Binding of Factor VIIa to Tissue Factor Permits Rapid Antithrombin III/Heparin Inhibition of Factor VIIa

By L. Vijaya Mohan Rao, Samuel I. Rapaport, and An D. Hoang

Because free factor VIIa is inactivated only very slowly by a plasma concentration of antithrombin III (AT III) even in the presence of heparin, it has been assumed that AT III plays no significant role in regulating the initiation of tissue factor-dependent blood coagulation. However, in the present study, we present evidence that factor VIIa bound to tissue factor, unlike free factor VIIa, is readily inactivated by AT III in the presence of heparin. In a reaction mixture containing calcium ions and approximately equimolar concentrations of relipidated tissue factor (8.9 nmol/L) and factor VIIa (10 nmol/L), AT III (100 μg/mL) plus heparin (1 U/mL) inhibited 50% of the factor VIIa coagulant activity of the reaction mixture within 5 minutes. AT III/heparin was also shown to inhibit the catalytic activity towards factor X of factor VIIa/tissue factor complexes formed on monolayers of an ovarian carcinoma cell line (OC-2008) that constitutively expresses surface membrane tissue factor. AT III, even in the absence of exogenously added heparin, substantially inhibited the functional activity of factor VIIa/cell surface tissue factor complexes on intact monolayers. AT III alone and AT III/heparin, to a greater extent, also inhibited factor VIIa on “nonfunctional” factor VIIa/tissue factor complexes on intact monolayers, with resultant inhibition of their expression of factor VIIa/tissue factor catalytic activity toward factor X after cell lysis. The potential physiologic significance of these findings is discussed.

© 1993 by The American Society of Hematology.

MATERIALS AND METHODS

Materials. Materials were obtained from the following sources: sodium [2H] and sodium boro [3H] hydride from Amersham Corp (Arlington Heights, IL); Iodo-Gen from Pierce Chemical Co (Rockford, IL); heparin (derived from porcine intestine) from Elkins-Sinn, Inc (Cherry Hill, NJ); and rabbit brain thromboplastin from Sigma (St. Louis, MO). Factor VII-deficient plasma was either purchased from George King Bio-Medical (Overland Park, KA) or prepared in the laboratory by passing normal human plasma over rabbit anti-human factor VII antibody immobilized on Affi-Gel 15 Bio-Rad, Richmond, CA). Low molecular weight heparin, Fragmin, was kindly provided by Dr Per Morten Sandset (University of Oslo, Oslo, Norway). Other chemicals, reagent grade or better, were from Fisher Scientific (Pittsburgh, PA) or Sigma.

Buffers. Buffer A contained 10 mmol/L HEPES, 0.15 mol/L NaCl, 4 mmol/L KCl, and 11 mmol/L glucose, pH 7.5. Buffer B, also referred to as calcium-containing buffer, was prepared by adding CaCl2 (5 mmol/L, final concentration) and 1 mg/mL bovine serum albumin (BSA; fatty acid-free) to buffer A.

Cell culture. An ovarian carcinoma cell line constitutively expressing surface membrane TF activity, OC-2008, was grown in T-75 flasks and subcultured into 12-well tissue culture plates as described earlier.

Tumor-shed membrane TF. Confluent monolayers of OC-2008 cells were washed twice with buffer A. Then, 0.5 mL of buffer A containing 1 mg/mL BSA was added to the each well and incubated at 37°C. After 2 hours, the supernate was removed and centrifuged for 1 hour at 20,000g. After centrifugation, the top half volume of the supernate was removed and the bottom half was vortexed to disperse sedimented membrane fragments evenly.

OC-2008 cell lysates. OC-2008 cell lysates were prepared essentially as described earlier.

