Activation of prothrombin in the presence of human umbilical-vein endothelial cells

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Addition of Factor Xa, Factor Va and prothrombin to immobilized cultured human umbilical-vein endothelial cells resulted after a time delay in thrombin formation. The prothrombin-converting (prothrombinase) activity, however, was not associated with the cell surface. Rather, perturbation by thrombin, either formed *in situ* or exogenously added, induced a procoagulant phospholipid surface in the fluid phase, which, in the presence of Factor Xa and Factor Va, enabled the assembly of prothrombinase.

INTRODUCTION

During blood coagulation, prothrombin is activated by prothrombinase, an enzymic complex composed of an enzyme, Factor Xa, a non-enzymic co-factor, Factor Va, and a procoagulant phospholipid surface [1]. At present it is generally believed that, during coagulation *in vivo*, activated platelets provide most of the procoagulant surface [2]. However, the role of vascular endothelium in normal haemostasis is becoming increasingly appreciated [3]. In this respect it has been reported that quiescent bovine endothelial cells are capable of binding Factor Xa [4,5], and human endothelial cells are capable of binding Factor Va [6]. This could permit a rapid assembly of prothrombinase at the site of a vascular injury, and, in support of these findings, it has been reported that cultured bovine aortic and human umbilical-vein endothelial (HUVE) cells support the activation of prothrombin by Factor Xa [7–10].

Recently it has been shown that the complement proteins C5b–9 induce vesiculation of the plasma membrane of HUVE cells. The membrane particles formed expressed binding sites for Factor Va and supported prothrombinase activity [11]. Although that report was not conclusive as regards the nature of the prothrombinase activity of cells in the absence of C5b–9, it did appear that, in this case, at least part of the activity was found in the supernatant of the endothelial cells.

Thus it seems that, on the one hand, prothrombinase can be assembled at the endothelial-cell surface, and, on the other hand, prothrombinase can be found in endothelial-cell supernatants. Partitioning of prothrombinase between the fluid phase and the surface could be an important feature in the regulation of prothrombinase. However, it is first of all important to know the relative importance of the cell and particle surface as assembly sites for prothrombinase under conditions where the endothelial surface is (initially) unperturbed. Here we report the further characterization of HUVE-cell-dependent prothrombin-activating activity.

EXPERIMENTAL

Proteins

Bovine Factor Va, human Factor Xa and human prothrombin were purified by earlier-reported methods [12]. Thrombin was isolated by chromatography of prothrombinase-activated pro-

thrombin on sulphopropyl-Sephadex (Pharmacia, Uppsala, Sweden), at pH 7.4 [13]. Its molar concentration was assessed by active-site titration with *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride [14]. Recombinant annexin V was prepared and purified as described previously [15]. All proteins were stored at –70 °C in 50 mM-Tris/HCl, pH 7.9, containing 175 mM-NaCl.

Endothelial-cell cultures

Human endothelial cells were isolated from umbilical-cord veins and cultured according to the method of Jaffe *et al.* [16], with a few modifications [17]. Second-passage HUVE cells were grown to confluence in fibronectin-coated 48-well plates (1.0 cm²/well). Endothelial cells were identified by their typical characteristics, such as the presence of von Willebrand factor. Monolayer status was monitored by phase-contrast microscopy, and contamination of the cultures by other cells was not noted. HUVE-cell cultures were used within 48 h after they reached confluence. Cells were never pooled for any experiments; thus in each case the results obtained reflect the activity of cells originating from one umbilical cord.

