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Research Article

Human thrombomodulin significantly inhibited the rate of prothrombin conversion to thrombin by Factor Xa in the presence of phospholipid or platelets, calcium, and Factor Va. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of ¹²⁵I-prothrombin activation revealed that thrombomodulin reduced the rate of prothrombin activation but did not alter the cleavage pattern. The inhibition was reversed by the inclusion of a highly specific rabbit antithrombomodulin antibody. If thrombomodulin was replaced by hirudin, the rate of thrombin generation was not decreased excluding the possibility that the inhibition by thrombomodulin was secondary to the binding of small amounts of thrombin formed early in the reaction and the prevention of feedback breakdown of prothrombin by thrombin. The inhibitory activity of thrombomodulin was overcome by increasing the concentration of Factor Xa and specific, saturable binding of thrombomodulin to Factor Xa was demonstrated. These results indicate that thrombomodulin binds to Factor Xa and thereby inhibits the activity of the prothrombinase complex.

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Inhibition by Human Thrombomodulin of Factor X_a-mediated Cleavage of Prothrombin

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Abstract

Human thrombomodulin significantly inhibited the rate of prothrombin conversion to thrombin by Factor X_a in the presence of phospholipid or platelets, calcium, and Factor V_a. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of ¹²⁵I-prothrombin activation revealed that thrombomodulin reduced the rate of prothrombin activation but did not alter the cleavage pattern. The inhibition was reversed by the inclusion of a highly specific rabbit antithrombomodulin antibody. If thrombomodulin was replaced by hirudin, the rate of thrombin generation was not decreased excluding the possibility that the inhibition by thrombomodulin was secondary to the binding of small amounts of thrombin formed early in the reaction and the prevention of feedback breakdown of prothrombin by thrombin. The inhibitory activity of thrombomodulin was overcome by increasing the concentration of Factor X_a and specific, saturable binding of thrombomodulin to Factor X_a was demonstrated. These results indicate that thrombomodulin binds to Factor X_a and thereby inhibits the activity of the prothrombinase complex.

Introduction

Hemostasis is a complex process requiring strict regulation of plasma and cellular proteins for normal function. The formation of thrombin, from the prothrombinase complex, and its subsequent actions, is a focal point in this process (1). The active enzyme in this complex is Factor X_a, which converts prothrombin to thrombin in the presence of calcium and lipid or a suitable surface (platelet or endothelial cells) (1, 2).

Thrombomodulin is an endothelial cell receptor for thrombin (3–5) and it has been well established that thrombin's procoagulant activities such as the clotting of fibrinogen, platelet activation, and the activation of Factor V, are inhibited by complex formation with thrombomodulin (6–8). Thrombin, when complexed to thrombomodulin, has over 1,000-fold greater ability to activate protein C when compared with thrombin alone (5). Activated protein C (APC)¹ functions as a potent natural anticoagulant, inactivating Factors Va (9, 10) and VIIIa (11, 12).

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1. *Abbreviations used in this paper:* APC, activated protein C; NP40, Nonidet P-40; PRP, platelet-rich plasma; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

We recently observed that thrombomodulin inhibits thrombin inactivation of protein S (an important cofactor in the inactivation of Factors Va and VIIIa by APC) (unpublished observations). Thrombin may thus facilitate or, when complexed with thrombomodulin, inhibit the coagulation reaction.

Most published studies on thrombomodulin have focused on its interaction with thrombin. In this report, we examine the effect of thrombomodulin on the ability of Factor X_a to activate prothrombin.

Methods

Materials

Chemicals were purchased from the following suppliers: hirudin, heparin (sodium salt), dithiothreitol, tris, ammonium persulfate and bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO), calcium chloride, sodium chloride and glycine (Ajax Chemicals, Australia), platelin (General Diagnostics, Morris Plains, NJ), acrylamide (Bio-Rad Laboratories Co., Richmond, CA), sodium dodecyl sulfate and iodogen (Pierce Chemical Co., Rockford, IL), nonidet (NP40) (BDH Chemicals Ltd., Poole, England) and D-Phe-pipecolyl-Arg-p-nitroanilide (S2238) (Kabi Diagnostics, Stockholm, Sweden).

Procedures

All proteins used were of human origin. Prothrombin (13), Factor X (14), Factor V (15), thrombin (14), and antithrombin III (16) were purified and/or activated as indicated. Thrombomodulin was prepared by a modification of the previously published procedure (17), replacing the DEAE-Sephacel chromatography by a second diisopropyl fluorophosphate thrombin affinity chromatography as described.

