Dissimilar interaction of factor VIII with endothelial cells and lipid vesicles during factor X activation

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A localized and regulated cascade of proteolytic events is a prerequisite for normal haemostasis. The activation of factor X by activated factor IX (factor IXa) in the presence of activated factor VIII (factor VIIIa) is essential for the formation of a fibrin clot at sites of vascular injury. We observed sustained activation of factor X on the surface of vascular endothelial cells, whereas, in agreement with others, on synthetic negatively charged phospholipid vesicles and activated blood platelets factor X activation is transient and starts to decline a few minutes after the onset of the reaction. We examined the mechanism responsible for these differences in factor X activation. Procoagulant membrane and solution were analysed separately for the occurrence of factor VIII and its activation fragments. On negatively charged phospholipid vesicles, on dissociation of

factor VIIIa, the 67 kDa light-chain fragment remains associated with the lipid membrane. As a result, factor VIII-binding sites remain occupied, and dampening of factor X activation occurs. In contrast, on monolayers of endothelial cells, no residual factor VIIIa fragments associated with the cell membrane were observed. During endothelial-cell-mediated activation of factor X, accumulation of factor VIIIa fragments was observed in the solution phase only. This finding suggests that, on endothelial cells, factor VIII-binding sites remain accessible for further factor VIII binding, guaranteeing sustained activation of factor X. These data demonstrate that the nature of the procoagulant membrane contributes to the regulation of the cofactor activity of factor VIII and thereby affects the progress of factor X activation.

INTRODUCTION

In blood coagulation, factor VIII functions as a cofactor in the activation of factor X by activated factor IX (factor IXa) [1]. Factor VIII is a heterodimeric protein consisting of a Me^{2+} -linked heavy chain and light chain [2–5]. The light chain (80 kDa) displays the domain structure sequence A3-C1-C2. The heavy chain (90–220 kDa) consists of the A1-A2-B domain structure. The heavy chain is heterogeneous as a result of limited proteolysis of its B domain. Activation of factor VIII is required for appropriate cofactor function in the factor X-activating complex [6–8]. Activation of factor VIII is achieved by proteolysis by thrombin or factor Xa at specific Arg residues in both the heavy and light chain [9]. The heavy chain is cleaved by both thrombin and factor Xa at residue 372 (A1–A2 junction) and residue 740 (A2–B junction). The light chain is cleaved near its N-terminus by thrombin and factor Xa at residue 1689 and 1721 respectively [10], resulting in fragments of 73 kDa and 67 kDa. Activated factor VIII (factor VIIIa) is a heterotrimer composed of the subunits A1, A2 and A3-C1-C2 [11–13]. The A1 subunit is associated with the A3-C1-C2 subunit via a Me^{2+} ion-dependent interaction [2,14]. The A2 subunit is loosely associated with the A1 subunit of the A1/A3-C1-C2 dimer electrostatically, independently of Me^{2+} ions [12,14]. Factor VIIIa is a cofactor with transient activity [11,15]. It is well documented that dissociation of the A2 subunit from the A1/A3-C1-C2 dimer is responsible for the spontaneous decay in factor VIIIa activity [14,16,17]. In addition, inactivation of factor VIIIa has also been associated with limited proteolysis within the A1 domain by activated protein C, factor Xa and factor IXa [9,18–21].

It is well established that biological membranes participate in

the factor VIIIa–IXa-dependent activation of factor X. It has been shown that the catalytic efficiency of the activation of factor X by factor IXa is dramatically increased when factor IXa is assembled with factor VIIIa on negatively charged phospholipid surfaces such as synthetic phospholipid vesicles and activated platelets [7,22–25]. Also cells of the vascular lining, especially endothelial cells, may actively participate in the factor VIIIa–IXa-dependent pathway of coagulation (for a review see reference [26]). As to the mechanism of endothelial-cell-mediated activation of factor X, it seems likely that, as for activated platelets, negatively charged phospholipids play a role in the assembly of functional factor VIIIa–IXa complexes. This view is supported by the observation that annexin V and prothrombin fragment 1, proteins that bind to negatively charged phospholipids, are able to inhibit factor VIIIa–IXa-mediated activation of factor X on endothelial cells [27,28]. In one respect endothelial-cell-mediated activation of factor X clearly differs from that supported by platelets or synthetic membranes. Recent studies indicate that the rate of endothelial-cell-mediated activation of factor X is essentially constant with time. In contrast, factor X activation supported by activated platelets or synthetic negatively charged phospholipid membranes declines at a significant rate soon after the onset of the reaction [28,29]. The nature of this difference is not clear. The present study was designed to investigate the role of the procoagulant membrane (either endothelial cells or synthetic negatively charged phospholipid vesicles) in the regulation of the cofactor function of factor VIII. Procoagulant membrane and solution were analysed separately for factor VIII and factor VIIIa fragments. These data were then correlated with data on factor X activation. From these experiments it became apparent that, during factor X activation, the interaction of factor VIIIa

