Regulation of Factor VIIa/Tissue Factor Functional Activity in an Umbilical Vein Model

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Activation of factor IX in an umbilical vein model was established to result solely from factor VIIa/tissue factor (TF) activity generated within the umbilical vein wall, and the model was then used to study regulation of such extravascular factor VIIa–TF complexes. Vein segments were filled with a reaction mixture containing factor VIIa, CaCl₂, a substrate, either [³H]factor IX or [³H]factor X, and a test material. Subsamples were assayed for activation peptide release. Test materials included defibrinated plasma or recombinant plasma as a source of TF pathway inhibitor (TFPI), recombinant factor VIIa to 10 times plasma factor VII concentrations, and annexin V. A plasma concentration of TFPI inhibited but did not totally suppress factor VIIa/TF activity. Reducing the TFPI concentration by 50% markedly reduced the inhibition. A 10-fold increase in the factor VIIa concentration in reaction mixtures failed to accelerate factor Xa generation. Annexin V, in contrast to its inhibition of factor VIIa/TF formed with TF reconstituted into mixed phospholipid vesicles, failed to inhibit factor VIIa–TF complexes formed within the vessel wall. We conclude that 1) moderate variation in plasma TFPI concentration or activity may affect TFPI's ability to inhibit factor VIIa/TF activity during hemostasis, 2) a plasma concentration of factor VIIa suffices to saturate TF sites exposed in a vessel after tissue injury, and 3) the resistance of factor VIIa–TF complexes to inhibition by annexin V suggests that they are formed in the umbilical vein model primarily on cell surfaces. (Arteriosclerosis and Thrombosis 1993;13:105–111)

Key Words • factor VIIa • tissue factor • tissue factor pathway inhibitor • annexin V • umbilical vein model • extravascular complexes • phospholipids • prothrombin

An umbilical vein model has been previously described in which the catalytic activity of factor VIIa–tissue factor (TF) complexes formed within the extravascular matrix of the umbilical vein may be studied. In contrast to factor VIIa–TF complexes formed with purified, reconstituted TF in suspension or TF expressed on the surfaces of cultured cells, which activate factor X more rapidly than they activate factor IX, factor VIIa–TF complexes formed within the umbilical vein wall were found to catalyze activation of factor X and factor IX at equivalent rates. This observation raised the possibility that other functional data obtained with factor VIIa–TF complexes that form on cultured cell surfaces may differ from those obtained with factor VIIa–TF complexes formed extravascularly in the umbilical vein wall. Therefore, in the experiments reported here, we have used the umbilical vein model to study the catalytic activity of factor VIIa–TF complexes in the presence of 1) TF pathway inhibitor (TFPI) (formerly called either extrinsic pathway inhibitor [EPI] or lipoprotein-associated coagulation inhibitor); 2) annexin V, a protein that binds to anionic phospholipid and interferes with its function in coagulation assays; and 3) a very high level of factor VIIa in the range of that achieved in plasma after administration of recombinant factor VIIa (rVIIa) to patients with a factor VIII inhibitor.

Methods

Coagulation Factors

Human brain TF apoprotein was purified to homogeneity as described earlier and incorporated into mixed phosphatidylserine/phosphatidylcholine (40:60, vol/vol) vesicles in a molar ratio of protein to phospholipid of 1:100,000 by the use of octylglucoside as a detergent. Mixed phosphatidylserine/phosphatidylcholine vesicles not containing TF were also prepared. Factor VII, factors IX and X and prothrombin, were purified from human plasma as described elsewhere.

Factor VIIa was prepared by incubating factor VII at a concentration of 100–150 µg/ml with factor Xa at a ratio of 50:1 (wt/wt) with the addition of 0.52 mM rabbit brain mixed phospholipid (cephalin) and 5 mM calcium for 20 minutes at 37°C, followed by addition of EDTA to a final concentration of 10 mM. The preparation was stored at −70°C in small aliquots. rVIIa, purchased from Novo BioLabs Inc., Danbury, Conn., was used in place of plasma factor VIIa in some experiments. Factor Va was prepared by incubating 1 mg/ml factor V with 6 units/ml thrombin in the presence of 5 mM CaCl₂ for 15 minutes at 37°C. The mixture was kept on ice and used within 30 minutes. Purified human thrombin was a gift from Dr. J.W. Fenton, New York State Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, Calif.