Coagulant proteins. Human clotting factors VII, IX, and X were purified to homogeneity essentially as described earlier. Factor VIIa was prepared by incubating factor VII with factor Xa at a 100:1 ratio (wt/wt) in the presence of cephalin and calcium ions for 15 to 20 minutes at 37°C. Recombinant factor VIIa was either purchased from Novo Bio-Labs Inc (Danbury, CT) or received as a gift from Novo-Nordisk (Gentofte, Denmark). AT III was purchased from Kabi Pharmacia (Franklin, OH). Human brain TF apoprotein was purified as previously described and the apoprotein was incorporated into phospholipid vesicles containing 40% phosphatidylserine (PS) and 60% phosphatidylcholine (PC) or 100% PC, as described earlier.

Factor VIIa clotting assay. Factor VIIa/VIIa was assayed by a standard one-stage assay using a Lancer coagulizer (Sherwood Medical, St Louis, MO). Briefly, 100 μL of sample was added to 100 μL

In the initiation of blood coagulation during hemostasis, plasma factor VII(a) binds to tissue factor (TF) on cell surface membranes and the factor VII bound to TF is rapidly activated to a two-chain serine protease. The resultant factor VIIa/TF enzyme/cofactor complexes catalyze activation of factors IX and X. A Kunitz-type protease inhibitor called TF pathway inhibitor (TFPI), formerly referred to as extrinsic pathway inhibitor (EPI) or lipoprotein-associated coagulation inhibitor (LACI), is thought to regulate factor VIIa/TF activity in a reaction requiring the participation of generated factor Xa.

In experiments in this laboratory in which AT III was used to prevent contaminating factor Xa from activating factor VII bound to TF, we obtained evidence suggesting that AT III could neutralize factor VIIa bound to TF. This led to the experiments reported here in which AT III/heparin is shown to be capable of inactivating factor VIIa bound either to relipidated TF in suspension or to TF expressed on a cell surface.

From the Department of Medicine, University of California, San Diego, La Jolla, CA.

Submitted October 5, 1992; accepted December 18, 1992.

Supported by Grants No. HL 42813 and HL 27234 from the National Heart, Lung, and Blood Institute. L.V.M.R. is the recipient of Research Career Development Award HL 02590 from the National Heart, Lung, and Blood Institute.

Address reprint requests to L. Vijaya Mohan Rao, PhD, UCSD Medical Center, Mail Code 8423, 200 W Arbor Dr, San Diego, CA 92103.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

0006-4971/93/8110-0011$3.00/0

Blood, Vol 81, No 10 (May 15), 1993: pp 2600-2607

From www.bloodjournal.org by guest on October 3, 2017. For personal use only.
of an equal part mixture of factor VII-deficient plasma and barium-absorbed bovine plasma in a cuvette and then loaded into the coagulometer. After the addition of 100 µL of rabbit brain thromboplastin, the reaction mixture was incubated for 3 minutes at 37°C, and then 100 µL of 25 mmol/L prewarmed CaCl₂ was added to initiate clotting. Factor VIIa activity in all samples was measured in duplicate, and the average of the two determinations was taken as the value for the specimen. Pooled citrated platelet-poor plasma obtained from 10 or more healthy volunteers was used to prepare a reference standard containing buffer (buffer B). After the addition of CaCl₂ (final concentration 5 mmol/L), 10 µL of serial subsamples was removed from the reaction mixture and diluted into 390 µL of TBS/BSA containing 5 mmol/L EDTA. The subsamples were further diluted fivefold in TBS/BSA before they were assayed for factor VIIa coagulant activity as described above. Variations in the concentrations of AT III and heparin in the above reaction mixture are described in Results and/or the figure legends. Unless specified otherwise, only TF incorporated into PC/PS vesicles and recombinant factor VIIa were used in all the experiments.