Abbreviations used: PS, L- α -phosphatidylserine; PC, L- α -phosphatidylcholine; factor VIIIa, activated factor VIII; factor IXa, activated factor IX; factor Xa, activated factor X.

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with the endothelial cell membrane is different from that with negatively charged lipid vesicles.

MATERIALS AND METHODS

Antibodies and purified proteins

The anti-(factor VIII light chain) monoclonal antibody CLB-CAG 69, directed against the sequence Lys¹⁶⁷³-Arg¹⁶⁸⁹, and the anti-(factor VIII heavy chain) monoclonal antibody CLB-CAG 9, directed against the A2 domain, have been described previously [30]. A polyclonal antibody against the 73 kDa factor VIII light-chain fragment was obtained as described [10]. Peroxidase-labelled horse anti-rabbit polyclonal antibodies were from the Division of Products of our institute.

Previously described methods were used to prepare the human coagulation factors X [31], Xa [32], IXa [32], VIII [28] and α -thrombin [7]. Purified factor VIII (10 μ g) was labelled with 36.6 MBq Na¹²⁵I (Amersham International, Amersham, Bucks., U.K.) in factor VIII storage buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM CaCl₂, 60% glycerol, pH 7.4) for 2.5 min using Iodo-Gen iodination reagent (Pierce, Rockford, IL, U.S.A.). Subsequently human serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to a final concentration of 0.25% (w/v) followed by dialysis against factor VIII storage buffer. Before use, iodinated factor VIII (1010 $\pm$ 30 MBq/mg; mean $\pm$ range of two preparations) was mixed with unlabelled factor VIII in a 1:9 ratio.

Cell-mediated activation of factor X

Human umbilical vein endothelial cells were isolated and cultured as previously described [28]. Layers of cells grown on fibronectin-coated culture well plates (2 cm²; Costar, Cambridge, MA, U.S.A.) were washed three times with prewarmed Hepes-buffered balanced salt (HBBS; 0.82 mM CaCl₂, 5.36 mM KCl, 0.61 mM MgCl₂, 138.3 mM NaCl, 25 mM Hepes, 1 mg/ml α ,D-glucose, pH 7.4). To the cell layers were added factor VIII and factor IXa diluted in HBBS supplemented with 5 mg/ml ovalbumin (NBS Biologicals) and CaCl₂ (5 mM). After 6 min at 37 °C, factor X activation was initiated by the addition of factor X and the activators of factor VIII, either factor Xa or thrombin. Concentrations of coagulation factors used are indicated in the Results section. At time intervals, samples of 10 μ l were withdrawn from the reaction mixture, diluted 25 times in buffer (65 mM Tris/HCl, 250 mM NaCl, 6.5 mM EDTA, 130 μ g/ml ovalbumin, pH 8.0) and analysed for factor Xa by using the chromogenic substrate S2337 (Chromogenix AB, Mölndal, Sweden). Substrate hydrolysis, monitored by recording A_{405} for 25 min, was correlated with factor Xa concentration using active-site-titrated factor Xa as standard [33]. In some experiments factor X activation was studied in the presence of suspensions of endothelial cells or activated blood platelets as described previously [28].

Factor Xa formation on phospholipid vesicles

Hydrogenated egg yolk L- α -phosphatidylcholine type 1-EH (PC) and bovine brain L- α -phosphatidylserine (PS) were purchased from Sigma Chemical Co. Lipid vesicles composed of PS and PC in a molar ratio of 50:50 or 20:80 were prepared as described [31] and diluted in HBBS supplemented with 5 mg/ml ovalbumin and 10 mM CaCl₂. After preincubation for 10 min at 37 °C, factor X activation was determined at a final Ca²⁺ concentration of 5 mM as described for cells in culture.

