Both Cellular and Soluble Forms of Thrombomodulin Inhibit Fibrinolysis by Potentiating the Activation of Thrombin-activable Fibrinolysis Inhibitor*

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Thrombin-activable fibrinolysis inhibitor (TAFI) is a recently described plasma zymogen that can be activated by thrombin to an enzyme with carboxypeptidase B-like activity. The enzyme, TAFIa, potentially attenuates fibrinolysis. TAFI activation, like protein C activation, is augmented about 1250-fold by thrombomodulin (TM). In this work, the effects of both soluble and cellular forms of TM on TAFI activation-dependent suppression of fibrinolysis were investigated. Soluble TM included in clots formed from purified components, barium citrate-adsorbed plasma, or normal human plasma maximally increased the tissue plasminogen activator-induced lysis time 2–3-fold, with saturation occurring at 5, 10, and 1 nM TM in the three respective systems. Soluble TM did not effect lysis in the system of purified components lacking TAFI or in plasmas immunodepleted of TAFI. In addition, the antifibrinolytic effect of TM was negated by monoclonal antibodies against either TM or TAFI. The inhibition of fibrinolysis by cellular TM was assessed by forming clots in dialyzed, barium citrate-adsorbed, or normal plasma over cultured human umbilical vein endothelial cells (HUVECs). Tissue plasminogen activator-induced lysis time was increased 2-fold, with both plasmas, in the presence of HUVECs. The antifibrinolytic effect of HUVECs was abolished 66% by specific anti-TAFI or anti-TM monoclonal antibodies. A newly developed functional assay demonstrated that HUVECs potentiate the thrombin-catalyzed, TM-dependent formation of activated TAFI. Thus, endothelial cell TM, in vitro at least, appears to participate in the regulation of not only coagulation but also fibrinolysis.

Thrombin and plasmin are the terminal proteases formed by the coagulation and fibrinolytic pathways (1, 2). Thrombin catalyzes the cleavage of fibrinogen to form a fibrin clot, while plasmin catalyzes the proteolysis of fibrin and subsequent clot degradation. Since both proteases appear to work antagonistically, the coagulation and the fibrinolytic systems presumably must be tightly regulated and interconnected. The failure to form a stable clot or its unregulated removal could result in exsanguination in the event of injury, whereas the failure to remove a clot or prevent its continuous formation could lead to thrombosis, heart attack, or stroke.

Thrombin regulates its own generation via the protein C pathway (3–5). Thrombin, in complex with thrombomodulin, catalyzes the proteolytic activation of protein C to produce the serine protease-activated protein C. Activated protein C attenuates further formation of thrombin by proteolytically inactivating two essential cofactors involved in the coagulation system, factor Va and factor VIIIa (6–8). In addition, high concentrations of thrombin can activate a thrombin-activable fibrinolysis inhibitor (TAFI)1 to activated TAFI (TAFIa), which subsequently inhibits fibrinolysis (9, 10). Therefore, activated protein C can promote fibrinolysis by attenuating the production of thrombin, thereby limiting the activation of TAFI and consequently enhancing fibrinolysis (9–12).

TAFIa is a carboxypeptidase B-like enzyme that is alternatively referred to as carboxypeptidase U (13) or plasma carboxypeptidase B (14). Activated TAFI probably inhibits fibrinolysis by catalyzing the removal of carboxy-terminal lysine residues produced by partial proteolysis of fibrin by plasmin (10, 15). Carboxy-terminal lysines augment the cofactor activity of fibrin in the tissue plasminogen activator (tPA)-mediated activation of plasminogen (16, 17). The ability of TAFIa to inhibit plasminogen activation is not exhibited with the plasmin-truncated form of plasminogen, Lys-plasminogen (10). Other means by which the antifibrinolytic effect of TAFIa might be attenuated include spontaneous decay (13, 15) and proteolytic inactivation by thrombin (10, 14) and plasmin (14, 18).

We recently demonstrated that the thrombin-catalyzed activation of TAFI is enhanced by a recombinant soluble form of thrombomodulin (15). The proposed mechanism, for which the kinetic parameters included $K_m = 1.0 \mu M$ and $k_{cat} = 1.24 \text{ s}^{-1}$, allows for the formation of the binary complexes thrombin-thrombomodulin and thrombin-TAFI, and subsequent formation of the thrombin-thrombomodulin-TAFI complex. Because protein C and TAFI are both substrates for thrombin-thrombomodulin, thrombomodulin may regulate not only coagulation, through activation of the protein C pathway, but also fibrinolysis, through the enhancement of TAFI activation. Thus, thrombomodulin potentially couples and regulates both the coagulation and fibrinolytic cascades. This possibility was tested in the present studies. The results indicate that a soluble form of thrombomodulin prolongs the lysis time of clots formed from both purified components and human plasma. These ef-