Washed platelets were prepared from whole blood collected into 1/10 vol of 4% disodium EDTA. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 g for 15 min. A platelet pellet was prepared from PRP by centrifugation at 700 g for 10 min. Platelets were washed three times in 20 mM tris buffer, pH 7.4, containing 150 mM NaCl, 5 mM glucose, 6 mM EDTA, and finally resuspended in the same buffer without EDTA.

Prothrombin was labeled with ¹²⁵I-Na (Amersham International, Buckinghamshire, England) using the "iodogen" method (following the manufacturer's instructions). Protein was separated from free iodide on a prepoured Sephadex G-25 column (Pharmacia Fine Chemicals, Uppsala, Sweden). Labeled prothrombin had a specific activity of 3,200 cpm/ng.

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed by the method of Laemmli (18) using 4% stacking gel and 10% acrylamide in the running gel. Samples were reduced by boiling in the presence of 0.5% dithiothreitol for 5 min. After electrophoresis, the gel was fixed, stained with Coomassie Brilliant Blue, dried, and autoradiographed.

Prothrombin activation by Factor X_a. Prothrombin (1.4 μM) was incubated at 37°C in the presence of phospholipid (platelin was prepared according to the manufacturer's instructions and using 100 μl/ml incubation mixture), calcium chloride (10 mM) and Factor X_a (0.05–10 nM). The reaction mixture was adjusted to a final volume of 1 ml using 20 mM tris buffer, pH 7.4, containing 0.15 M NaCl and 5 mg/ml BSA. Aliquots (50 μl) were removed at various time intervals and the amount

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of thrombin formed assayed by measuring the rate of hydrolysis of the chromogenic substrate S2238. The amidolytic reaction (600 μ l) contained 0.2 mM S2238 in the buffer described above. The concentration of thrombin formed was determined by reference to a standard curve constructed using known amounts of thrombin.

Thrombin generation in the presence of thrombomodulin was evaluated by adding thrombomodulin (prepared in 20 mM tris/100 mM NaCl/0.2% NP40 buffer, pH 7.4) or an equal volume of tris/NaCl/NP40 control buffer to the incubation mixture.

In some experiments, Factor V_a was included in the incubation and/or plateletin was replaced with washed platelets.

Antithrombomodulin antibody was obtained from a rabbit immunized with 60 μ g thrombomodulin injected at multiple intradermal sites. The antithrombomodulin IgG was isolated from the rabbit antiserum using protein A-Sepharose.

Factor X_a binding to thrombomodulin. Microtiter plates (Flow Laboratories, Zwamenburg, The Netherlands) were coated with 50 μ l of Factor X_a (80 nM) in 0.1 M NaHCO₃ buffer, pH 8.3, at 4°C for 16 h. The wells were then blocked with 20 mM tris buffer, pH 7.4, containing 50 mM NaCl and 5 mg/ml BSA for 1 h at 37°C. Thrombomodulin (50 μ l) at a final concentration between 30 nM and 550 nM (or buffer as control) was added to the wells and incubated for 1 h in the presence or absence of thrombin (50 μ M). After washing with 20 mM tris buffer, pH 7.4, containing 50 mM NaCl and 0.2% NP40, the wells were incubated with 50 μ l culture supernatant from hybridoma cells secreting an antithrombomodulin monoclonal antibody. Control experiments were also performed using culture supernatant from an antiprotein S hybridoma line.

After 1 h at 37°C, the plates were thoroughly washed with the tris/NP40 buffer and 50 μ l (500,000 cpm/well) of ¹²⁵I-goat anti-mouse IgG (Biorad, Sydney, Australia; affinity purified and labeled by the chloramine T method to a specific activity of 4,800 cpm/ng) was added. The plate was incubated at 37°C for 1 h, washed and the ¹²⁵I-antibody remaining on the plate quantitated (LKB gamma counter, Stockholm, Sweden).

Nonspecific binding was determined by the inclusion of a 100-fold molar excess of unlabeled Factor X_a (8 μ M) and the specific binding was calculated by deducting the nonspecific from the total binding.