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Antibodies
Specific polyclonal anti-TF antiserum was raised in a goat. The immunoglobulin G (IgG) fraction was separated by precipitation at 45% (NH₄)₂SO₄ saturation, followed by DEAE-Affi-Gel blue chromatography (Bio-Rad Laboratories, Richmond, Calif.). A concentration of 4.0 μg/ml of the anti-TF IgG neutralized more than 95% of the activity of 0.05 μg/ml of reconstituted, purified TF apoprotein. An antiserum to human rTFPI was raised in a rabbit. The immunoglobulin G (IgG) fraction was separated by precipitation at 45% (NH₄)₂SO₄ saturation, followed by DEAE-Affi-Gel blue chromatography (Bio-Rad Laboratories, Richmond, Calif.).

Annexin V
Annexin V was purified from human placenta as described and was kindly provided by Dr. J.F. Tait, University of Washington, Seattle, Wash.

Radicalabelling
Sialyl [³H]factor IX, with a specific radioactivity of 3.2x10⁶ cpm/mg, and sialyl [³H]factor X, with a specific radioactivity 2.0x10⁶ cpm/mg, were prepared by the general technique of Van Lenten and Ashwell with slight modifications as described earlier.

Defibrinated Plasma
Platelet-poor plasma was prepared from blood drawn into a balanced-citrate anticoagulant (0.06 mM sodium citrate and 0.04 mM citric acid) from normal fasting donors and from a patient with severe hereditary factor XI deficiency. Plasma was stored at -20°C until used. The plasma was defibrinated by adding either purified human thrombin or bovine thrombin in a final concentration of 15 units thrombin per milliliter of plasma. After 1 hour at room temperature, the fibrin clot was removed and the solution centrifuged for 10 minutes at 10,000g to separate the residual fibrin. TFPI activity in the defibrinated plasma was 0.95 unit/ml.

Reaction Buffer
This calcium-containing buffer consisted of 10 mM N-hydroxysuccinimide, 140 mM NaCl, 4 mM KCl, 11 mM glucose (pH 7.45) plus 1 mg/ml bovine serum albumin (ICN Pharmaceuticals, Cleveland, Ohio), and either 5 or 10 mM CaCl₂.

Assay of TFPI Activity
TFPI activity of a test sample was measured in a capacity assay by its ability to inhibit factor VIIa/TF-catalyzed activation of [³H]factor IX in the presence of factor X. The reaction mixture contained the test sample; a saturating concentration of factor VII; a limiting concentration of TF that resulted in an essentially full factor VII occupancy of TF binding sites; factor X as the precursor for the factor Xa that is needed as a cofactor for TFPI; [³H]factor IX as the substrate; and calcium ions. Details of the assay have been provided earlier. Activation of factor IX was measured from the percent trichloroacetic acid (TCA)-soluble [³H]factor IX at 2.0 in a subsample taken after 120 minutes and converted to units of TFPI in the test sample from a reference curve prepared with 0.5-8% dilutions of a pooled plasma standard.

Measurement of Activation of Factors IX and X by Factor VIIa–TF Within Umbilical Vein Segments
Human umbilical cords obtained from the Department of Reproductive Medicine at University of California, San Diego Medical Center were cut into two (rarely three) segments 10 cm long. For each experimental observation, one vein segment of a cord served as the experimental segment and the other as the control segment. Veins were processed and washed under sterile conditions with phosphate-buffered saline. Vein segments were then filled by means of a syringe with 3.5 ml reaction buffer containing 0.5 μg/ml factor VIIa, 5 μg/ml of either [³H]factor IX or [³H]factor X, and other reactants as described in specific experiments. Seventy-microliter subsamples were removed over time for measurement of TCA-soluble [³H] counts indicative of activation peptide release from [³H]factor IX or [³H]factor X. The initial linear portions of the time-activation curves were used to calculate activation rates. Details have been previously provided.