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1 The abbreviations used are: TAFI, Thrombin-activable fibrinolysis inhibitor; TAFIa, activated TAFI; tPA, tissue plasminogen activator; mAb, monoclonal antibody; PC, phosphatidylcholine; PS, phosphatidylserine; NHP, normal human plasma; BAP, barium citrate-adsorbed plasma; TdNHP, TAFI-deficient NHP; TdBAP, TAFI-deficient BAP, rTdBAP, TdBAP reconstituted with 50 nM TAFI.
Effects require TAFI activation. In addition, thrombomodulin expressed on HUVECs potentiates the activation of TAFI, thereby retarding the lysis of clots formed in their presence. These data indicate that the thrombomodulin-thrombin complex is relevant as a regulator not only of protein C activation but also of TAFI activation, thereby contributing to the downregulation of both coagulation and fibrinolysis.

**Experimental Procedures**

**Proteins and Reagents**—Human proteins including fibrinogen (330 kDa, \( \varepsilon \text{16.0} \)), plasminogen, (91 kDa, \( \varepsilon \text{16.1} \)), protein C (100 nM), thrombin, (98 kDa, \( \varepsilon \text{14.5} \)), antithrombin III, (57 kDa, \( \varepsilon \text{7.0} \)), recombinant \( \alpha \)2-antiplasmin, (65 kDa, \( \varepsilon \text{26.4} \)), TAFI (48 kDa, \( \varepsilon \text{26.4} \)), and factor V (330 kDa, \( \varepsilon \text{9.6} \)) were purified and activated, when required, as described previously (10). The activity of thrombin was 2900 units/mg. Recombinant tPA was a generous gift of Dr. Gordon Vehar of Genentech (S. San Francisco, CA). Recombinant soluble thrombomodulin (Solulin) and mAb TM-531 were gifts provided by Berlex Biosciences (Richmond, CA). Vesicles of phosphatidylycholine/phosphatidylserine (PC/PS; 3:1) were prepared as described by Barenholz et al. (19). The fluorescent substrate 11,12-dihydroxy-9,10-epoxy-9,10-dihydro-stearic acid was provided by Drs. S. Butenas and K. G. Mann of the University of Vermont, and placenta were kindly provided by the Birthing Ward at Fletcher Allen Medical Center (Burlington, VT). All other reagents were of analytical quality.

**Measurement of Modulation of the Fibrinolytic Cascade**—The degradation of fibrin by the actions of the fibrinolytic cascade and modulation of the process by substances such as TAFI, thrombomodulin, antibodies directed against them, and protein C were assessed by lysis assays, using either purified components or various plasmas as the source of required components, as described previously (10, 20). In the system of purified components, a solution was prepared in 20 mM HEPES, pH 7.4, 0.15 M NaCl containing fibrinogen (2.7 μM), plasminogen (0.6 mM), PC/PS (10 μM), thrombin (0.7 μM), factor V (8.3 nM), antithrombin III (1.3 μM), \( \alpha \)2-antiplasmin (0.5 μM), and, when included, TAFI (30 nM) and protein C (100 nM). To assess fibrinolysis, aliquots (100 μL) were pipetted into the wells of a microtiter plate containing separated, small aliquots of thrombin (6.0 μM final concentration), tPA (294 pM final) and Ca2+ (10 mM final). Immediate clotting and subsequent fibrinolysis were monitored over time by continual absorbance (turbidity) measurements at 405 nm, at 37 °C, in a Thermomax plate reader (Molecular Devices, Sunnyvale, CA). Lysis time is defined as the time to the midpoint of the turbid-to-clear transition that characterizes the lysis of fibrin. In plasma-based assays, the solution of purified components was replaced with one of several plasmas, diluted 1:3 (\( A_{\text{μM}} = 16.0 \)). The plasmas included normal human plasma (NHP), barium citrate-adsorbed plasma (BAP), and TAFI-deficient samples of NHP (TdBAP) and BAP (TdBAP). NHP and BAP were prepared as described previously (20) from fresh frozen human plasma that had been thawed at 37 °C for 16 h. Wells were washed 0.15 M NaCl, pH 7.4, 50 units/ml penicillin, and 50 μg/ml streptomycin. Collagenase was then maintained within the vein for 15 min, and released endothelial cells were recovered. The vein was then rinsed with Hanks’ buffered salt solution containing 10% heat-inactivated fetal bovine serum. The rinsed solution was combined with the collagenase extract. Cells were then pelleted and resuspended in M-199 medium (Sigma) with 20% fetal bovine serum at 105 cells/ml. Wells of a 24-well plate (Falcon from VWR) were seeded with 100,000 or 10,000 HUVECs/well, respectively, and maintained in M-199 and either TAFI (400 nM) or protein C (100 nM). An aliquot (200 μL) was then added to washed HUVECs that had been grown to confluence in the wells of a 24-well plate (Falcon from VWR). The mixture was incubated at 37 °C, and at regular intervals 15-μl aliquots were withdrawn and placed on ice. At the end of the experiment, 10-μl aliquots of the samples were added to 190 μl of TdNHP supplemented with 10 μM PC/PS vesicles. Two 100-μl aliquots of these samples were then added