Results

The activation of purified prothrombin (1.4 μ M) by Factor X_a (0.05 nM) in the presence of Factor V_a (0.07 nM), calcium and phospholipids is shown in Fig. 1. After a slight lag period, a rapid formation of thrombin amidolytic activity is observed at a rate of 1,000 mol thrombin/mol Factor X_a per min, with all the prothrombin being converted to thrombin after 90 min. Inclusion of thrombomodulin (8 nM) in the incubation mixture produced a significant inhibition in the rate of thrombin generation to 125 mol thrombin/mol Factor X_a per min.

Fig. 2 shows the effects of different thrombomodulin concentrations on the rate of prothrombin activation. In this experiment, Factor V_a was excluded and Factor X_a was used at a concentration of 0.5 nM to obtain a rate of prothrombin activation similar to that in Fig. 1. Only a slight inhibition of thrombin formation was obtained with thrombomodulin values of 0.4 nM or less while concentrations of 1.6 nM thrombomodulin produced >90% inhibition.

The activation products obtained upon Factor X_a action on ¹²⁵I-prothrombin were analyzed by SDS-PAGE (Fig. 3). In the absence of thrombomodulin, the proteolysis of the ¹²⁵I-prothrombin was almost complete by 90 min with the formation of thrombin via two major intermediates, prothrombin 1.2 and prethrombin 2 (19). Thrombomodulin significantly reduced the rate of proteolysis of ¹²⁵I-prothrombin with no accumulation of any of the thrombin intermediates being observed.

To confirm that the observed inhibitory activity was related

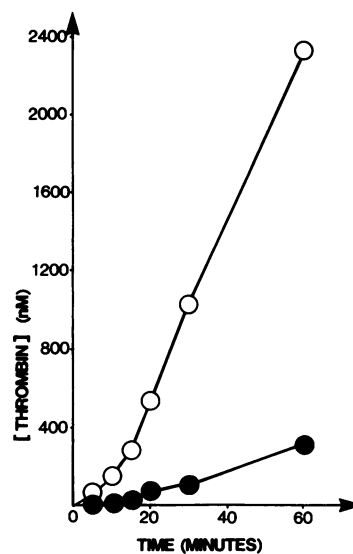


Figure 1. Inhibition of thrombin production by thrombomodulin. Reaction mixtures contained prothrombin (1.4 μ M), phospholipid (plateletin) 100 μ l, CaCl₂ (10 mM), and Factor V_a (0.07 nM) in the presence ● and absence ○ of thrombomodulin (8 nM). The buffer used was 0.02 M tris, pH 7.4, containing 0.15 M NaCl and 5 mg/ml BSA at a final incubation volume of 1 ml. The reaction was started by the addition of Factor X_a (0.05 nM) at 37°C and amidolytic activity measured at the indicated time intervals.

to thrombomodulin and not a minor contaminant in the preparation, the effect of a rabbit antithrombomodulin antibody was examined. This antibody is highly specific for thrombomodulin as previously reported. Fig. 4 shows that the inclusion of the antibody reduced the inhibitory activity of thrombomodulin on prothrombin activation. A higher concentration of antithrombomodulin IgG completely blocked the ability of thrombomodulin to inhibit thrombin formation (data not shown). Control IgG, on the other hand, had no effect. These studies confirm that the inhibition of prothrombin cleavage is in fact related to thrombomodulin.

Thrombomodulin forms a 1:1 molar complex with thrombin. The effects of thrombomodulin on prothrombin activation could be related to the binding and subsequent inactivation of small amounts of thrombin formed early in the course of the reaction

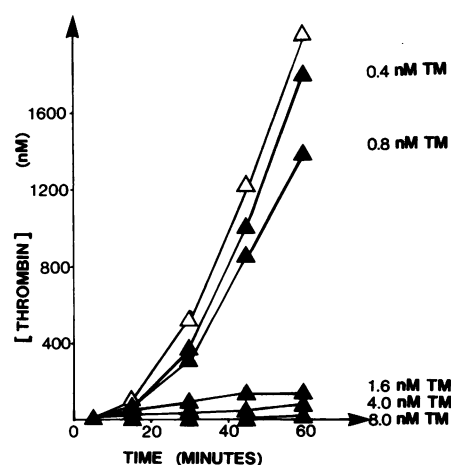


Figure 2. Effect of thrombomodulin concentration on thrombin generation. Prothrombin (1.4 μ M) was incubated at 37°C with washed platelets (5×10^6 /ml) in the absence Δ or presence of thrombomodulin (TM): 0.4; 0.8; 1.6; 4.0; 8.0 nM \blacktriangle . The buffer used was 0.02 M tris, pH 7.4, containing 0.15 M NaCl, 5 mg/ml BSA and CaCl₂ (10 mM) at a final incubation volume of 1 ml. The reaction was started with Factor X_a (0.5 nM) and the amidolytic activity measured at the indicated time intervals.