HUVE-cell-dependent activation of prothrombin

Cultures were gently washed with five 200 μ l portions of prewarmed (37 °C) 10 mM-Hepes (pH 7.45), containing 135 mM-NaCl, 4.0 mM-KCl, 4.0 mM-CaCl₂, 1.0 mM-MgCl₂, 11 mM-D-(+)-glucose and human serum albumin (fatty-acid-free) (2.5 mg/ml), hereafter referred to as 'Hepes buffer'. After the last washing step, the cells were equilibrated for 10 min in Hepes buffer at 37 °C. Then 200 μ l of a mixture of Factor Xa and Factor Va, diluted in Hepes buffer, was applied to the cells. Shortly thereafter, an equal volume of prothrombin was added. The final concentrations of Factor Xa and Factor Va were variable, and the final prothrombin concentration was 1.0 μ M. Every few minutes the reaction mixtures were gently whirled. At timed intervals, 10 μ l samples were withdrawn and assayed for thrombin by adding them to a cuvette containing 440 μ l of 50 mM-Tris/HCl, pH 7.9, 175 mM-NaCl, 20 mM-EDTA, human serum albumin (0.5 mg/ml) and 50 μ l of the chromogenic substrate D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride (S2238). The final concentration of S2238 was 0.22 mM. The conversion of S2238 was monitored on a dual-wavelength spectrophotometer at 405 nm (reference wavelength 500 nm). Thrombin concentrations were calculated from a standard curve

Abbreviations used: HUVE, human umbilical-vein endothelial; S2238, D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride.

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of known amounts of thrombin constructed under identical conditions. All procedures were performed at 37 °C.

Assay for prothrombinase activity

Samples were tested for prothrombinase activity by adding 50 μ l aliquots to a mixture of Factor Xa, Factor Va and CaCl_2 in 50 mM-Tris/HCl, pH 7.9, containing 175 mM-NaCl and human serum albumin (0.5 mg/ml). Thrombin generation was initiated at 37 °C by the addition of prothrombin. The final concentrations were 50 pM-Factor Xa, 0.50 nM-Factor Va, 5.0 mM- CaCl_2 and 1.0 μ M-prothrombin in a final volume of 200 μ l. Thrombin was determined as described above. The rates of thrombin generation obtained are used as indicators for prothrombinase activity. Serial dilutions of samples showed a linear relationship between sample volume and thrombin-generation rate.

RESULTS AND DISCUSSION

Time course of HUVE-cell-dependent thrombin generation: effect of Factor Va concentration

Endothelial cells were incubated with Factor Xa (50 pM), Factor Va (50 pM) and prothrombin (1.0 μ M) as described in the Experimental section. Thrombin generation was monitored in two wells, and the mean values are shown in Fig. 1. During the first 2 min, no thrombin is measurable, then thrombin gradually starts to appear, until after about 10 min a rate of thrombin generation of 2.0 nM/min is obtained. These characteristics of prothrombin activation were identical for all endothelial-cell cultures studied. The final rate of thrombin generation, however, varied over a 4-fold range. In the absence of endothelial cells (Fig. 1), no measurable thrombin formation occurred.

It has recently been reported that, in the absence of exogenous Factor Va, significant thrombin formation occurred in the presence of HUVE cells [10]. This was attributed to endothelial-cell-associated Factor V, which became activated as a result of a thrombin-dependent feedback mechanism. The presence of

HUVE-cell-bound Factor V could provide a rationale for the observed thrombin-generation curves. First of all it must be noted that, at the Factor Xa concentrations employed, the Factor Va-independent activation rate of prothrombin is, in both the absence or presence of a procoagulant phospholipid surface, negligible. In the presence of exogenous Factor Va, the rate of prothrombin activation in the solution could become sufficiently high to result in enough thrombin necessary for activation of the endothelial Factor V (if present), which in turn enables the assembly of prothrombinase and subsequent rapid activation of prothrombin at the endothelial surface. In this situation the observed time delay in the rapid prothrombin activation is caused by the time necessary to generate sufficient endothelial Factor Va. Consequently, this model predicts that increasing amounts of exogenous Factor Va will shorten the time delay, by increasing the rate of thrombin generation in the fluid phase, whereas the final rate of prothrombin activation will be relatively independent of the exogenous Factor Va concentration.