Factor VIII proteolysis

Factor VIII proteolysis was measured in identical incubations parallel to those used for factor X activation. Proteolysis was terminated by the addition of one-third volume of sample buffer (0.25 M Tris/HCl, 8% SDS, 40% glycerol, 0.004% Bromophenol Blue, 2 M ureum, pH 6.8) used for SDS/PAGE. Samples were reduced with 1,4-dithiothreitol (Merck, Darmstadt, Germany) and subjected to SDS/PAGE on a 7.5% (w/v) gel. In experiments using radiolabelled factor VIII, protein was visualized by autoradiography. Otherwise, proteins were immunoblotted after electrophoresis using the anti-(factor VIII light chain) polyclonal antibody or the monoclonal antibodies CLB-CAG 9 (directed against the A2 domain) and CLB-CAG 69 (directed against the sequence Lys¹⁶⁷³-Arg¹⁶⁸⁹ on the light chain) labelled with ¹²⁵I. Bands were visualized using a peroxidase-labelled horse anti-rabbit polyclonal antibody or by autoradiography respectively. Factor VIII fragments were quantified by excising the radioactive spots from the immunoblots and counting for radioactivity. Free and cell-bound factor VIII were separated by pipetting off the supernatant from the cells, leaving less than 3% of the supernatant in the culture well. Washing was omitted in order not to disturb the established binding equilibrium between factor VIII fragments and the cell surface. Separation of free and lipid-vesicle-bound factor VIII was achieved by centrifugation in siliconized microsedimentation tubes (Sarstedt, Numbrecht, Germany). After centrifugation in a horizontal rotor at 8000 *g* for 5 min, the supernatant was removed, leaving less than 1% of the total sample volume at the bottom of the tube. Supernatants were collected in one-third volume of sample buffer. Cells and lipid sediment were suspended in one-third volume of sample buffer and 1 vol. of HBBS/0.5% ovalbumin. The time required for complete lipid sedimentation at 8000 *g*, assessed as the time to spin down lipid-bound ¹²⁵I-labelled prothrombin, was 2.5 min.

Data analysis

Initial rates of factor X activation were calculated from the steepest part of the factor X activation curves. For endothelial cells, this was performed by linear regression. For lipid vesicles, the rapid decrease in the rate of factor X activation allows calculation of initial rates only over the first 6 min. The decline in factor X activation was quantified as described by Jesty [34], employing the following exponential function:

$$[Xa]_t = [Xa]_{\max}(1 - e^{-tk_x}) \quad (1)$$

where $[Xa]_t$ is the concentration of factor Xa at time *t* and $[Xa]_{\max}$ is the final concentration of factor X activated. The rate of the decline of factor X activation is given by k_x , the apparent first-order rate constant of factor X activation. Cleavage rates of factor VIII heavy and light chain by factor Xa were determined by employing the following equation, which describes the single exponential decay of intact factor VIII:

$$[VIII]_t = [VIII]_0 e^{-tk_{h,1}} \quad (2)$$

where $[VIII]_t$ and $[VIII]_0$ are the amounts of radioactivity measured in excised radioactive spots on immunoblots of samples taken at time *t* and *t* = 0 respectively, and $k_{h,1}$ is the apparent first-order rate constant of either factor VIII light-chain or factor VIII heavy-chain cleavage.

RESULTS

Limitations in factor VIIIa-IXa-dependent activation of factor X

Studies on the mechanism of factor VIIIa-IXa-dependent ac-

Figure 1 Limitations of intrinsic factor X activation on lipid vesicles

(A) Factor VIII (0.3 nM), factor IXa (0.6 nM), factor X (200 nM) and factor Xa (1 nM) were added to either 5 μ M (closed symbols) or 50 μ M (open symbols) lipid vesicles (PS/PC, 50:50). Samples were withdrawn at timed intervals for factor Xa measurement as described in the Materials and methods section. After 30 min, incubation mixtures were supplemented ($\downarrow$) with 0.1 vol. of buffer containing sufficient amounts of factor VIII ($\bullet$, $\circ$), factor IXa ($\blacktriangle$, $\triangle$) or factor X ($\blacksquare$, $\square$) to double their concentration. (B) Initial conditions were as for (A). After 30 min, incubations were supplemented ($\downarrow$) with 0.1 vol. of buffer ($\blacktriangledown$, $\triangledown$) or lipids ($\blacklozenge$, $\lozenge$) to give a 50 μ M increase in lipid concentration. The amount of factor Xa added initially was subtracted from the data obtained.