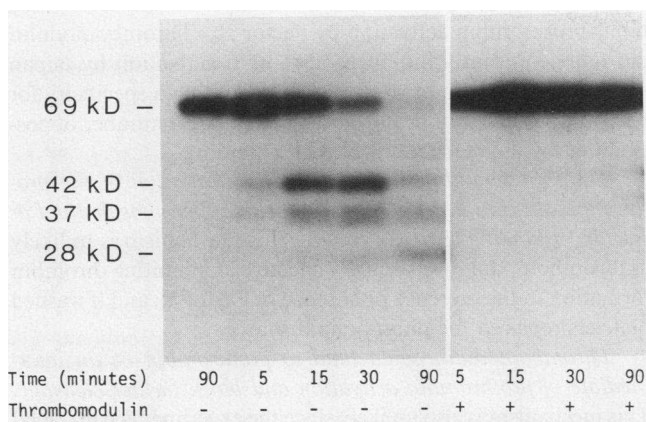


Figure 3. The effect of thrombomodulin on the activation of ^{125}I -prothrombin by Factor X_a . Cleavage of ^{125}I -prothrombin (5.5 nM) and cold prothrombin (1.4 μM) by Factor X_a (0.5 nM) was studied at 37°C in the presence of platelin and Factor V_a (0.07 nM). The buffer used was 0.02 M tris, pH 7.4, containing 0.15 M NaCl, 5 mg/ml BSA, and CaCl_2 (10 mM). At the indicated times, 50- μl samples were removed and reduced by boiling as described in Methods and 50,000 cpm applied to each well. (Lane 1) ^{125}I -prothrombin at 90'. Reactions were performed in the presence (lanes 2–5) or absence of 8 nM thrombomodulin (lanes 6–9) after 5, 15, 30, and 60 min incubation, respectively.

and thus prevention of feedback autocatalysis of prothrombin by thrombin. To address this possibility, thrombomodulin was replaced by the specific thrombin inhibitor, hirudin (40 U/ml, a concentration that is capable of inhibiting ~ 400 nM thrombin). Fig. 5 shows that prothrombin cleavage was not decreased in the presence of hirudin, thus excluding the possibility that the inhibitory effects of thrombomodulin were secondary to

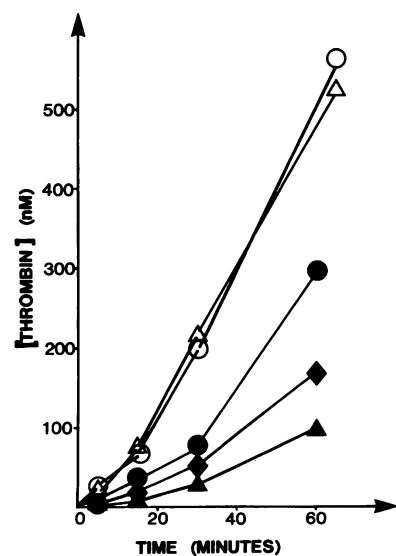


Figure 4. Effect of antithrombomodulin antibody on thrombomodulin inhibition of thrombin generation. Activation of prothrombin (1.4 μM) with 0.5 nM Factor X_a was performed as described in Methods. Control Δ . Control plus antithrombomodulin IgG (1.3 μM) \circ . Thrombomodulin (8 nM) \blacktriangle . Thrombomodulin plus antithrombomodulin IgG (0.3 μM) \blacklozenge . Thrombomodulin plus antithrombomodulin IgG (1.3 μM) \bullet .