Inactivation of factor VIIa complexed with cell surface TF. Activation peptide release from [3H] factor X was used to monitor the AT III/heparin inactivation of factor VIIa complexed with cell surface TF. Briefly, confluent OC-2008 monolayers were washed with 1 mL of buffer A supplemented with 5 mmol/L EDTA and then washed two more times with 1 mL of buffer A not containing EDTA. The monolayers were incubated with factor VIIa (10 ng/mL) for 1 hour at 37°C in a final volume of 0.5 mL of calcium-containing buffer (buffer B). After 1 hour, the monolayers were washed three times in ice-cold buffer B to remove the unbound factor VIIa, and AT III (100 µg/mL) and/or heparin (1 U/mL), in a volume of 0.5 mL buffer B, was added. [3H]Factor X, at a final concentration of 175 nmol/L (10 µg/mL), was then immediately added. Serial aliquots were removed over the next hour for determination of tri-chloroacetic acid soluble [3H]-labeled activation peptide release as described in detail earlier. Modifications to the above protocol, where necessary, are described in Results and/or in the figure legends.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Weber and Osborn using 8% polyacrylamide vertical slab gels. Factor VIIa (labeled by using Iodo-Gen according to the manufacturer's technical bulletin) was used so that free factor VIIa and factor VIIa-AI complex on the gels could be identified by autoradiography.

RESULTS

Inhibition of free factor VIIa and factor VIIa bound to TF by AT III and heparin. As expected from earlier reports, AT III/heparin was found to inhibit free factor VIIa activity only very slowly. The time required for 50% inactivation of free factor VIIa (10 nmol/L) activity by AT III (100 µg/mL) and heparin (1 U/mL) was 145 ± 26 minutes (n = 4). Incubation for 3 hours of factor VIIa with either AT III or heparin alone failed to inactivate factor VIIa beyond the small loss of activity (about 20%) observed on incubation of factor VIIa with a control buffer (Fig 1).

In contrast, when a mixture of purified, reconstituted human TF (8.9 nmol/L), rVIIa (10 nmol/L), and CaCl₂ (5 mmol/L) was incubated with AT III (100 µg/mL) and heparin (1 U/mL), the mean time required for 50% inactivation of the factor VIIa present in the reaction mixture was shortened to 5.25 ± 0.75 minutes (n = 6). Under these conditions, about 80% of the factor VIIa in the reaction mixture was present as factor VIIa/TF complexes. If calcium ions, AT III, or heparin was omitted from the reaction mixture, then factor VIIa activity was not lost (Fig 2). Although linear rates of inactivation of factor VIIa were observed at all concentrations of TF, decreasing the concentration of TF in the reaction mixture proportionately decreased the rate of inactivation of factor VIIa. In a reaction mixture containing 0.55 nmol/L TF and 10 nmol/L factor VIIa, about 20 minutes was required to inhibit about 50% of factor VIIa activity. These observations imply that the binding of factor VIIa to TF is essential for the rapid inactivation of factor VIIa by AT III and heparin.

Similar rates of inactivation of factor VIIa were observed when TF apoprotein reconstituted into vesicles containing 100% neutral phospholipid, PC, was substituted for TF apoprotein reconstituted into vesicles containing mixed phospholipids, 60% PC, and 40% negatively charged phospholipid, PS. Similar rates of inactivation were also observed when purified plasma factor VIIa was substituted for recombinant factor VIIa.

The rate of inactivation of factor VIIa bound to TF by AT III depended on the final concentration of heparin in the reaction mixture (Fig 3). If the heparin final concentration was increased to 5 U/mL, factor VIIa bound to TF was inactivated very rapidly by AT III. The time required for 50% inactivation of factor VIIa activity was just 100 seconds. If
the heparin final concentration was decreased to a 0.25 U/mL, the time required for 50% inactivation of factor VIIa was about 20 minutes. In the presence of either 1 U/mL of a low molecular weight heparin, fragmin, or 1 U/mL of unfractionated heparin, AT III inactivated factor VIIa bound to TF at a similar rate.