Measurement of Thrombin Generation Within Umbilical Vein Segments
The ability of the phospholipid-like activity within umbilical vein segments to support the generation of thrombin was measured in the reaction buffer containing 100 μg/ml prothrombin, 0.1 μg/ml factor Xa, and 3 μg/ml factor Va. Serial 30-μl subsamples were removed, added to 270 μl of a Tris-HCl–buffered saline/bovine serum albumin stopping buffer containing 5 mM EDTA, and assayed for amidolytic activity with a chromogenic substrate for thrombin (Chromozym TH, Boehringer Mannheim) at a 405-nm wavelength in a Molecular Devices microplate reader. Thrombin concentration was calculated from a standard curve made with known concentrations of purified human α-thrombin. The rate of thrombin generation was calculated from the initial linear portion of the time–thrombin generation curves.

Results
Evidence That the Vein Wall Does Not Shed Significant Amounts of TF Activity or Anionic Phospholipid Activity Into Reaction Mixtures
We have previously presented evidence that the TF-supporting factor VIIa catalytic activity in reaction

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mixtures in umbilical vein segments is located within fenestrations of the muscularis media of the vein and in the underlying Wharton’s jelly.1 This evidence consisted of the following: 1) histological demonstration that the endothelial cell lining of the vein was disrupted; 2) the absence of TF apoprotein on endothelial cells after immunostaining; and 3) the presence of TF apoprotein on cells within fenestrations of the underlying vein wall after immunostaining. For the present study, it was necessary to characterize the system further to examine whether the TF on cell membrane vesicles that might be shed into the reaction mixture during mixing could also contribute to the factor VIIa-TF activity measured in the umbilical vein model. On seven occasions, an umbilical vein segment was filled with 3.5 ml reaction buffer containing no reactants. During a 2-hour incubation period, the buffer was treated as though it were a reaction mixture, i.e., it was mixed periodically by back-and-forth suction with plastic syringes that had been inserted into the flexible tubing at each end of the cord segment. Then the reaction buffer was drained from the vein, and 0.5 μg/ml factor VIIa and 5 μg/ml of either [3H]factor X or [3H]factor IX were added to the buffer. Subsamples removed over time were assayed for TCA-soluble H counts. Activation peptide release from the substrates was not measurable after 1 hour on four occasions and was less than 5% on three occasions. These data established that our technique for mixing reaction mixtures within vein segments did not cause shedding from the vein wall of material that could support factor VIIa-catalyzed activation of the substrates, i.e., of material possessing significant TF activity.

In a supplemental experiment performed with five cords, factor Xa, factor Va, and prothrombin were added to an aliquot of the reaction buffer that was drained from the vein after incubation and mixed as described above. Subsamples were assayed for thrombin, which for its generation requires shedding from the vein wall of material with anionic phospholipid or equivalent activity. The mean rate of thrombin generation was 1.6 units • ml⁻¹ • min⁻¹ as contrasted to a mean rate of 25.5 units • ml⁻¹ • min⁻¹ (n=4) in experiments described below, in which prothrombin and factors Xa and Va were added to the reaction buffer before it was injected into the vein segment. Thus, under our experimental conditions, cell membrane vesicles that possess anionic phospholipid or equivalent coagulant activity were not shed in significant concentrations into the reaction mixtures.

Inhibition by TFPI of Factor VIIa/TF Activity in Vein Segments

The ability of plasma TFPI to inhibit factor VIIa/TF activity in umbilical vein segments was studied by comparing the activation of factor IX in paired reaction mixtures made in defibrinated plasma or in reaction buffer. On three occasions, one segment of a vein pair was filled with a reaction mixture made in undiluted defibrinated plasma, and the other segment was filled with a reaction mixture made in buffer to which cold factors IX (4 μg/ml) and X (8 μg/ml) had been added to maintain equivalent concentrations of cold substrates in both vein segments. The reaction mixture in each vein segment contained 0.5 μg/ml factor VIIa, 5 μg/ml [3H]factor IX, 10 mM CaCl₂, and 0.5 μg/ml factor Xa.