tivation of factor X are usually performed in systems using synthetic negatively charged phospholipid vesicles. Experiments were performed to explore some of the restrictions of such a system. Factor Xa, in 3.3-fold molar excess, was included in the reaction mixture to activate factor VIII. On lipid vesicles composed of PS/PC in equimolar ratio (Figure 1) as well as at a PS/PC ratio of 20:80 (not shown), a decline in the rate of factor X activation was observed a few minutes after the reaction was started. After 30 min, factor X activation had reached a plateau. Subsequently, reaction mixtures were supplemented with factor VIII, factor IXa, factor X or lipids separately. Figure 1(A) shows that at 50 μ M PS/PC, factor X activation was restored if the reaction mixture was supplemented with factor VIII. Supplementation with factor IXa or factor X was without effect (Figure 1A), as was the addition of extra lipids (Figure 1B). These data indicate that factor VIII is the limiting factor in the activation of factor X by factor IXa on synthetic phospholipid vesicles. In contrast, at 5 μ M PS/PC, no further factor X activation was observed after supplementation of the reaction mixtures with factor IXa, factor X or factor VIII (Figure 1A). It appears that under these conditions the phospholipid vesicles are the limiting compound, as supplementation of incubations with extra lipids caused further activation of factor X (Figure 1B). Thus, both a depletion of factor VIII and a depletion of binding sites for functional factor VIIIa/IXa/X complex assembly on PS/PC vesicles appear to contribute to the decline in the rate of factor X activation.

When factor X activation on the membrane of cultured endothelial cells was examined, two striking differences from the PS/PC-mediated reactions were observed. First, a steady increase in factor Xa over time was observed, which was in contrast with reactions mediated by synthetic phospholipid vesicles (Figure 2). Endothelial-cell-mediated activation of factor X by factor IXa in the presence of factor VIII proceeded for at least 2 h showing only a slight decline. A similar difference in the time course of factor X activation was observed between endothelial cells and activated blood platelets (Figure 2, inset). The second difference between factor X activation on endothelial cells and lipid vesicles is shown in Figure 3, in which the initial rate of factor X activation is plotted against the amount of procoagulant con-

Figure 2 Comparison between factor VIIIa-IXa-mediated activation of factor X on endothelial cells and lipid vesicles

Endothelial cell monolayers ($\circ$) and lipid vesicles (50 μ M PS/PC, 50:50) ($\triangle$) were incubated with factor VIII (0.3 nM) and factor IXa (0.3 nM). After 6 min, factor X (200 nM) and factor Xa (1 nM) were added. At timed intervals samples were taken for factor Xa measurement as described in the Materials and methods section. The inset shows factor X activation curves obtained under similar conditions using suspensions of 1×10^6 endothelial cells/ml ($\circ$) and 3×10^6 activated blood platelets/ml ($\square$). The amount of factor Xa added at $t = 0$ was subtracted from the data obtained. Curves were obtained by fitting data to eqn. (1) (see the Materials and methods section). Viability of the endothelial cells under the experimental conditions used, as measured by the amount of lactate dehydrogenase [35] released during a 1 h incubation in HBBS/0.5% ovalbumin, was $97 \pm 2\%$ and $93 \pm 6\%$ for cells in monolayer and suspension respectively (mean $\pm$ S.D. for three separate experiments).

stitute added (either the number of endothelial cells or the molar concentration of PS). As shown in Figure 3(A), initial rates of factor X activation were linear with the number of endothelial cells, with rates exceeding those on lipid vesicles. On PS/PC vesicles with a molar ratio of 50:50 as well as 20:80, saturation was observed at 20 μ M PS. Thus under our experimental conditions in which the number of endothelial cells was rate-limiting, factor X activation was linear with time. In contrast, when a rate-limiting concentration of lipids (5 μ M PS/PC, 50:50) was used, a rapid decline in factor X activation was observed.

