, dotted line) or α-thrombin, 8.8 μg/ml, in 5% NBCS/Waymouth’s medium (closed square, solid line). Alpha-thrombin, 8.8 μg/ml, was then added to both untreated and pretreated cells and FVIII-Ag release again determined. Values represent means of 3 replicates ± 1 SEM. The arrows indicate addition of α-thrombin.

with α-thrombin compared to control values (Table 1).

Effect of Protein Synthesis Inhibition

Treatment of HEC with cycloheximide did not decrease α-thrombin-mediated FVIII-Ag release by HEC at 4 hr. The percent increase in FVIII-Ag release produced by α-thrombin (8.8 μg/ml) alone was 319.5% ± 66.5% compared to 485.8% ± 91.9% with α-thrombin (8.8 μg/ml) and cycloheximide (10 μg/ml); the increase is significant at p = 0.02.

Role of Serine Protease Activity

In contrast to α-thrombin, DIP-thrombin, TLCK-thrombin, and hirudin-treated thrombin failed to increase the release of FVIII-Ag over control studies. At 4 hr, α-thrombin (8.8 μg/ml) caused a 113% ± 22.6% increase over control (p < 0.001) compared to 1.3% (range 0%–6%) with DIP-thrombin (8.8 μg/ml), 4.1% (range 0%–15%) with TLCK-thrombin (8.8 μg/ml), and 20.9% ± 14.8% with hirudin-treated thrombin.

Effect of Pretreatment With α- and DIP-Thrombin

Alpha-thrombin-mediated FVIII-Ag release was not inhibited by preincubation of HEC with a 50-fold excess of DIP-thrombin (Fig. 2). Pretreatment with α-thrombin, however, prevented subsequent FVIII-Ag release by additional α-thrombin. (Fig. 3).

Effect of α-Thrombin on Endothelial ³¹Cr-Release

To determine if α-thrombin-mediated FVIII-Ag release was a consequence of HEC lysis, ³¹Cr release was measured after a 4-hr incubation with α-thrombin. The percent specific release with α-thrombin was 1.6% ± 1.1% compared to 0.7% ± 1.0% with DIP-thrombin (p > 0.05).

DISCUSSION

These studies demonstrated that human α-thrombin mediates a time and dose-dependent release of FVIII-Ag from HEC. The release of FVIII-Ag by α-thrombin was not dependent on de novo protein synthesis, since it was not prevented by cycloheximide. Since HEC contained sufficient preformed FVIII-Ag to account for that released into the medium over the first 4 hr, we conclude that the FVIII-Ag released is derived from cellular stores. It is noteworthy that α-thrombin-mediated FVIII-Ag release was not due to disruption of the HEC by lysis, as shown by the lack of significant ³¹Cr-release following the addition of α-thrombin.

Thrombin-mediated FVIII-Ag release was clearly dependent on intact proteolytic activity of thrombin, since DIP-, TLCK-, and hirudin-treated thrombin did not induce release. Further studies will be required to determine whether this effect is specific for α-thrombin or whether other serine proteases of the coagulation cascade will also induce nonlytic FVIII-Ag release from HEC.

Although DIP-thrombin binds reversibly to HEC with the same affinity as α-thrombin, DIP-thrombin did not induce FVIII-Ag release. Furthermore, excess DIP-thrombin did not inhibit subsequent FVIII-Ag release by α-thrombin. Thus, thrombin-mediated FVIII-Ag release, like thrombin-mediated PGI₃ release,¹⁴ does not appear to be receptor-mediated.

The physiologic relevance of these in vitro observations remains to be established. They do suggest, however, that elevated FVIII-Ag levels in the plasma of patients with acute or chronic vascular diseases may sometimes reflect nonlytic, specific thrombin-mediated release rather than the discharge of FVIII-Ag from disrupted endothelial cells.¹⁵

REFERENCES

Thrombin-mediated release of factor VIII antigen from human umbilical vein endothelial cells in culture

JD Levine, JM Harlan, LA Harker, ML Joseph and RB Counts