The factor Xa was added to facilitate formation of the factor Xa-TFPI complexes required for neutralization of factor VIIa/TF activity. Percent activation values of [3H]factor IX in the reaction mixtures of these paired segments at 90 minutes were as follows: pair 1, buffer 42.5%, plasma 9.5%; pair 2, buffer 53.5%, plasma 22.5%; and pair 3, buffer 41.0%, plasma 10%. Average percent inhibition with undiluted plasma was about 70%. The progress curves of factor IX activation observed in one experiment are illustrated in Figure 1A.

In an additional experiment, the control segment of a vein pair was filled with the reaction mixture made in undiluted, defibrinated plasma, and the experimental segment was filled with the reaction mixture made in undiluted, defibrinated plasma to which anti-TFPI antibodies had been added 30 minutes earlier. The resultant progress curves of [3H]factor IX activation (Figure 1B) provide evidence that TFPI is primarily responsible for the inhibitory effect of undiluted, defibrinated plasma. Nevertheless, this experiment does not rule out the existence of additional inhibitory activity in plasma, since an additional control, a third vein segment from the same cord containing a reaction mixture made in reaction buffer, is lacking. (It was rarely possible to obtain umbilical cords long enough to prepare more than two vein segments from that portion of the cord far enough away from the ends to minimize the possibility of vein trauma.)

On two occasions, an experiment was carried out in which the experimental vein segment contained reactants made in 50%-diluted, defibrinated plasma, and the control segment contained reactants made in reac-
fibrinated plasma containing 500 ug/ml anti-TF immunoglobulin G and added to a vein segment previously treated with anti-TF immunoglobulin G. All reaction mixtures contained 0.5 ug/ml factor VIIa, 0.5 ug/ml factor Xa, 10 mM CaCl$_2$, and 5 ug/ml $[^{3}H]$factor IX. The buffer reaction mixture (c) also contained unlabeled factors IX and X in umbilical vein segments.

The limited inhibition observed with 50%-diluted plasma contrasted strikingly with our earlier observation that plasma diluted to 5-10% suppressed factor VIIa/TF catalytic activity formed with TF expressed on perturbed, cultured, endothelial cells. Because of this and despite the data from our first study with the umbilical vein model that showed that antibodies to TF or factor VII prevented activation of factors IX and X in umbilical vein segments, we carried out two additional experiments.

For the first experiment we obtained a cord long enough to prepare three vein segments. These were used for 1) a control segment containing reaction buffer, factor Xa, factor VIIa, the appropriate concentrations of cold factors IX and X, and $[^{3}H]$factor X; 2) an experimental segment containing 50%-diluted, defibrinated plasma, 10 mM CaCl$_2$, and $[^{3}H]$factor IX, and 3) a second experimental segment identical to the first, except that the reaction mixture of 50%-diluted, defibrinated plasma contained anti-human TF antibodies and the vein segment was filled with buffer containing anti-human TF antibodies, incubated for 15 minutes, and drained of buffer before being filled with the reaction mixture. Figure 2 illustrates the progress curves of factor IX activation observed in this experiment. In the segment treated with anti-TF antibodies, activation of factor IX was totally suppressed. In contrast, activation of factor IX was only moderately suppressed in the vein segment containing 50%-diluted, defibrinated plasma but not treated with antibodies to TF.

FIGURE 2. Line plot providing evidence that residual activation of $[^{3}H]$factor (F) IX observed in vein segment containing 50%-diluted, defibrinated plasma is not due to factor VIIa/tissue factor (TF)-independent activation of factor IX. Symbols denote reaction mixtures made in (c) buffer, (o) 50%-diluted, defibrinated plasma, and (△) 50%-diluted, defibrinated plasma containing 500 ug/ml anti-TF immunoglobulin G and added to a vein segment previously treated with anti-TF immunoglobulin G. All reaction mixtures contained 0.5 ug/ml factor VIIa, 0.5 ug/ml factor Xa, 10 mM CaCl$_2$, and 5 ug/ml $[^{3}H]$factor IX. The buffer reaction mixture (c) also contained unlabeled factors IX and X (4 ug/ml) to keep the total concentrations of factors IX and X the same as in the 50%-diluted, defibrinated plasma.