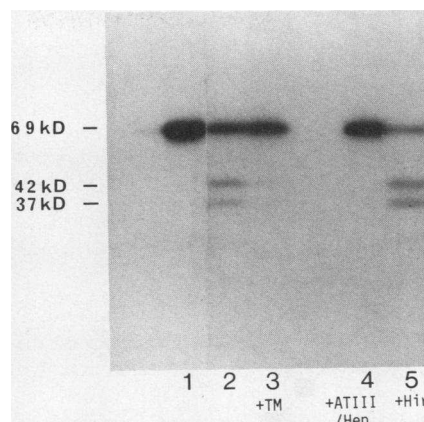


Figure 5. Effect of thrombin inhibitors and thrombomodulin on prothrombin activation by Factor X_a . Cleavage of ^{125}I -prothrombin (5.5 nM) and cold prothrombin (1.4 μM) by Factor X_a (0.5 nM), in the presence or absence of various thrombin inhibitors was studied at 37°C. The buffer used was 0.02 M tris, pH 7.4, containing 0.15 M NaCl, 5 mg/ml BSA, CaCl_2 , and platelin. At 120 min, 50- μl samples were removed and reduced by boiling and subjected to SDS-PAGE. (1) ^{125}I -prothrombin alone. (2) ^{125}I -prothrombin plus Factor X_a (0.5 nM). (3) ^{125}I -prothrombin plus Factor X_a plus thrombomodulin (8 nM). (4) ^{125}I -prothrombin plus Factor X_a plus antithrombin III (0.82 μM) and heparin (0.28 U/ml). (5) ^{125}I -prothrombin + Factor X_a + hirudin (40 U/ml).

binding of the small amounts of thrombin formed early in the course of the reaction.

On the other hand, if antithrombin III (0.82 μM) and heparin (0.28 U/ml) were included (inhibiting both thrombin and Factor X_a), total inhibition of prothrombin conversion is observed similar to that obtained with high concentrations of thrombomodulin.

Effect of increasing Factor X_a concentration on the inhibition of thrombin generation by thrombomodulin. Thrombin generation was measured following a 60-min incubation of prothrombin with various Factor X_a concentrations in the presence or absence of thrombomodulin. The inhibition by thrombomodulin was expressed as a percentage of the control. Fig. 6 shows that thrombomodulin inhibitory activity decreased as the concentration of the enzyme Factor X_a was increased. In the experiment, thrombomodulin was used at a concentration of 8 nM and it can be seen that maximum thrombomodulin inhibition was obtained at Factor X_a concentrations < 2.0 nM while only 2% inhibition was found when 10 nM Factor X_a was used.

Factor X_a binding to thrombomodulin. The finding that thrombomodulin inhibition of prothrombin activation is decreased as the concentration of Factor X_a increased suggests that thrombomodulin exerts its inhibitory activity via binding to Factor X_a . Fig. 7 demonstrates that indeed thrombomodulin (50–550 nM) binds to Factor X_a (80 nM) and the binding is specific and saturable. When Factor X instead of Factor X_a was used, no binding could be demonstrated and similarly, specific binding was not observed when the culture supernatant containing antithrombomodulin antibody was replaced with supernatant from an anti-protein S hybridoma line.

To determine the relationship between the Factor X_a and thrombin binding sites on thrombomodulin, binding studies were performed in the presence of 100-fold molar excess of thrombin.

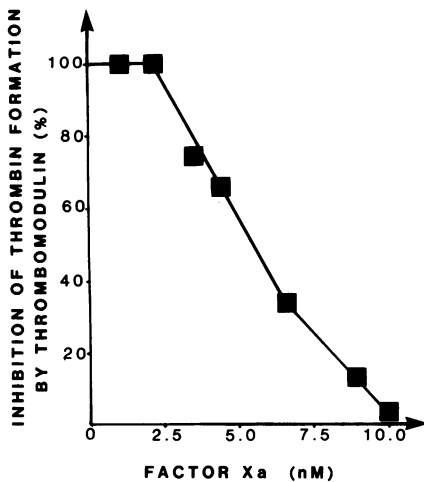


Figure 6. Effect of increasing Factor X_a concentration on thrombomodulin inhibition of thrombin generation. Activation of prothrombin (1.4 μM) in the presence of platelin and calcium was performed as described in Methods using various Factor X_a concentrations (0.1–10 nM) in the presence and absence of thrombomodulin (8 nM). After 60 min, the thrombin amidolytic activity was measured and the results expressed as percentage inhibition of control values.

As shown in Fig. 7, thrombin abolished the binding of thrombomodulin to Factor X_a, suggesting that both thrombin and Factor X_a bind to the same or very close sites on thrombomodulin.

Discussion

Prothrombin is converted to thrombin by the serine protease, Factor X_a via the intermediates prothrombin 1.2 and prethrombin 2, in a calcium-dependent reaction (19). The very slow reaction rate is increased 20,000-fold by the inclusion of phospholipid and the cofactor, Factor V_a (14).