Figure 3 Relationship between membrane concentration and factor X activation rates

Suspensions of endothelial cells (A; $\bullet$) and lipid vesicles [B; PS/PC either 50:50 ($\blacksquare$) or 20:80 ($\blacktriangle$)] were incubated with factor VIII (0.3 nM) and factor IXa (0.3 nM). After 6 min, factor X (200 nM) supplemented with factor Xa (1 nM) was added. At timed intervals samples were taken for factor Xa measurement as described in the Materials and methods section. Initial rates of factor X activation (v_i) were calculated and plotted against the number of endothelial cells and amount of phospholipids (expressed as the molar concentration of PS) respectively. Data given are means $\pm$ range for two separate experiments.

Figure 4 Membrane-dependent proteolysis of factor VIII by factor Xa during factor X activation

Monolayers of endothelial cells (○) and phospholipid vesicles composed of equimolar concentrations of PS and PC, 5 μ M (▽) and 50 μ M (△), were incubated with factor VIII (1.2 nM) and factor IXa (1.2 nM). After 6 min, the reaction was started by the addition of factor X (400 nM) and factor Xa (2 nM). □, Control incubation without membrane. Samples were taken at timed intervals and factor Xa was measured (A) as described in the Materials and methods section. Curves were obtained by fitting data to eqn. (1). Data were corrected for the amount of factor Xa added at $t = 0$. Parallel incubations were run to monitor factor VIII light chain (LC) (B) and heavy chain (HC) (C) cleavage. At the time points indicated, sample buffer for SDS/PAGE was added to the incubations. Factor VIII was analysed on immunoblots employing 125 I-labelled murine monoclonal antibodies CLB-Cag 69 and CLB-Cag 9 as outlined in the Materials and methods section. Radioactive spots on the immunoblot corresponding to the heavy chain (90–220 kDa) and uncleaved light chain (80 kDa) subunits were excised and counted for radioactivity. Data are expressed as a percentage of uncleaved factor VIII light chain and heavy chain added. Curves were obtained by fitting data to eqn. (2). Results shown in this experiment are from one of the experiments listed in Table 1.

These data indicate that the constitution of the procoagulant membrane defines the decline in factor X activation.

Proteolysis of factor VIII

To examine whether the observed difference in the time course of factor X activation on endothelial cells and phospholipid vesicles is reflected by differences in factor VIII proteolysis, we examined the extent of limited proteolysis of factor VIII by factor Xa during factor X activation on both endothelial cells and PS/PC vesicles (Figure 4). At an initial factor Xa/factor VIII molar ratio of about 2, cleavage of the heavy and light chain of factor VIII was strictly membrane-dependent (Figures 4B and 4C). In

Table 1 Kinetic parameters of factor X activation and factor VIII proteolysis

Monolayers of endothelial cells and lipid vesicles (5 μ M and 50 μ M, PS/PC, 50:50) were incubated with factor VIII (1.2 nM), factor IXa (1.2 nM), factor X (400 nM) and factor Xa (2 nM). None indicates incubations performed in the absence of lipids or endothelial cells. Samples were withdrawn at timed intervals and factor X activation was measured. In parallel incubations, factor VIII light chain and heavy chain cleavage was monitored (see the legend of Figure 4 for details). The apparent first-order rate constants k_x , k_h and k_l and initial rate of factor X activation v_i were calculated as outlined in the Materials and methods section. Data are mean values $\pm$ S.D. for three separate experiments. ND, not detectable.

	Factor X activation		Factor VIII proteolysis	
	v_i (nM/min)	k_x (min ⁻¹)	Light chain (k_l , min ⁻¹)	Heavy chain (k_h , min ⁻¹)
Endothelial cells	1.13 ± 0.25	0.016 ± 0.004	0.022 ± 0.001	0.040 ± 0.001
50 μ M PS/PC	4.60 ± 1.95	0.109 ± 0.029	0.216 ± 0.123	0.220 ± 0.140
5 μ M PS/PC	0.77 ± 0.37	0.082 ± 0.007	0.019 ± 0.004	0.034 ± 0.028
None	ND		ND	0.003 ± 0.001