Thus we studied thrombin-generation curves, obtained with cultures of HUVE cells originating from five different umbilical cords, in the presence of increasing Factor Va concentrations, which varied between 50 and 500 pM. Under all conditions thrombin became measurable only after 2 min, and it took 10–15 min before the full prothrombinase activity became apparent. Thus increasing amounts of exogenous Factor Va do not shorten the time delay in prothrombin activation. The final rates of thrombin generation are shown in Fig. 2 as function of the Factor Va concentration. These data suggest that the rate of thrombin generation is, over this range of Factor Va concentrations, linearly dependent on the Factor Va concentration. Linear-regression analysis of the data revealed a statistically non-significant intercept of 0.45 ± 0.80 (S.E.M.) nM-thrombin/min. Thus these findings imply that, under our conditions, endothelial Factor V does not contribute to the generation of HUVE-cell-dependent prothrombinase.

Localization of HUVE-cell-dependent prothrombinase activity

What then causes the time delay observed in the generation of thrombin? A 15 min incubation of the endothelial cells with Factor Xa and Factor Va prior to the addition of prothrombin could not abolish the time delay. Thus the time delay is not due to a time-consuming process of prothrombinase assembly at the HUVE-cell monolayer, and consequently, HUVE cells in their unperturbed state are not ready to form prothrombinase activity. Apparently such an activity has to be induced, and, as Factor Xa and Factor Va are present, it is quite feasible that the generation of a procoagulant phospholipid surface is the limiting step in the generation of prothrombinase activity. Therefore we further investigated the generation of procoagulant activity of HUVE cells during thrombin generation.

Endothelial-cell-dependent thrombin generation was monitored for 30 min, then the cell culture was gently washed with HEPES buffer, and prothrombin was added to the culture again. It is clearly seen in Fig. 3 that, in this case, thrombin generation was negligible after the washing procedure. Significant thrombin generation required the re-addition of Factor Xa and Factor Va, which is again characterized by a time delay. Identical curves were obtained when Factor Va (0.25 nM) was present in the washing buffer and the prothrombin solution. Thus these results cannot be explained by assuming a dissociation of prothrombinase after washing, but rather it seems that the prothrombinase activity is removable. In addition, as the prothrombinase activity has to be regenerated upon re-addition of Factor Xa and Factor Va, it seems that the HUVE-cell surface itself has not become procoagulant after thrombin generation.

Fig. 1. HUVE-cell-dependent activation of prothrombin

Confluent cultures of HUVE cells were incubated with 50 pM-Factor Xa, 50 pM-Factor Va and 1.0 μ M-prothrombin as described in the Experimental section. Thrombin generation was monitored in two different wells, and the thrombin generation data obtained were averaged (○). Alternatively, Factor Xa, Factor Va and prothrombin were incubated in the absence of endothelial cells (△).

Fig. 2. Rate of thrombin generation as function of Factor Va concentration

Confluent cultures of HUVE cells were incubated with 50 pM-Factor Xa, Factor Va at the indicated concentrations and 1.0 μ M-prothrombin, as described in the Experimental section. Steady-state rates of thrombin generation were obtained after 10–15 min (cf. Fig. 1) and are plotted as function of Factor Va concentration. The data point at 50 pM-Factor Va (○) is the mean of 20 experiments performed with HUVE cells originating from five umbilical cords; the error bar indicates the 95% confidence interval. The other experiments (●) were performed with HUVE cells originating from one of these cords.

Fig. 3. Procoagulant activity of HUVE cells after thrombin generation

Confluent HUVE cells were incubated with 50 pM-Factor Xa, 0.25 nM-Factor Va and 1.0 μ M-prothrombin as described in the Experimental section. Thrombin generation was monitored during 30 min. The culture was then washed with prewarmed (37 °C) Hepes buffer, and 1.0 μ M-prothrombin was added at 35 min. Also, at 60 min, Factor Xa (50 pM) and Factor Va (0.25 nM) were added again.

So we further investigated the HUVE-cell supernatant for prothrombinase activity.