In the second experiment, we tested whether the factor XI in 50%-defibrinated plasma contributed measurably to the activation of factor IX. The control segment of a vein pair contained a reaction mixture of calcium ions, factor VIIa, factor Xa, and $[^{3}H]$factor IX made in 50%-diluted, defibrinated, normal plasma, and the experimental segment contained the same reaction mixture made in 50%-diluted, defibrinated plasma from a patient with hereditary factor XI deficiency. Percent factor IX activation at 90 minutes was 23% for the control segment containing defibrinated normal plasma and 28% for the experimental segment containing factor XI-deficient plasma.

The above data extend and confirm our earlier data from studies with purified reaction mixtures. The residual activation of $[^{3}H]$factor IX observed in vein segments containing 50%-diluted, defibrinated plasma does not represent activation of factor IX by a mechanism independent of factor VIIa/TF. This conclusion received further support from an experiment in which rTFPI was added to reaction mixtures at a final concentration of 100 ng/ml (1 unit/ml), which is equivalent to the TFPI concentration in defibrinated plasma. Factor VIIa/TF catalytic activity was markedly suppressed: for factor IX, by ~70% of the activity measured in the control segment of a vein pair and for factor X, by ~90% of the activity measured in the control segment of a vein pair (Figure 3). As expected, inhibition induced by rTFPI of factor IX activation was dependent on the addition of exogenous factor Xa to the reaction mixture. Activation rates measured in subsamples from paired vein segments containing 0.5 mg/ml factor VIIa, 5 ug/ml $[^{3}H]$factor IX, 100 mg/ml rTFPI, and either 0.5 mg/ml factor Xa or no factor Xa were 4.6 nM/hr for the reaction mixture with factor Xa and 22.4 nM/hr for the reaction mixture without factor Xa.

Increasing rVIIa Concentration 10-Fold in the Presence or Absence of rTFPI Failed to Affect Activation of Factor X

On two occasions, a vein pair was filled with reaction buffer containing $[^{3}H]$factor X as the substrate and rVIIa at a final concentration of 0.5 ug/ml (plasma concentration of factor VII) in the control segment and 5 ug/ml in the experimental segment. Activation rates of $[^{3}H]$factor X were as follows: in the first experiment, 33.4 nM/hr for the control segment and 32.1 nM/hr for
the experimental segment; in the second experiment, 48.4 nM/hr for the control segment and 38.4 nM/hr for the experimental segment. These differences were not significant. The experiment was then carried out on four occasions with reaction mixtures that also contained 1 unit/ml rTFPI. Mean activation rates for factor X were as follows: with rVIIa at 0.5 μg/ml, 7.7±5.0 nM/hr and with rVIIa at 5 μg/ml, 7.5±1.5 nM/hr. Increasing rVIIa concentration to 10 times that of the plasma factor VII concentration did not enhance the catalytic activity toward factor X of factor VIIa–TF complexes formed in the wall of human umbilical vein segments in the presence of a plasma concentration of rTFPI.

Effect of Annexin V on Factor VIIa/TF Activity Formed in the Umbilical Vein Model

Paired vein segments were prepared in which reaction mixtures were made with 0.5 μg/ml rVIIa and either [3H]factor IX or [3H]factor X as the substrate. Annexin V at a 10 μg/ml final concentration was added to the reaction mixture of one pair. Mean activation rates were as follows: for factor IX in the absence of annexin V, 25.0±2.7 nM/hr and in the presence of annexin V, 20.0±2.5 nM/hr (n=3); for factor X in the absence of annexin V, 32.0±16.9 nM/hr and in the presence of annexin V, 21.7±7.8 nM/hr (n=5). Although mean activation rates were lower for both substrates in the presence of annexin V, the differences were not statistically significant. As illustrated in Figure 4A, the inability of annexin V to significantly suppress factor VIIa/TF activity formed in the umbilical vein segment differed strikingly from the known ability of annexin V to suppress factor VIIa/TF activity formed with purified TF apoprotein reconstituted into mixed phosphatidylserine/phosphatidylcholine vesicles.21