**Assessment of Fibrinolysis**—Plasmas that included BAP, TdBAP, NHP, or TdNHP were diluted 2:3 with HBS, and tPA was added (183 pM final concentration to either of the barium citrate-adsorbed plasma samples or 367 pM final concentration to either of the normal human plasma samples). After rinsing the wells of a 96-well microtiter plate containing the confluent monolayer of HUVECs with HBS, an aliquot (25 μL) containing both thrombin (6 nM, final concentration) and CaCl2 (10 mM, final) in HBS was added to each well, immediately before the addition of 25 μL of 2:3 diluted plasma. Clot formation and subsequent fibrinolysis thus were initiated by the addition of the plasma to the well. Turbidity, monitored at 405 nm, was determined at 2.5-min intervals using a Thermomax plate reader (Molecular Devices, Sunnyvale, CA) maintained at 37 °C. Lysis time was defined as the time required to achieve a 50% reduction in the maximum absorbance produced by the initial formation of a fibrin clot. Either mAb TAF16 or mAb TM-531 (100 nM, final concentration), when required, was added to the plasma prior to its addition to each well to verify both TAFI- and thrombomodulin-dependent prolongation of lysis times.

**Assay of TAFI**—Samples (10 μL) containing TAFI were added to 190 μl of TdNHP, diluted 1:3 (\( A_{\text{μM}} = 16.0 \)), containing PC/PS vesicles (10 μL). One hundred μl of the mixture was then pipetted into the well of a microtiter plate that contained separated, 2-μl aliquots of thrombin, activated protein C, tPA, and CaCl2. Their respective final concentrations were 6.0 nM, 10 nM, 294 pM, and 5.0 mM. Turbidity was continually monitored at 405 nm and 37 °C in a Thermomax plate reader, and lysis time was determined as described previously (10, 20). Activated TAFI was produced from purified TAFI (1.0 μg) by incubating it at 22 °C for 15 minutes in a solution of 0.02 M HEPES, 0.15 M NaCl, 5.0 mM CaCl2, pH 7.4, containing thrombin (200 nM) and soluble thrombomodulin (50 nM). The sample was serially diluted to generate the standard curve. The standard curve is approximately linear up to 2.0 nM TAFI and then tends toward saturation at higher TAFI concentrations (Fig. 1). The lower and upper limits of lysis times are 60 and 185 min under these conditions. Because activated protein C is included in the assay, endogenous thrombin formation in the plasma is prevented (9, 10); therefore, unactivated TAFI is not converted to TAFI by the assay during the assay. As a consequence, the assay is specific for TAFI, and mixtures of TAFI and TAFI can be assessed without interference from the latter.

**The Activation of TAFI and Protein C on HUVECs**—A solution was prepared containing HBS, CaCl2 (5.0 mM), bovine serum albumin (1% w/v), and either TAFI (400 nM) or protein C (100 nM). An aliquot (200 μL) was then added to washed HUVECs that had been grown to confluence in the wells of a 24-well plate (Falcon from VWR). The mixture was incubated at 37 °C, and at regular intervals 15-μl aliquots were withdrawn and placed on ice. At the end of the experiment, 10-μl aliquots of the samples were added to 190 μl of TdNHP supplemented with 10 μM PC/PS vesicles. Two 100-μl aliquots of these samples were then added

**FIG. 1. Standard curve for quantitation of TAFI.** Lysis times were determined for clots formed in TdBAP in the presence of 10 nM activated protein C. TAFI was included at the concentrations indicated. Lysis times exhibited a TAFIs concentration-dependent prolongation. TAFI concentrations in various samples were determined by reference to this curve. Activated protein C was included to prevent quantitative activation of prothrombin in situ, which would contribute to the further activation of TAFI in samples that might contain both TAFI and TAFI.

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protein C, the ability of the latter to competitively inhibit TAFI activation was assessed by comparing the effect of soluble thrombomodulin on lysis time in the presence and absence of protein C at its plasma concentration (Fig. 2). The data demonstrated that protein C had negligible effect, indicating that thrombomodulin was able to prolong lysis time in a TAFI-dependent manner despite the presence of the alternate substrate at its plasma concentration. The substrate concentration dependence for a substrate S is given by the equation, $r = k_{cat}(E_0)[S_1]/(1 + [S_1]/K_{m1} + [S_2]/K_{m2})$. If $[S_1] \ll K_{m1}$, which is the case here for protein C ($K_{m1} \approx 5.0$ nM, [PC] = 0.1 μM; Refs. 24 and 25), then the effect of the competing substrate on the rate of conversion of the first substrate is very small.