The studies presented here show that thrombomodulin in-

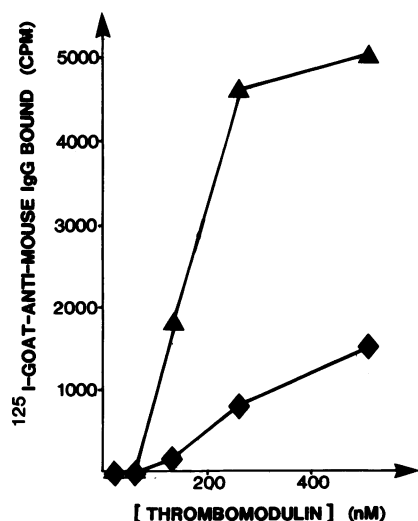


Figure 7. Specific binding of thrombomodulin to Factor X_a in the presence or absence of thrombin. See Methods for experimental details. Thrombomodulin alone ▲. Thrombomodulin plus thrombin (50 μM) ♦.

hibits prothrombin activation by Factor X_a. Thrombomodulin did not significantly inhibit prothrombin activation by taipan snake venom (data not shown), demonstrating a specificity for Factor X_a cleavage of prothrombin. There are a number of possibilities for the mechanism of this inhibition.

Thrombomodulin could prevent the formation of the prothrombinase complex by interfering with either phospholipid or Factor V_a binding to prothrombin. This mechanism is unlikely as thrombomodulin was equally effective at inhibiting thrombin formation in the presence or absence of Factor V_a and if washed platelets replaced the phospholipid source.

Thrombomodulin could bind to prothrombin or an intermediate of prothrombin activation and block further cleavage. This mechanism is also unlikely since the experiments performed used a 100-fold molar excess of prothrombin to thrombomodulin. Furthermore, analysis of prothrombin activation products by SDS-PAGE showed no alteration in the cleavage pattern nor accumulation of the intermediates, prothrombin 1.2 or prethrombin 2 in the presence of thrombomodulin.

The inhibitory effect of thrombomodulin could be secondary to binding of small amounts of thrombin formed early in the course of the reaction and prevention of feedback autocatalysis of prothrombin by thrombin. This possibility was excluded since inclusion of hirudin, a specific thrombin inhibitor, did not alter prothrombin activation by Factor X_a (Fig. 7). Furthermore, cleavage of prothrombin by thrombin, in concentrations up to 10 U/ml (100 nM), could not be demonstrated under the experimental conditions used (data not shown).

Thrombomodulin could bind Factor X_a in a manner that prevents the protease from binding to or cleaving prothrombin. Several lines of evidence support the suggestion that inhibition of prothrombin activation by thrombomodulin is via interaction with Factor X_a. (a) The inhibition could be reversed by increasing the concentration of Factor X_a. An increase in Factor X_a concentration produced a decrease in the inhibition observed with thrombomodulin. (b) Specific and saturable binding of thrombomodulin to Factor X_a could be demonstrated in a microtiter plate assay.

Thrombin and Factor X_a are structurally similar with sequence homology between the β-chain of thrombin and the β form of the heavy chain of Factor X_a, both containing the active site amino acid residues—serine, histidine, aspartic acid, and aspartic acid in homologous positions (1, 20). It is of interest that the binding of thrombomodulin to Factor X_a was inhibited by a 100-fold molar excess of thrombin. Based on these results, one can conjecture that the binding site of Factor X_a on thrombomodulin is identical or in close proximity to the thrombin binding site. Full binding studies to ascertain the exact nature of the thrombomodulin binding sites on thrombin and Factor X_a and the relative binding affinities are in progress. Whether other vitamin K-dependent clotting factors also interact with thrombomodulin remains to be determined.

The relevance of our observation that thrombomodulin can inhibit the procoagulant activity of Factor X_a is emphasized by the reports of Rogers and Shuman (21, 22) that prothrombin can be activated on the surface of vascular endothelium by Factor X_a. The presence of thrombomodulin at these sites may represent a local regulatory mechanism thus avoiding excess thrombin formation.

It has become evident that the role of thrombomodulin in the modulation of hemostasis is significantly more complex than

originally suspected. The anticoagulant activities of thrombomodulin include acceleration of thrombin-dependent protein C activation by over 1,000-fold, inhibition of thrombin's ability to clot fibrinogen, activate platelets and activate Factor V, the inhibition of thrombin inactivation of protein S and as demonstrated in this study, thrombomodulin reduces thrombin generation itself by inhibiting Factor X_a cleavage of prothrombin. By acting at different levels, thrombomodulin thus serves as a potent modulator of the coagulation system.

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