At a fixed concentration of heparin (1 U/mL), factor VIIa bound to TF was inhibited by AT III in a dose-dependent manner (Fig 4). In reaction mixtures containing factor VIIa (10 nmol/L), purified reconstituted TF (8.9 nmol/L), calcium ions (5 mmol/L), a heparin (1 U/mL), and AT III, increasing the final concentration of AT III from 5 μg/mL to 250 μg/mL progressively increased the rate of inactivation of factor VIIa until a plateau was reached at 50 μg/mL AT III. This is well below the plasma concentration of AT III of 150 to 250 μg/mL.

Analysis of the data of inhibition of factor VIIa bound to TF by AT III (100 μg/mL) and heparin (1 U/mL) yielded a second-order rate constant of $6.5 \times 10^9$ mol/L$^{-1}$ min$^{-1}$. However, one should note that the concentration of 1 U/mL heparin does not fully saturate the available AT III in the reaction mixture (Fig 3). When the concentration of heparin was increased to a saturating concentration of 10 U/mL, AT III/heparin inhibited the factor VIIa bound to TF with a second-order rate constant of $4 \times 10^5$ mol/L$^{-1}$ min$^{-1}$.

**Factor VIIa-AT III complex formation.** $^{125}$I-factor VIIa was incubated with AT III and heparin in the presence and absence of TF as described in Fig 2. Serial subsamples were removed from the reaction mixtures and subjected to SDS-PAGE. As shown in Fig 5, in the presence of TF a significant amount of factor VIIa was complexed with AT III as early as first time point (i.e., 2 minutes) and the complex was progressively increased with incubation period. At the end of a 10-minute incubation period, most of factor VIIa was present as factor VIIa-AT III complex. The molecular weight of the factor VIIa-AT III complex was about 100 Kd. In the absence
of either AT III or heparin in the reaction mixture, factor VIIa was migrated as a single band with an apparent molecular weight of 50 Kd (figure not shown). As expected from the data of Fig 1, either no or only minimal amounts of factor VIIa was complexed with AT III within this time period if TF was omitted from the reaction mixture (Fig 5).

The ability of AT III/heparin to inhibit factor VIIa bound to cell surface TF. The evidence that AT III/heparin could inactivate factor VIIa bound to reconstituted purified TF in suspension led us to determine whether AT III in the presence of heparin could also inactivate factor VIIa bound to TF expressed on cell surfaces. Monolayers of OC-2008 cells were incubated at 37°C with factor VIIa (10 nmol/L) in buffer B for 1 hour to allow saturation binding of factor VIIa to TF sites on the monolayers. After this, the monolayers were washed three times with ice-cold buffer B to remove unbound factor VIIa. Then, buffer B containing [1H] factor X (10 μg/mL) in the presence or absence of AT III (100 μg/mL), heparin (1 U/mL), or both was added to the monolayers. The time course of factor X activation was determined by measuring activation peptide release as trichloroacetic acid-soluble counts in serial subsamples. As expected from earlier experiments, the addition of heparin alone to the reaction mixture yielded an enhanced rate of factor VIIa/cell surface TF catalyzed activation of factor X. In contrast to the above experiments with purified reconstituted TF, adding AT III alone resulted in a variable, but consistent inhibition of factor VIIa/cell surface TF catalyzed activation of factor X. However, in all experiments, maximum inhibition of factor VIIa bound to cell surface TF was observed in reaction mixtures containing AT III plus exogenously added heparin (Fig 6).

Because trace concentrations of TFPI, possibly present either as a contaminant in our purified reagents or secreted endogenously by the carcinoma cells, could have accounted for the above results, additional experiments were performed in which a mixture of AT III and heparin was incubated for 30 minutes with anti-TFPI IgG and then added to the monolayers. As also shown in Fig 6, the addition of anti-TFPI antibodies failed to reverse AT III/heparin inhibition of factor VIIa/TF activity. In control experiments, the addition of anti-TFPI antibodies reversed TFPI/factor Xa-induced inhibition of factor VIIa/cell surface TF activity (data not shown).