Similar results (Fig. 3) were obtained in assessing the effect of thrombomodulin on the lysis times of clots formed from BAP, TdBAP, and TdBAP reconstituted with 50 nM purified TAFI (rTdBAP). In this system, the vitamin K-dependent proteins are absent, and prothrombin activation cannot occur during the lysis assay. Again, by increasing the concentration of thrombomodulin in the assay (from 0 to 20 nM), lysis times of clots formed from BAP or rTdBAP minimally doubled (from 60 to either 150 or 135 min, respectively), whereas thrombomodulin had no effect on the lysis time of clots formed from TdBAP. The doubling of lysis time observed was comparable with that observed in a system comprising purified components (Fig. 2). The dependence of lysis time on the thrombomodulin concentration exhibited saturation between 5 and 10 nM (EC_{50} = 1.5 nM). These data show that in a plasma-based system thrombomodulin prolongs lysis time through a mechanism that is TAFI-dependent and does not require the production of high concentrations of thrombin in situ.

To determine whether thrombomodulin prolongs clot lysis time through a TAFI-dependent mechanism, two monoclonal antibodies, mAb TAFI16 and mAb TM-531, were examined for their ability to inhibit both the TAFI- and the thrombomodulin-dependent prolongation of lysis time. mAb TAFI16 specifically blocks the activation of TAFI by both thrombin and the thrombin-thrombomodulin complex (12), whereas mAb TM-531 inhibits protein C activation catalyzed by the thrombin-thrombomodulin complex. mAb TAFI16 (100 nM) inhibited the prolongation in lysis time produced by 20 nM thrombomodulin.

FIG. 2. The effect of thrombomodulin on lysis times of clots formed from purified components. Lysis times were determined for clots produced in a system of purified human components present at approximately 1/3 their plasma concentrations and supplemented with 10 μl PC/PS vesicles, in the presence (open symbols) and absence (closed symbols) of 30 nM TAFI. To determine whether TAFI and protein C compete in their thrombin-thrombomodulin-dependent activation, lysis times were determined in the presence (○, □) and absence (○, ●) of 100 nM protein C. No competition for TAFI activation by protein C was evident.

RESULTS

Thrombomodulin Prolongs the Lysis Time of Clots Formed from Purified Plasma-derived Components or BAP in the Presence of TAFI—Fibrin clots were formed from a solution comprising purified components including fibrinogen, plasminogen, prothrombin, factor V, antithrombin III, and α_{2}-antiplasmin at approximately 1/3 their respective plasma concentrations. These experiments were performed in the presence of PC/PS vesicles and of soluble thrombomodulin at various concentrations. The tPA-induced lysis times were then determined (Fig. 2). Because factor Xa is absent in this system, prothrombin activation cannot occur. Thus, the only thrombin present is that used to initiate clot formation. In the absence of thrombomodulin, TAFI elicited a modest increase in lysis time (from 36 to 50 min), most likely due to its partial activation during the lysis assay. Notably, however, the addition of as little as 2.5 nM thrombomodulin to the assay system caused an approximately doubling of the lysis time to 100 min. The maximum increase in lysis time was observed at 5 nM thrombomodulin, and an EC_{50} value of 1.0 nM was obtained. In the absence of TAFI, however, lysis time was unaffected by thrombomodulin at any concentration tested.

Since the thrombin-thrombomodulin complex also activates...
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**Fig. 4.** Thrombomodulin and TAFI-dependent prolongation of lysis times of clots formed from BAP. A, hatched bars indicate lysis times of clots formed from BAP, TdBAP, and rTdBAP in the absence of thrombomodulin. The effects on lysis times of 20 nM thrombomodulin are depicted by the filled bars, and lysis times in the presence of both 20 nM thrombomodulin and 100 nM mAb TAFI16 are represented by the open bars. Shown in Fig. 4B are the effects of mAb TM-531 on lysis times of clots formed from BAP in the presence (●) and absence (○) of 20 nM thrombomodulin.

in both BAP and rTdBAP (Fig. 4A) without affecting the lysis time of TdBAP in both the presence and absence of thrombomodulin. Similarly, mAb TM-531 at approximately 25 nM completely neutralized the inhibitory effects of 20 nM thrombomodulin but had no effect on lysis time in the absence of thrombomodulin (Fig. 4B). These data indicate that mAb TAFI16 and mAb TM-531 specifically inhibit TAFI- and thrombomodulin-dependent prolongation of lysis times and are, therefore, suitable tools to assess the contributions of TAFI and thrombomodulin to lysis times in complex systems.