Factor Xa, Factor Va and prothrombin were added to a HUVE-cell culture. After 15 min the supernatant was removed and transferred to a clean test tube. Samples were removed from the test tube during an additional 25 min period. It is observed (Fig. 4) that the rate of thrombin generation in the supernatant is exactly the same as that in the presence of the HUVE cells before removing the supernatant. Thus, under our conditions, HUVE-cell-dependent prothrombinase activity is due to the generation of prothrombinase assembly sites in the fluid phase. Circumstantial evidence that these sites are generated by a thrombin-dependent feedback mechanism was obtained when it

Fig. 4. Localization of HUVE-cell-dependent prothrombinase activity

Confluent HUVE cells were incubated with 50 pM-Factor Xa, 0.25 nM-Factor Va and 1.0 μ M-prothrombin as described in the Experimental section. Thrombin generation was monitored over 15 min. The supernatant was then removed, and thrombin activity was monitored in the supernatant.

Fig. 5. Neutralization of prothrombinase activity by annexin V

Cultures of HUVE cells were incubated with 20 pM-Factor Xa, 0.20 nM-Factor Va and 1.0 μ M-prothrombin as described in the Experimental section. After 6 min annexin V was added to a final concentration of 10 μ g/ml (Δ). Experiments were performed in duplicate, and the thrombin-generation data were averaged; ○, control without the addition of annexin V.

was observed that incubation of HUVE cells with purified thrombin (20 min, 30 nM) resulted in a fluid-phase prothrombinase activity comparable with that obtained with thrombin formed *in situ* (results not shown).

Inhibition of fluid-phase prothrombinase activity by annexin V

In view of our current knowledge on the prothrombinase-catalysed activation of prothrombin, it is difficult to reconcile that a procoagulant phospholipid surface would not be involved in generation of the prothrombinase activity. To test this, we studied the effect of annexin V, a phospholipid-binding protein, on HUVE-cell-dependent prothrombin activation. Annexin V predominantly binds to negatively charged (procoagulant) phospholipids, and in this way disables the proper assembly of prothrombinase in model systems utilizing phospholipid vesicles [18,19].

Thrombin generation was studied in two wells containing HUVE cells (20 pM-Factor Xa, 0.20 nM-Factor Va, 1.0 μ M-prothrombin) as described in the Experimental section (Fig. 5). The steady-state rate of thrombin generation was 4.3 nm/min. In two other wells, thrombin generation was initiated under the same conditions, and, after 6 min (a very small portion of)

recombinant annexin V was added to a final concentration of 10 $\mu\text{g/ml}$. The thrombin generation rate almost immediately decreased to about 0.18 nm/min (96% inhibition). These experiments thus give good evidence that the endothelial-cell-dependent prothrombinase activity is related to the generation of a procoagulant phospholipid surface in the bulk solution. As endothelial-cell vesicles induced by the complement proteins C5b-9 express binding sites for Factor Va [11], and thus sites for the association of prothrombinase, it is tempting to speculate that, during HUVE-cell-dependent thrombin generation, vesiculation occurs, which is the fluid-phase source for the prothrombinase activity.

Contribution of microparticles to prothrombinase activity

As with platelet-derived membrane vesicles, it might therefore be expected that the prothrombinase activity can be isolated through centrifugation at high, but not at low, relative centrifugal forces [20]. Therefore we performed the following experiments. Four wells containing HUVE cells were incubated with 500 μl of purified thrombin (30 nm) in Hepes buffer at 37 °C. After 20 min the supernatants were removed and pooled. Prothrombinase activity was assayed as described in the Experimental section. With a 50 μl sample the rate of thrombin generation was 6.4 nm/min. The solution was then centrifuged for 10 min at 950 g (25 °C). The resulting supernatant was centrifuged at 40000 g for 45 min (4 °C), and the pellet was resuspended in Hepes buffer (100 μl). After the first centrifugation the supernatant was responsible for a rate of thrombin generation of 4.4 nm/min. After the second centrifugation, almost no activity was recovered in the supernatant (thrombin generation rate 0.6 nm/min), whereas the resuspended pellet was highly active (thrombin generation rate 21 nm/min). Thus it is clear that the majority of the prothrombinase activity can be sedimented at high, but not at low, relative centrifugal forces, which supports our notion that we are dealing with HUVE-cell-derived membrane particles.

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