The ability of AT III/heparin to inhibit factor VIIa/cell surface TF activity was also assessed using a different experimental protocol in which free factor VIIa was not removed from the reaction mixtures. In these experiments, OC-2008
monolayers were incubated with factor VIIa (10 nmol/L) in the presence or absence of AT III (100 μg/mL), heparin (1 U/mL), or both for 60 minutes in buffer B. (During this incubation period the following occurred: [1] increasing binding of factor VIIa to TF sites, [2] neutralization by AT III of factor VIIa bound to TF sites, and [3] presumed exchange on TF sites of AT III/factor VIIa complexes with free factor VIIa.) Then, [3H] factor X (10 μg/mL) was added to the reaction mixture and the time course of factor X activation was determined by measuring activation peptide release. The results obtained from these experiments were similar to those obtained in the earlier experiments (Fig 6) in which free factor VIIa was removed from reaction mixtures before the addition of test materials and the substrate. The mean factor X activation rates from two experiments were as follows: control (buffer), 1.12 nmol/L/min; in the presence of heparin alone, 2.41 nmol/L/min; in the presence of AT III alone, 0.60 nmol/L/min; and in the presence of both AT III and heparin, 0.34 nmol/L/min.

In further experiments, the ability of AT III/heparin to inactivate factor VIIa/TF complexes was tested in reaction mixtures in which TF was provided by a cell lysate prepared by freeze-thawing OC-2008 cells (Fig 7) or by membrane fragments shed over a 2-hour incubation period into buffer overlying monolayers of OC-2008 cells (figure not shown). The following reactants were incubated together in buffer for 15 minutes: cell lysate or shed membrane TF, factor VIIa, AT III, and/or heparin. Then, [3H] factor X was added and serial subsamples were removed to measure activation peptide release. AT III/heparin markedly inhibited factor VIIa/TF activation of factor X. However, in contrast to the inhibition induced by AT III alone of factor VIIa/TF activity on intact monolayers, AT III without exogenous heparin did not inhibit the ability to activate factor X of factor VIIa/TF complexes formed with cell lysate TF or shed membrane TF.

In a recent study from this laboratory, it was found that only a small fraction of factor VIIa/TF complexes forming on intact monolayer surfaces were catalytically active towards factor X. The majority of factor VIIa/TF complexes were inactive, but became fully functional if the cells were disrupted by lysis. The following experiment was performed to determine whether AT III/heparin could inactivate “nonfunctional” factor VIIa/TF complexes on intact monolayers. Parallel sets of monolayers were incubated for 90 minutes with 10 nmol/L factor VIIa in the presence or absence of AT III/heparin and washed three times with cold buffer B to remove unbound factor VIIa and AT III/heparin. Then, one set of monolayers was left intact and the other set was lysed by freeze-thawing. After this, [3H] factor X was added to both sets and the time course of activation of factor X was measured. As shown in Fig 8A, when the catalytic activity towards factor X of factor VIIa/TF complexes on intact monolayers was measured, inhibition was similar to that observed earlier with intact monolayers under slightly different experimental conditions (eg, Fig 6), except that, because the heparin had been washed away before the substrate was added, reaction mixtures incubated with heparin and buffer gave similar time courses of factor X activation. AT III alone induced substantial inhibition of factor X activation and AT III in the presence of exogenous heparin induced yet a greater degree of inhibition.

The rates of activation of factor X catalyzed by the factor VIIa/TF complexes of the lysed cells are shown in Fig 8B. As expected from earlier experiments with OC-2008 cells, lysis induced a marked increase in the functional activity of the factor VIIa/TF complexes formed on intact monolayers. (One should note that the cell lysate was diluted 1/10 before assay and that the times listed on the abscissa of Fig 8B are much shorter than those of Fig 8A.) Incubating the intact monolayers with AT III alone or with AT III in the presence of exogenous heparin markedly inhibited the enhanced factor VIIa/TF activity after cell lysis. This means that, under our experimental conditions, AT III, in the presence and also in the absence of exogenously added heparin, must have inhibited the factor VIIa bound to both functional and nonfunctional factor VIIa/TF complexes on intact monolayers.