the absence of factor Xa, no proteolysis of factor VIII was detected. At a 20-fold molar excess of factor Xa over factor VIII, factor VIII was also cleaved by factor Xa in the absence of membranes (not shown). In the presence of 50 μ M PS/PC vesicles, a rapid decline in uncleaved factor VIII light chain (Figure 4B) and heavy chain (Figure 4C) with concomitant decay in factor X activation (Figure 4A) was observed. On endothelial cells, the decline in uncleaved factor VIII light chain and heavy chain was similar to that observed in incubations using 5 μ M PS/PC (Figures 4B and 4C). Most intriguing is the observation that 30 min after the onset of the reaction, factor X activation had stopped in the incubation using 5 μ M PS/PC but not on endothelial cells (Figure 4A), whereas in both cases after 30 min about 50% uncleaved factor VIII light chain and heavy chain was left (Figures 4B and 4C). In order to quantify the decline in factor X activation as well as the proteolysis of factor VIII, data were fitted to eqns. (1) and (2) respectively (see the Materials and methods section). Data are listed in Table 1. With respect to k_l , k_h and v_i , the endothelial-cell-mediated processes matched those in incubations using 5 μ M PS/PC. The major difference, however, was the rate of decline of factor X activation (k_x). In the presence of 5 and 50 μ M PS/PC, k_x values were similar. On endothelial cells, k_x was 5 times lower than on lipid vesicles.

In Figure 5 a representative experiment is shown in which procoagulant surface and bulk solution were analysed separately for factor VIII cleavage products 30 min after factor X activation had been initiated. At this stage factor X activation had reached its plateau on lipid vesicles but not on endothelial cells. The experiment was performed with 125 I-labelled factor VIII. In the presence of phospholipid vesicles a decrease in factor VIII heavy chain (90–220 kDa) and light chain (80 kDa) and a concomitant increase in factor Xa cleaved light chain (67 kDa) and A2 fragment was observed. The extent of factor VIII cleavage was proportional to the lipid concentration used. The 67 kDa subunit of factor VIII was equally distributed between lipid membrane and solution, whereas the A2 subunit of factor VIII was predominantly present in the supernatant. On endothelial cells, however, both the 67 kDa and A2 subunits of factor VIII were present in the supernatant rather than associated with the cells. Similar results were obtained using unlabelled factor VIII and monitoring cleavage on immunoblot using antibody CLB-Cag 9 (directed against A2) and the polyclonal antibody against the 73 kDa light chain fragment (not shown). These data indicate that PS/PC vesicles, but not endothelial cells, appear to retain

Figure 5 Detection of membrane-associated and free factor VIII subunits

Monolayers of endothelial cells and suspensions of lipid vesicles composed of PS/PC in an equimolar ratio at a final concentration of 5 μ M and 50 μ M were incubated with 125 I-factor VIII (1.2 nM) and factor IXa (1.2 nM). After 6 min, the reaction was started by the addition of factor X (400 nM) and factor Xa (2 nM). After 30 min, sample buffer for SDS/PAGE was added. In parallel incubations cells and lipids were separated from the bulk solution before the addition of sample buffer, as outlined in the Materials and Methods section. Samples were reduced and subjected to SDS/PAGE on a 7.5% (w/v) polyacrylamide gel. Shown is an autoradiograph. TOTAL, total incubation mixture including cells or lipids; SEDIMENT, pellet after centrifugation of total incubation mixture containing lipid vesicles; CELLS, cells after removal of supernatant; FREE, supernatant above cells and lipid sediment after centrifugation; HUVEC, human umbilical vein endothelial cells. As a control, 1.2 nM factor VIII was incubated for 12 min with 2 nM thrombin alone (lane 2). Probably because of proteolysis within the A1 domain by factor Xa [9,21], this factor VIII fragment was not visible, except in samples from incubations containing thrombin. Factor X activation was monitored in a separate set of incubations run in parallel with those used to monitor factor VIII proteolysis, employing identical experimental conditions. Kinetic parameters for factor X activation were similar to those listed in Table 1.

the 67 kDa factor VIII light chain fragment. This phenomenon probably accounts for the enhanced decline of factor X activation on lipid vesicles compared with that on endothelial cells.

DISCUSSION

Vascular endothelial cells provide appropriate binding sites for functional factor VIIIa–IXa complex assembly [27–29,36,37]. In contrast with those on blood platelets, these sites are constitutively expressed [28]. On activated platelets, the exposure of negatively charged phospholipids on the outer leaflet of the plasma membrane is essential for efficient factor X activation [23,24]. On platelets as well as on synthetic negatively charged phospholipid vesicles, significant dampening of factor X activation is observed. On endothelial cells, however, a steady increase in factor Xa is observed over a prolonged period of time (Figure 2) [28,29]. In the present study, evidence is provided that the nature of the procoagulant membrane contributes to the regulation of factor VIIIa activity, thereby affecting the course of factor X activation.