**Thrombomodulin Increases Lysis Times of Clots Formed from NHP—**Thrombomodulin prolongs lysis times of clots formed in a defined system comprising purified components both in the absence and presence of protein C at its plasma concentration, as well as in clots formed from BAP (plasma depleted of the vitamin K-dependent proteins including protein C). The effect of protein C on thrombomodulin-dependent prolongation of lysis times in clots formed from normal human plasma, however, is unknown. The results, shown in Fig. 5, indicate that as little as 1 nM thrombomodulin increases the lysis time from 120 to 180 min maximally in normal human plasma. Notably, in the absence of thrombomodulin, the lysis time was 120 min, which is twice as long as that observed in BAP in the absence of thrombomodulin (Fig. 4A). This observation is consistent with partial activation of TAFI by thrombin at the high concentrations formed during the lysis assay in clots formed from NHP but not in clots formed from BAP (9, 26). Again, the effect of thrombomodulin in clots formed from NHP was eliminated by mAb TAFI16 (100 nM), indicating the dependence of this effect on TAFI.

This shows that the lysis time of clots formed from NHP is prolonged in a TAFI-dependent manner by thrombomodulin. Most likely, the formation of additional thrombin in NHP during the assay contributes to the activation of TAFI and reduces the requirement for thrombomodulin. This is reflected in the observation that both the EC₅₀ (0.5 nM) and the concentration of thrombomodulin required to achieve saturation are reduced when compared with those determined for clots produced from BAP. Furthermore, protein C (present in NHP but not in BAP) did not negate the effect of thrombomodulin by acting as a competing substrate for the thrombin-thrombomodulin complex.

**Cultured HUVECs Inhibit Fibrinolysis through a Mechanism Dependent on both TAFI and Thrombomodulin—**Although soluble thrombomodulin prolongs lysis times in a TAFI-dependent manner, no evidence exists indicating that cellular thrombomodulin is capable of inducing a similar response. Therefore, cultured HUVECs, as a cellular source of thrombomodulin, were examined for their effects on lysis times of clots formed from either BAP or TdBAP. BAP and TdBAP, rather than NHP and TdNHP, were used in initial experiments to avoid generation of additional thrombin during the experiment, which would contribute to TAFI activation and perhaps obscure the effects of cellular thrombomodulin on lysis times. Data shown in Table I indicate that HUVECs prolong lysis times of clots formed from BAP (containing TAFI) from 50 to 115 min. The data also indicate that the observed prolongation of lysis time is attenuated by both mAb TAFI16 and mAb TM-531, either individually or in combination. The antibodies did not affect the lysis times of clots formed in the absence of HUVECs. This indicates that the effect of HUVECs on lysis time is both TAFI- and thrombomodulin-dependent. Further-
Table I

Effect of cultured HUVECs on tPA-induced fibrinolysis

Lysis times of tPA (183 pm)-induced fibrinolysis were determined for clots made from either BAP or TdBAP, supplemented with 5 mM Ca\(^{2+}\), in the presence and absence of cultured HUVECs. TAFI and thrombomodulin dependence was assessed by determining the lysis times in the absence and presence of either mAb TAFI16 (100 nM) or mAb TM531 (100 nM), alone or in combination.

| Conditions                  | Lysis time ± S.E. (n = 6) |
|-----------------------------|---------------------------|
| No HUVECs                   |                           |
| BAP                         | 45 ± 2                    | 60 ± 5                     |
| BAP and mAb TAFI16          | 40 ± 4                    | 62 ± 7                     |
| BAP and mAb TM531           | 45 ± 3                    | 58 ± 4                     |
| TdBAP                       | 38 ± 4                    | 60 ± 5                     |
| TdBAP and mAb TAFI16        | 42 ± 4                    | 50 ± 6                     |
| TdBAP and mAb TM531         | 40 ± 4                    | 55 ± 7                     |
| TdBAP and both mAbs         | 40 ± 6                    | 53 ± 4                     |

more, since the lysis time observed in the presence of both antibodies was identical to that observed in the presence of each antibody individually, prolongation of lysis time involves a single pathway including both TAFI and thrombomodulin.

To determine whether the antibodies influence the HUVECs in some undefined way and thereby shorten the lysis time independently of TAFI, clots were formed from TdBAP, rather than BAP, and lysis times were determined in the presence and absence of both HUVECs and the antibodies (Table I). In TdBAP, a slight prolongation in lysis time was observed in the presence of HUVECs (38–60 min), most likely because the release of plasminogen activator inhibitor-1 from the cultured endothelial cells (27, 28). This modest prolongation, however, was not due to either TAFI or thrombomodulin, since neither mAb TAFI16 nor mAb TM-531 alone, or in combination, overcame this effect. In addition, since the magnitude of the prolongation in lysis time of clots formed from TdBAP (22 min) was substantially less than that observed in clots formed from BAP (65 min), the majority of the prolongation of the lysis time by HUVECs (−60%) was dependent on both TAFI and thrombomodulin.