**DISCUSSION**

It is known from the earlier work of others and confirmed here that AT III alone cannot inhibit the activity of free factor VIIa and that AT III in the presence of heparin inhibits the activity of free factor VIIa only slowly. Previous data on the ability of AT III/heparin to inhibit factor VIIa when it is present in reaction mixtures containing TF and calcium ions are limited. Jesty, using purified bovine factor VIIa, reported that both free factor VIIa and factor VIIa/TF complexes were not significantly inhibited by plasma levels of AT III either in the absence or in the presence of heparin in a concentration of 0.1 U/mL. Broze and Majerus stated, without provided experimental details, that the addition of TF and calcium ions to factor VIIa “did not prevent its inactivation” by AT III in the presence of a high concentration of heparin.
ANTITHROMBIN III INHIBITION OF FACTOR VIIa

The present data establish that factor VIIa bound to TF, in contrast to free factor VIIa, is rapidly inactivated by AT III in the presence of heparin. For example, 50% of the factor VIIa activity of the reaction mixture was inhibited within 5 minutes (Fig 2) when factor VIIa at a 10 nmol/L concentration (plasma factor VII concentration) was incubated in calcium-containing buffer with an almost 10 nmol/L concentration of relipidated TF in suspension, a plasma concentration of AT III (100 µg/mL), and heparin (1 U/mL). The increased rate of inhibition by AT III/heparin of factor VIIa functional activity when factor VIIa is bound to TF was confirmed by autoradiography after SDS-PAGE, which showed the formation of enzyme-inhibitor (factor VIIa-AT III) complex in reaction mixtures when TF was present but not when TF was absent (Fig 5). We interpret these data to mean that binding to TF markedly enhances the ability of the reactive site of factor VIIa to interact with the reactive center of AT III in the presence of heparin. This fits with earlier evidence that the binding of factor VIIa to TF alters the activity of factor VIIa's active site, eg, enhances its ability to hydrolyze small synthetic substrates.20,21

In the experiments summarized in Figs 1 to 4, EDTA was added to reaction mixtures before the residual factor VIIa activity of the mixture was assayed. Because the EDTA caused the factor VIIa/TF complexes to dissociate, all factor VIIa in the reaction mixtures before assay was free factor VIIa, either active or inactivated as AT III/factor VIIa complexes. Therefore, one may conclude from the data of Fig 1 through 4 that the factor VIIa remained inhibited even after it was dissociated from TF.

After obtaining the above cited data with factor VIIa bound to relipidated TF in suspension, we examined the ability of AT III/heparin to inhibit the catalytic activity of factor VIIa bound to TF expressed on a cell surface. When AT III/heparin was added to intact monolayers of OC-2008 cells that had first been incubated with 10 nmol/L factor VIIa to form factor VIIa/cell surface TF complexes and then washed to remove unbound factor VIIa, the factor VIIa/TF activity of the monolayers towards factor X was markedly inhibited (Fig 6). Moreover, when AT III was added without heparin, the functional activity of factor VIIa/cell surface TF complexes was also inhibited substantially, although not as extensively as when the AT III was added with heparin (Figs 6 and 8A). Because AT III alone did not inhibit factor VIIa bound to TF in suspension (Fig 2), the inhibition of factor VIIa/cell surface TF complexes by AT III alone is thought to reflect the participation of glycosaminoglycans present on the surface membrane of OC-2008. In an earlier study, we also obtained indirect functional evidence for the presence of glycosaminoglycans on intact monolayers of OC-2008 cells.11