Effect of Annexin V on Thrombin Generation in Umbilical Vein Segments

Since the aforementioned experiments with annexin V yielded negative results, an additional control experiment was carried out in which annexin V was added to reaction mixtures containing factors Xa and Va, prothrombin as the substrate, and no source of anionic phospholipid other than that provided by the umbilical vein segment. Mean values for rates of thrombin generation for four vein pairs were 25.5±10 units • ml⁻¹ • min⁻¹ in the absence of annexin V and 4.4±1.7 units • ml⁻¹ • min⁻¹ in the presence of 10 μg/ml annexin V. In further experiments annexin V in concentrations as low as 1 μg/ml yielded a similar degree of inhibition. As illustrated in Figure 4B, annexin V inhibited thrombin generation comparably when the source of anionic phospholipid was either the umbilical vein segment or mixed phospholipid vesicles.

Discussion

TFPI has been established as the major plasma inhibitor of the factor VIIa/TF activity formed either in vitro with TF in suspension (as reviewed in Reference 22) or with TF expressed on the surface membrane of monolayers of cultured human umbilical vein endothelial cells,23 fibroblasts,24,25 and carcinoma cell lines.14,25,26 TFPI has also been shown to function in vivo in the rabbit as a natural anticoagulant that can inhibit the factor VIIa/TF catalytic activity formed in circulating blood exposed to a low concentration of TF.27,28
The data presented here establish that plasma TFPI can also diminish the catalytic activity of factor VIIa-TF complexes formed extravascularly within the wall of the umbilical vein. However, it was unexpected to find that reaction mixtures made in undiluted, defibrinated plasma retained about 30% and reaction mixtures made in 50%-diluted, defibrinated plasma retained about 75% of the factor VIIa/TF catalytic activity toward factor IX of that measured in paired control vein segments containing reaction mixtures made in buffer. In earlier experiments with monolayers of cultured human umbilical vein cells perturbed to express TF activity, we had found that the TFPI activity in plasma diluted to 5% markedly suppressed factor VIIa/TF activation of factor X. In experiments with monolayers of OC-2008 carcinoma cells, which constitutively express more TF activity than do perturbed endothelial cells, it was found that a concentration of rTFPI equivalent to that of 50% plasma suppressed factor VIIa/TF activity totally, and a concentration of rTFPI equivalent to that of 5% plasma suppressed factor VIIa/TF activity by about 70%. The ability of undiluted plasma to inhibit activation of factor IX in umbilical vein segments was shown to stem primarily from plasma TFPI activity, since anti-TFPI antibodies added to plasma markedly reduced its inhibitory effect (Figure 1B). Moreover, when 1 unit/ml rTFPI was added to reaction mixtures made in buffer, the degree of inhibition was similar to that observed when reaction mixtures were made in undiluted plasma. The residual factor IX activation observed in vein segments containing defibrinated plasma was not the result of a factor VIIa/TF-independent activation of factor IX. It was not diminished when defibrinated plasma from a patient with hereditary factor XI deficiency was substituted for normal defibrinated plasma, but it was abolished when a vein segment that had been exposed to anti-TF antibodies was filled with a reaction mixture made in defibrinated plasma that also contained anti-TF antibodies (Figure 2).

It is not yet known why a higher concentration of plasma TFPI is needed to suppress factor VIIa/TF activity in umbilical vein segments than to suppress TF expressed by stimulated cultured monolayers of umbilical vein endothelial cells. Conceivably, a qualitative difference in the milieu of TF in the umbilical vein wall impairs the ability of TFPI–factor Xa complexes to bind to factor VIIa–TF complexes in the vein wall. However, it seems more likely to us that the difference simply reflects the formation of more factor VIIa–TF complexes within the umbilical vein wall than were formed in our earlier experiments with monolayers of perturbed, cultured, umbilical vein cells. TFPI stoichiometrically neutralizes factor VIIa/TF activity, and the ability of a plasma concentration of TFPI to neutralize factor VIIa/TF activity is known to be limited from earlier experimental data. Thus, in early in vitro experiments, increasing the concentration of a crude TF activating factor VIII inhibitor.