These experiments were repeated (n = 6) with NHP and TdBHP with the tissue plasminogen activator concentration doubled to achieve lysis in under 3 h. Under these conditions, the lysis time of clots formed from NHP in the presence of HUVECs (154 ± 40 min) was prolonged in a TAFI- and thrombomodulin-dependent manner, as was demonstrated by the decreased lysis times obtained with both mAb TAFI16 and mAb TM-531, alone or in combination, individually, or both antibodies in combination (40 ± 9 min). The finding that both antibodies together exhibited a slightly shorter lysis time than either alone probably reflects less than complete saturation by the antibodies when used individually. The average lysis time of clots formed in TdBHP was 40 ± 6 min regardless of the presence or absence of both HUVECs and either antibody, indicating that the prolongation of lysis time is dependent on TAFI. In addition, these data indicate that the thrombomodulin of HUVECs is able to prolong lysis time in clots formed from human plasma even in the presence of protein C.

The Thrombomodulin-dependent Activation of TAFI on HUVECs—The ability of thrombomodulin expressed on the surface of HUVECs to potentiate the thrombin-catalyzed activation of TAFI was studied using the assay for TAFIa detailed under “Experimental Procedures.” TAFI in HBS, 1% bovine serum albumin, and 5 mM Ca\(^{2+}\) was incubated with either thrombin or HUVECs or both. At various times, aliquots were removed and assayed for TAFIa by its ability to prolong the lysis time of clots formed from TdBHP in the presence of activated protein C. Since the assay was performed in the presence of activated protein C, thrombin formation during the lysis assay was prevented, and the lysis time observed was solely dependent upon the amount of TAFIa generated during incubation.

Fig. 6 displays the time course of TAFIa activation, where the measured concentration of TAFIa is plotted as a function of incubation time. Activation of TAFIa appears to terminate by 30 min at a level equal to the initial TAFI concentration, indicating quantitative conversion of TAFI to the activated form. The TAFIa also appears stable for a further 30 min. In the absence of HUVECs or thrombin, levels of TAFIa were 5–10-fold lower than in the presence of both.

As a test of the ability of HUVECs to participate in another thrombomodulin-dependent reaction, activation of protein C also was determined under similar conditions using the same HUVEC isolate (Fig. 6, inset). Activation of protein C showed a time course similar to that of TAFI; however, a lag was evident, and quantitative conversion did not occur until approximately 55 min. The concentrations of both TAFI and protein C, although different, were below the K_m values for their activation (15, 24). These data indicate that thrombomodulin on HUVECs can probably participate in the thrombin-catalyzed activation of both TAFI and protein C without interference from each other and with relative kinetics similar to those observed with soluble thrombomodulin (15, 24).

To determine whether the activation of TAFI on HUVECs is thrombomodulin-dependent, an experiment similar to that shown in Fig. 6 was initiated in which TAFI was incubated with HUVECs alone; HUVECs plus thrombin; or the combination of HUVECs, thrombin, and mAb TM-531. Shown in Fig. 7 are the time courses of TAFIa activation. Activation of TAFIa by thrombin plus HUVECs exhibited a time course similar to that shown in Fig. 6. Furthermore, potentiation of the thrombin-dependent activation of TAFI by HUVECs was completely inhibited by the antibody directed against thrombomodulin. Interestingly, with this particular isolate of HUVECs, the lysis times in the assay for TAFIa showed a progressive increase with time of incubation of TAFI with the cells, even when thrombin was not present in the incubation. When these values were interpreted as reflecting TAFIa levels, a value of 100 nM, or slightly less than 30% of the initial TAFI, was inferred by 30 min of incubation. This phenomenon was not inhibited by mAb TM-531 and thus implies that the cells either elaborated an inhibitor of the assay (e.g. PAI-1) or they activated TAFI by another, thrombomodulin-independent protease. Determination of which of these accounts for the apparent activation of TAFI in the absence of added thrombin will require further work. This phenomenon was not as evident with the isolate of HUVECs depicted in Fig. 6.

**DISCUSSION**

Previous work identified TAFI as a plasma zymogen that is converted to an enzyme with carboxypeptidase B-like activity following proteolytic cleavage of the bond at Arg^{52}–Ala^{53} (10, 14, 29). The activated enzyme very potently attenuates fibrinolysis (15). Because cleavage can be catalyzed by thrombin at the high levels typically found transiently in normal plasma following activation of the coagulation system, fibrinolysis is attenuated when prothrombin activation occurs in clotted plasma. This effect is overcome by activated protein C, and thus this anticoagulant enzyme appears pro fibrinolytic in plasma because it can attenuate TAFI activation (10, 12). Further studies (15) showed that thrombomodulin greatly enhances TAFI activation by thrombin (1250-fold). The studies in which this was demonstrated, however, were performed with a soluble form of thrombomodulin. Although those studies
clearly showed that TAFI activation can be enhanced by thrombomodulin, they did not indicate specifically whether the phenomenon is exhibited by cellular thrombomodulin. The present work shows that cellular thrombomodulin also is effective in promoting TAFI activation, thus supporting the notion that thrombomodulin-dependent activation of TAFI is relevant in vivo. In addition, the current work indicates that fibrinolysis in the presence of endothelial cells can be attenuated in a manner that is dependent on both TAFI and thrombomodulin. Therefore, physiological and/or pathophysiological modulation of fibrinolysis involving the endothelium is a likely possibility.