Evidences has recently been presented from this laboratory that the majority of factor VIIa/TF complexes formed on intact monolayers of OC-2008 cells are not functional towards their physiologic substrate, factor X, until the cells are disrupted by lysis. However, the active site of the factor VIIa in these "nonfunctional" complexes of intact monolayers can catalyze hydrolysis of a small synthetic substrate.19 It was not surprising, therefore, to obtain evidence in the present study (Fig 8B) that AT III/heparin could bind to and inactivate the factor VIIa of such "nonfunctional" factor VIIa/TF complexes. This observation has potential physiologic significance, because there are reasons, summarized elsewhere,22 for believing that such "nonfunctional" factor VIIa/TF complexes may be present extravascularly and could potentially become functional when cells are perturbed after tissue injury.

It is well known that factor VIIa bound to TF that has been reconstituted into vesicles containing negatively charged phospholipids activates factors X and IX much more rapidly than factor VIIa bound to TF that has been reconstituted into vesicles containing only the neutral phospholipid, PC.23

Fig 8. Inhibition of both "functional" and "nonfunctional" cell surface-factor VIIa/TF complexes by AT III and heparin. Parallel sets of cells were incubated for 90 minutes with 10 nmol/L factor VIIa in the presence of buffer (○); heparin (1 U/mL) (■); or AT III (100 µg/mL) (▲); or AT III plus heparin (▲). In calcium-containing buffer. The cells were then washed three times with buffer B to remove unbound factor VIIa, AT III, and heparin. One set of monolayers was left intact and the other set of monolayers was lysed by freeze-thawing. The residual factor VIIa/TF activity of intact monolayers (A) and lysed monolayers (B) was measured by its ability to activate [3H] factor X. Note that all lysed cell extracts were diluted 1 0-fold in buffer B before they were assayed, and that the time course of assay was much shorter than with the intact monolayers.
The reasons are not clear, but do not appear to be related to an effect of the phospholipids upon the catalytic site of TF-bound factor VIIa. This is consistent with the observation here that the type of phospholipid used to relipidate TF did not affect the rate of AT III/heparin inactivation of TF-bound factor VIIa.

TFPI has been considered the primary regulator of factor VIIa/TF activity during hemostasis. Whether AT III in the presence of glycosaminoglycans on cell surfaces expressing TF can function as an auxiliary second physiologic regulator of factor VIIa/TF activity is not yet known. Each protease inhibitor shares the key property that it can inhibit the activity of factor VIIa only when factor VIIa is bound to TF. In other important respects their inhibitory mechanisms differ. Whereas AT III/heparin inhibition of factor VIIa requires only that factor VIIa bind to TF, a plasma concentration of TFPI can not inhibit factor VIIa/TF activity until some factor Xa is generated, with resultant formation of TFPI/Xa complexes that can bind to and inhibit the functional activity of factor VIIa/TF complexes. A plasma concentration of TFPI in the presence of factor Xa will rapidly and “irreversibly” inhibit all factor VIIa/TF catalytic activity of a reaction mixture containing a limiting concentration of TF and a much higher concentration of factor VIIa. This reflects the final very high affinity binding of TFPI/Xa (the inhibitor complex) to factor VIIa/TF (the enzyme complex). In contrast, adding AT III at a plasma concentration plus 1 U/mL heparin to a reaction mixture containing a limiting TF concentration and a much higher factor VIIa concentration will result in little or no inhibition of factor VIIa/TF activity. Thus, when monolayers of OC-2008 cells (limiting TF source) were overlaid with a reaction mixture in buffer B of factor VIIa (0.5 μg/mL), AT III (100 μg/mL), heparin (1 U/mL), and [3H]factor X and measurement of the time course of activation of factor Xa was begun without delay, the inhibitory effect of AT III/heparin did not become evident until after 30 minutes had elapsed (data not shown). This fits with the observation cited in Results that about 20 minutes was required for a similar concentration of AT III/heparin to inhibit 50% of the factor VIIa activity in a reaction mixture containing 0.55 nmol/L relipidated TF in suspension and 10 nmol/L factor VIIa. These observations led us to conclude that AT III/factor VIIa complexes formed on TF sites can exchange with free factor VIIa. Moreover, it is also conceivable that factor VIIa dissociates much more rapidly from factor VIIa/TF complexes after its reaction with AT III. Factor VIIa activity must be depleted by such continuing exchange/dissociation before factor VIIa/TF catalytic activity is effectively suppressed. For example, in an earlier study from this laboratory in which AT III and heparin were used to block back-activation of factor VII by factor Xa, we found no inhibition of factor VIIa/TF activation of factor X by AT III/heparin. At the time we assumed that this stemmed from the inability of AT III/heparin to inactivate factor VIIa. However, it is now clear that the short time period of the experiment and the presence in the reaction mixture of a 250-fold molar excess of factor VIIa over TF accounted for the result.