Moreover, in patients with severe Gram-negative endotoxicemia, TF-induced DIC may continue despite elevated plasma concentrations of TFPI. Nevertheless, we had assumed from the ability of plasma diluted to 5% to suppress the activity of factor VIIa–TF complexes formed on cultured cell monolayers that moderate variation in plasma TFPI concentration would have little effect on the ability of TFPI to regulate TF-dependent coagulation during hemostasis at sites of tissue injury. The present data cast doubt on this assumption. If the response of factor VIIa–TF complexes formed in the umbilical vein wall to TFPI in 50%-diluted plasma is representative of the response to be expected at other vessel wall sites, then modest variations in plasma TFPI concentration or activity might well influence the regulation of TF-dependent coagulation during hemostasis.

There is growing evidence that the intravenous injection of a pharmacological concentration of rVIIa can stop bleeding in patients with factor VIII inhibitors, but how it does so is unknown. In the experiments reported here, concentrations of rVIIa in reaction mixtures that were 10 times those of the plasma factor VII concentration failed to increase the rate at which factor VIIa–TF complexes formed in the umbilical vein wall activated factor X. When TFPI was added to reaction mixtures, similar results were obtained. Since the bleeding of hemophilia is thought to stem from TFPI-induced inhibition of factor VIIa/TF activation of factor X during hemostasis, these negative data provide little further insight into how a high plasma concentration of rVIIa controls bleeding in a patient with a factor VIII inhibitor.

Annexin V in the presence of Ca²⁺ binds to anionic phospholipids and interferes with their functions in in vitro coagulation assays. This includes the ability of purified TF apoprotein reconstituted into mixed phospholipid vesicles to support factor VIIa–catalyzed activation of its substrates. Annexin V binds to activated platelets and to monolayers of an ovarian carcinoma cell line with a dissociation constant of approximately 10 nM. Annexin V at a concentration of about 200 nM was reported to inhibit TF activity expressed on monolayers of a fibroblast cell line, but in another report, a very high concentration of annexin V (16 μM) was needed to inhibit TF activity expressed on intact monolayers of perturbed, cultured, saphenous vein endothelial cells. In the present experiments, annexin V at a 10 μg/ml concentration (about 270 nM) readily inhibited the activity of purified TF reconstituted in mixed phospholipid vesicles (see Figure 4B). This concentration of annexin V also inhibited thrombin generation in vein segments filled with a reaction mixture containing factors Xa and Va, prothrombin, and Ca²⁺ but lacking a source of anionic phospholipid or equivalent activity other than that provided by the umbilical vein wall (Figure 4A). In contrast, an approximately 270 nM concentration of annexin V failed to substantially inhibit the activity of factor VIIa–TF complexes formed in the umbilical vein wall to activate factor IX (Figure 4A). These data fit with earlier data from this laboratory in which annexin V could not be demonstrated to inhibit factor VIIa/TF activity formed on intact monolayers of a cultured ovarian carcinoma cell line that constituatively expresses surface membrane TF activity. In working...
with this and other cell lines, we have found that either monolayers must be disrupted or TF must be shed in membrane vesicles into the overlying medium before annexin V can substantially inhibit factor VIIa/TF catalytic activity. It is unknown why annexin V, at a concentration exceeding that needed to suppress the ability of cell surfaces to support prothrombin generation, fails to suppress the ability of TF expressed on cultured cell monolayers or in the umbilical vein wall to support factor VIIa-catalyzed activation of its substrates. In summary, the experiments reported here confirm and extend the evidence that activation of factor IX in the umbilical vein model stems solely from factor VIIa/TF catalytic activity generated extravascularly in the umbilical vein wall. The failure of annexin V to suppress factor VIIa/TF activity supports the hypothesis that the factor VIIa-TF complexes formed within the umbilical vein wall are present primarily on cell surfaces. The failure of a 10-fold higher rVIIa concentration (5 μg/ml) than plasma factor VII concentration (0.5 μg/ml) to enhance factor X activation in reaction mixtures represents further evidence that a plasma concentration of factor VII can fully saturate TF sites exposed after tissue injury. The experiments with TFPI suggest that variation in the concentration and/or activity of TFPI present in plasma could influence the ability of TFPI to regulate the function of factor VIIa-TF complexes formed extravascularly during hemostasis.

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