TAFI can be activated at a rate sufficient to impair fibrinolysis by either high levels of thrombin in the absence of thrombomodulin or low levels in the presence of thrombomodulin. When coagulation is triggered in normal plasma, thrombin is generated at a level far in excess of that needed to clot fibrinogen (9, 26, 30), and suppression of fibrinolysis occurs. Attenuation of fibrinolysis does not occur, however, when coagulation is triggered in hemophilic plasmas or factor XI-deficient plasma (31, 32). Because attenuated fibrinolysis does not occur in hemophilic plasma, the hypothesis has been presented that bleeding in hemophilia might be due to failure to suppress fibrinolysis as much as a failure to clot fibrin (31). Such might be the case, especially in the vicinity of relatively large vessels, where the surface area, and thus thrombomodulin density, is low relative to the local volume. In smaller vessels, the impact of thrombomodulin would increase because of an increased surface area:volume ratio, and thus TAFI activation might occur even with relatively low levels of thrombin generation.

The current work, previous work from our group (33), work by Broze and Higuchi (31), and work by Sakharov et al. (34) all show that added soluble thrombomodulin prolongs the time needed to achieve fibrinolysis in clotted human plasma. In addition, Sakharov et al. (34) showed that adding thrombomodulin to plasma enhanced TAFI activation and diminished plasminogen association with fibrin; they thus concluded that the antifibrinolytic effect of TAFI is probably expressed through the removal of COOH-terminal lysines from fibrin, which would be expected to diminish its affinity for plasminogen. A soluble form of thrombomodulin has been found and measured in human plasma (35, 36). In addition, a form found in urine has anticoagulant properties (37), and functionally active thrombomodulin (protein C activation) is reported in platelets (38). The existence of these invites the concept that they can enhance anticoagulation through protein C activation and would tend to be antithrombotic. However, they also might contribute to TAFI activation and thereby attenuate fibrinolysis and confer a tendency to thrombose. Whether this happens is not currently known, but, in principle at least, platelet and plasma thrombomodulin could contribute to antifibrinolytic as well as anticoagulant potential. Under pathological conditions, tendencies toward either bleeding or thrombosis could prevail.

Since TAFI and protein C both are substrates for the thrombin-thrombomodulin complex, each through competition could severely compromise the activation of the other. The magnitude of such effects, however, would probably be minimal physiologically, because the plasma concentrations of protein C and TAFI (each about 100 nm) are 10–50-fold lower than their respective \( K_m \) values for activation (12, 15, 24, 25). That thrombomodulin-dependent TAFI activation occurs in the presence of protein C is clearly demonstrated by the thrombomodulin-promoted antifibrinolytic effect of TAFI activation observed in normal human plasma (Fig. 5). Since protein C activation clearly occurs when coagulation is triggered in vivo (39), TAFI
does not, at least absolutely, suppress protein C activation. Thus, both pathways (TAI and protein C activation) can occur simultaneously without seriously impeding each other.

Based on currently available data, thrombin generation in the presence of thrombomodulin would lead to activation of both protein C and TAI. The activated protein C and TAIa would down-regulate the formation of thrombin and plasmin, respectively. The physiologic significance of regulatory pathways involving the thrombin-thrombomodulin complex that attenuate both coagulation and fibrinolysis is not immediately obvious. If, however, the vascular system responds to fibrin deposition by activating the fibrinolytic cascade, TAI activation might function to stabilize a clot when such stabilization is appropriate. Furthermore, pathologically attenuated TAI activation might be associated with a tendency to bleed, whereas exaggerated TAI activation might be associated with a tendency to thrombose. Determination of whether these are realistic inferences will require further study.