It would appear that at least three factors could influence the ability of a given concentration of AT III to inhibit factor VIIa/TF complexes formed during hemostasis. One factor would be the glycosaminoglycan composition of cell surfaces expressing TF. A second factor would be the concentration of factor VII(a) in the milieu of immediate vicinity to the cell surface. A third factor would be the presence in the milieu of other serine proteases of coagulation, eg, thrombin, that could compete for binding to AT III.

The bleeding of hemophilia is thought to result from the failure of factor IXa/factor VIIa/phospholipid complexes to maintain generation of factor Xa during hemostasis after an initial factor VIIa/TF-induced generation of factor Xa is suppressed by TFPI. Infusing rVIIa in doses that increase plasma rVIIa levels to many times the plasma factor VII levels has been shown to control bleeding in patients with hemophilia A refractory to infusion of factor VIII because of factor VIII antibodies. The reason for this is not understood. If, as has been thought, TFPI is the only physiologic inhibitor of factor VIIa/TF that functions during hemostasis, then infusion of rVIIa should not reverse the inhibition because plasma levels of factor VII are more than sufficient to saturate TF sites in an area of tissue injury. However, if the factor VIIa on a fraction of TF sites has been inhibited not by TFPI but by AT III, then increasing the concentration of plasma-free factor VIIa to a very high level could facilitate exchange on TF sites of free factor VIIa for inhibited AT III/factor VIIa complexes.

Trousseau’s syndrome is a complication of malignancy in which a patient develops a continuing low-grade intravascular coagulation and repeated thrombo-embolic episodes. Evidence has been summarized elsewhere to support the hypothesis that Trousseau’s syndrome results from a continuing exposure of the circulating blood to TF expressed on tumor cells. It is now well documented that anticoagulation with heparin can prevent continuing intravascular coagulation and thrombosis in Trousseau’s syndrome, whereas anticoagulation with warfarin, even to levels exceeding recommended therapeutic ranges, fails to prevent new thromboses. One possible reason why heparin but not warfarin could be antithrombotic in Trousseau’s syndrome became apparent with the recently reported evidence that heparin can facilitate TFPI/factor Xa-induced inactivation of factor VIIa/TF through increasing both plasma TFPI levels and TFPI activity. The data presented herein raise the possibility of an additional explanation, which is that heparin would also facilitate the ability of AT III to neutralize the activity of factor VIIa that is bound to TF.

ACKNOWLEDGMENT

The authors thank Dr Bonnie Warn-Cramer and Steve Maki of this laboratory for providing anti-TFPI antibodies used in the present study.

REFERENCES


30. Wun T-C: Lipoprotein associated coagulation inhibitor (LACI) is a cofactor for heparin. Synergistic anticoagulant action between LACI and sulfated polysaccharide. Blood 79:430, 1992
Binding of factor VIIa to tissue factor permits rapid antithrombin III/heparin inhibition of factor VIIa

LV Rao, SI Rapaport and AD Hoang