Using a PS/PC concentration that was not rate-limiting (50 μ M PS/PC, 50:50), we confirmed the suggestion [38] that the decline in factor X activation is due to loss of factor VIII activity (Figure 1). Analysis of solution and lipid membrane separately revealed that the A2 fragment of the heavy chain of activated factor VIII was present in solution rather than associated with the lipid vesicles (Figure 5). The 67 kDa fragment of the light chain of factor VIIIa was detectable both in solution and associated with the lipid membrane (Figure 5). This observation supports the current view that dissociation of the A2 fragment from factor VIIIa is associated with factor VIIIa inactivation with concomitant decay in factor X activation [12–14,16,17]. A novel finding is that the lipid-associated 67 kDa fragment is not

readily substituted for intact factor VIII from the solution. This was concluded from the following observations using PS/PC vesicles present at a rate-limiting concentration (5 μ M PS/PC 50:50): (1) factor X activation stopped within 30 min even though uncleaved factor VIII was still detectable (Figures 4 and 5); (2) addition of factor VIII was ineffective in restoring factor X activation (Figure 1A), whereas the addition of extra lipids did restore factor X activation (Figure 1B). Thus our results strongly suggest that during factor X activation the procoagulant PS/PC vesicle becomes ineffective in promoting functional factor VIIIa–IXa complex assembly. The initially formed factor VIIIa–IXa complexes on the lipid vesicle become inactivated as the result of dissociation of the A2 fragment of activated factor VIII.

The situation with endothelial cells, however, is clearly different from that with phospholipid vesicles. Few, if any, factor VIIIa polypeptides were detected associated with the cell membrane (Figure 5), and rates of factor X activation were even higher than on lipid vesicles (Figure 3). This suggests that endothelial-cell-bound factor VIIIa has a higher cofactor activity than PS/PC-bound factor VIIIa. The presence of both A2 and 67 kDa light chain fragment in the buffer above the cells (Figure 5) indicates that either the intact factor VIIIa molecule is dissociated from the cell surface, or, in a two-step model, the 67 kDa light chain loses its affinity for the cell membrane after dissociation of the A2 fragment. This suggests a continuous exchange of cell-bound factor VIIIa or its subunits with free factor VIII and, consequently, ongoing activation of factor X. On the other hand, the A2 and 67 kDa light chain fragment detected in the supernatant may also originate, at least in part, from cleavage of factor VIII by factor Xa in solution that contains factor Xa in excess over factor VIII [39]. We indeed observed membrane-independent proteolysis of factor VIII at a 20-fold molar excess of enzyme. Membrane-independent cleavage of factor VIII by factor Xa

may explain why ongoing factor VIII proteolysis was observed on 5 μ M PS/PC vesicles, whereas factor X activation had stopped within 30 min (Figure 4).

Recently we reported that factor VIII as well as its 80 kDa light chain bind with high affinity to PS/PC membranes (K_d 0.2 nM) [40]. The present study indicates preferential binding of the 67 kDa light chain fragment, suggesting that this fragment binds to PS-containing lipid membranes with a higher affinity than the uncleaved light chain. It seems likely that once lipid-bound factor VIIIa has lost its cofactor activity as the result of dissociation of A2, free factor VIII cannot bind to the vesicles because of competition from the already bound 67 kDa light chain fragment. Similarly, factor X (and factor IXa) could occupy factor VIII-binding sites. As a result, factor X activation on lipid vesicles stops. The persistent factor X activation on endothelial cells might be due to the exposure of multiple classes of binding site on the cell membrane. Indeed, it has been reported that factor X and factor IXa bind to different sites on the endothelial cell membrane [41,42]. Furthermore binding of factor IXa appears to be independent of negatively charged membrane phospholipids [43]. The existence of multiple classes of binding site on the endothelial cell membrane would allow a continuous exchange of cell-bound factor VIII fragments with factor VIII in solution, guaranteeing a constant level of factor X activation until all factor VIII is consumed. This mechanism, in which the nature of the procoagulant membrane defines the progress of factor VIIIa-IXa-mediated activation of factor X, could be of importance in the regulation of thrombin formation at sites of vascular injury.

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