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REFERENCES
1. Collen, D., and Liljestrand, H. (1991) Blood 78, 3114–3124
2. Astrup, T. (1991) Semin. Thromb. Hemostasis 17, 161–174
3. Emon, C. T., and Emon, N. L. (1984) Semin. Thromb. Hemostasis 10, 122–130
4. Emon, C. T., Johnson, A. E., and Emon, N. L. (1991) Ann. N. Y. Acad. Sci. 614, 39–43
5. Walker, F. J., and Fay, P. J. (1992) FASEB J. 6, 2561–2567
6. Suzuki, K., Stenflo, J., Dahlback, B., and Teodorsson, B. (1983) J. Biol. Chem. 258, 1914–1920
7. Fulcher, C. A., Gardiner, J. E., Griffin, J. H., and Zimmerman, T. S. (1984) Blood 63, 486–489
8. Kalafatis, M., Rand, M. D., and Mann, K. G. (1994) J. Biol. Chem. 269, 31869–31880
9. Bajzar, L., and Nesheim, M. (1993) J. Biol. Chem. 268, 8608–8616
10. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 14477–14484
11. Cote, H. C. F., Stevens, W. K., Bajzar, L., Banfield, D. K., Nesheim, M. E., and MacGillivray, R. T. A. (1994) J. Biol. Chem. 269, 11374–11380
12. Bajzar, L., Nesheim, M. E., and Tracy, P. B. (1996) Blood 88, 2993–2999
13. Wang, W., Hendriks, D. F., and Scharpe, S. S. (1994) J. Biol. Chem. 269, 15937–15944
14. Eaton, D. L., Malloy, B. E., Tsai, S. P., Hanks, W., and Drayna, D. (1991) J. Biol. Chem. 266, 21833–21838
15. Bajzar, L., Morser, J., and Nesheim, M. (1996) J. Biol. Chem. 271, 16603–16608
16. Suenson, E., Lutzen, O., and Thorsen, S. (1984) Eur. J. Biochem. 140, 513–522
17. de Vries, C., Veerman, H., and Pannekoek, H. (1989) J. Biol. Chem. 264, 12604–12610
18. Nesheim, M., Wang, W., Boffa, M., Nagashima, M., Morser, J., and Bajzar, L. (1997) Thromb. Haemostasis 78, 386–391
19. Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F. D. (1987) Biochemistry 16, 2806–2810
20. Bajzar, L., Fredenburgh, J. C., and Nesheim, M. (1990) J. Biol. Chem. 265, 16948–16954
21. Shatos, M. A., Doherty, J. M., Stump, D. C., Thompson, E. A., and Collen, D. (1992) J. Biol. Chem. 267, 25443–25448
22. Stone, S. R., and Hofsteenge, J. (1986) Biochemistry 25, 4622–4628
23. Butenas, S., Drungilaite, V., and Mann, K. G. (1995) Anal. Biochem. 225, 231–241
24. Le Bonnier, B. F., MacGillivray, R. T. A., and Emon, C. T. (1991) J. Biol. Chem. 266, 13796–13803
25. Griffin, J. H. (1984) Semin. Thromb. Hemostasis 10, 162–166
26. Hemker, H. C., Wielders, S., Kessels, H., and Bregun, S. (1983) Thromb. Haemostasis 70, 617–624
27. Levin, E. G., Stern, D., Nawroth, P. P., Marlar, R. A., Fair, D. S., Fenton, J. W., and Harker, L. A. (1986) Thromb. Haemostasis 56, 115–119
28. Schleef, R. R., Polor, T. J., Dunne, E., Mimura, J., and Lokutudil, D. J. (1990) Cell 110, 155–163
29. Tan, A. K., and Eaton, D. L. (1995) Biochemistry 34, 5811–5816
30. Tans, G., Janssen-Claessen, T., Hemker, H. C., Zwaal, R. F. A., and Rosing, J. (1991) J. Biol. Chem. 266, 21864–21873
31. Brode, G. J., and Hiratani, D. A. (1996) Blood 88, 3815–3823
32. von dem Borne, P. A. K., Bajzar, L., Meijers, J. C. M., Nesheim, M. E., and Bouma, B. N. (1997) J. Clin. Invest. 99, 2323–2327
33. Bajzar, L., Nesheim, M. E., and Tracy, P. B. (1996) Fibrinolysis 10, 81 (Abstr. 10)
34. Sakharov, D. V., Plow, E. F., and Rijken, D. C. (1997) J. Biol. Chem. 272, 14477–14482
35. Ishii, H., and Majerus, F. W. (1985) J. Clin. Invest. 76, 2178–2181
36. Kodama, S., Uchijima, E., Nagai, M., Mikawatani, K., Hayashi, T., and Suzuki, K. (1997) J. Clin. Invest. 104, 2522–2527
37. Takahashi, Y., Hosaka, Y., Niina, H., Nagasawa, K., Naotsuka, M., Sakai, K., and Uemura, A. (1995) Thromb. Haemostasis 73, 805–811
38. Suzuki, K., Nishikawa, J., Hayashi, T., and Kusaka, Y. (1988) J. Biochem. (Tokyo) 104, 628–632
39. Hoogendoorn, H., Nesheim, M. E., and Giles, A. R. (1990) Blood 75, 2164–2171
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