The Profibrinolytic Effect of Activated Protein C in Clots Formed From Plasma Is TAFI-Dependent

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Thrombin-activatable fibrinolysis inhibitor (TAFI) is the precursor of an exopeptidase that is identical to plasma carboxypeptidase B. Upon activation by thrombin, activated TAFI (TAFIa) attenuates fibrinolysis, presumably by catalyzing the removal of C-terminal lysines from partially degraded fibrin. Activated protein C (APC) proteolytically inactivates the essential cofactor in prothrombinase, factor Va, and limits both the formation of thrombin and subsequent activation of TAFI, thereby appearing profibrinolytic. TAFI is able to reconstitute an APC-dependent shortening of lysis time in a purified system; however, it remained to be determined the extent to which TAFI is involved in the profibrinolytic effect of APC in a plasma-based system. To aid in addressing this question, two monoclonal antibodies (MoAbTAFI16 and #13) and a polyclonal antibody were produced against purified TAFI. MoAbTAFI16 was shown to inhibit TAFI activation and thereby appears to stimulate fibrinolysis. Furthermore, an enzyme-linked immunosorbent assay was developed using MoAbTAFI13 and the polyclonal antibody. Through its use, the plasma concentration of TAFI was determined to be 73 nmol/L. In addition, a turbidity assay was used to determine the effect of APC on tissue plasminogen activator-induced fibrinolysis of clots produced from normal human plasma (NHP), plasma immunodepleted of TAFI (TdP), and TdP reconstituted with purified TAFI. APC shortened lysis time of clots produced from NHP in a saturable and concentration-dependent manner. However, APC had no effect on lysis time of clots formed from either TdP or NHP in the presence of 80 nmol/L MoAbTAFI16. The APC effect could be reconstituted in TdP by the addition of purified TAFI. The lysis time in TdP was increased from 50 to 180 minutes in a TAFI concentration-dependent manner. The EC50 was 15 nmol/L and saturation was approached at physiologically relevant concentrations (60 nmol/L). The profibrinolytic effect of APC was also compared with that of MoAbTAFI16 and two competitive inhibitors, an inhibitor of the carboxypeptidase A and B family purified from potato tubers and 2-Guadinodithiolemercaptoacetic acid (GEMSA). All were able to reduce lysis time of clots formed from normal human plasma by 90 minutes, yielding respective EC50 values of 5 nmol/L, 15 nmol/L, 50 nmol/L, and 90 nmol/L. Therefore, the majority of the profibrinolytic effect of APC, in an in vitro plasma system, is dependent on TAFI. Because TAFIa dramatically influences lysis time, inhibitors of TAFI or TAFI activation may prove to be important adjuncts for thrombolytic therapy. © 1996 by The American Society of Hematology.

THE STRUCTURAL INTEGRITY of a clot is provided by fibrin, which is an insoluble mesh comprising the single most abundant protein within a clot.1 Fibrin monomers, formed subsequent to thrombin-catalyzed cleavage of fibrinogen and release of fibrinopeptides A and B, spontaneously polymerize to form a fibrin clot.2 Activation of the zymogen prothrombin to thrombin is facilitated by the tetrameric complex known as prothrombinase. Prothrombinase is composed of a negatively charged surface (supplied by platelets physiologically and phosphatidylcholine/phosphatidylserine [PCPS] vesicles experimentally); Ca2++; the enzyme, factor Xa; and the essential cofactor, factor Va. Loss of factor Va from the complete prothrombinase complex results in a 10,000-fold reduction in the rate of prothrombin activation.1 Deposition of fibrin by the coagulation cascade is opposed by the removal of fibrin, which is accomplished by the fibrinolytic cascade. Fibrin is proteolytically solubilized by plasmin, the terminal serine protease of the fibrinolytic pathway.3 Plasminogen is proteolytically activated by tissue plasminogen activator (tPA). However, tPA is a poor activator in the absence of fibrin. Fibrin, particularly that which has been partially degraded by plasmin to expose C-terminal lysines, is a potent, although transient, cofactor for tPA-induced activation of plasminogen.4 A balance between clot formation and dissolution is required to protect an organism by providing the capability of mounting an immediate response to injury to prevent catastrophic loss of blood and by maintaining a continuous flow of blood throughout the vasculature by preventing or removing inappropriately deposited fibrin.5

The coagulation response is regulated, in part, through the inactivation of factor Va by the serine protease activated protein C (APC).6 APC is formed by limited proteolysis of protein C by thrombin in the presence of the cofactor thrombomodulin.7 The thrombin-thrombomodulin complex, therefore, participates in downregulating coagulation. When thrombin is produced during fibrinolysis in a system comprising clots formed from plasma, it inhibits fibrinolysis and the magnitude of the effect correlates directly with the thrombin concentration.8 In an effort to reconstitute a thrombin-dependent antifibrinolytic effect in a system composed of purified components, we were able to both postulate the presence of and purify a thrombin-activatable fibrinolysis inhibitor (TAFI).9,10 TAFI is also referred to as carboxypeptidase U11 and plasma carboxypeptidase B.12 It is hypothesized that activation of TAFI yields an exopeptidase with carboxypeptidase B-like activity that is able to remove C-terminal lysines from partially degraded fibrin, thereby inhibiting fibrinolysis.12,13 Proteolytic activation of TAFI requires relatively high levels of thrombin. However, activation by thrombin is accelerated 1,250-fold by thrombo-

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and MoAbTAFI#16 were found to be most potent. We, therefore, propose that APC promotes fibrinolysis of clots formed from plasma by preventing the formation of thrombin during (Richmond, CA). The sheep polyclonal anti-TAFI antibody was reconstituted in TAFI-deficient plasma supplemented with TAFI. The ability of APC to promote fibrinolysis was compared with that of inhibitors of the carboxypeptidase A- and B-like exopeptidase (PTI), an inhibitor of carboxypeptidase A- and B-like exopeptidases, Solulin, was provided by Dr. a specific inhibitor of the carboxypeptidase B-like protease family, thrombomodulin, Solulin, was provided by Dr. G. Morser of Berlex Canada). PCPs vesicles (PC:PS 3:1) were prepared according to the method of Lundblad et al., as described previously.20 Prothrombin was purified for quantification of MoAbTAFI using a periodate coupling method. The HRP-coupled polyclonal anti-TAFI, which was stored at -20°C. Development of an enzyme-linked immunosorbent assay (ELISA) for quantification of TAFI. Wells of a 96-well microtiter plate, 1 X 10^6 cells/mouse.22 Concentrates enriched in MoAbTAFI#13 and MoAbTAFI#16, were prepared in pristane-primed BALB/c mice by injecting 1 X 10^6 cells/mouse.23 Two milliliters of 2.5 mg/mL MoAbTAFI#16, was incubated with the activated Sepharose 4B (Pharmacia, La Jolla, CA) by modification of the procedure of Cuatrecasas, as reported previously.10 Two milliliters of 2.5 mg/mL MoAbTAFI#16 was incubated with the activated Sepharose 4B for 6 hours at 4°C and for a further 8 hours after the addition of 0.4 mL 1.0 mol/L Tris, pH 8.0. This resulted in an 82% coupling efficiency producing a 2.0 mL column with 2.05 mg MoAbTAFI#16 per milliliter of Sepharose 4B resin.

Affinity purification and HRP conjugation of polyclonal anti-TAFI. The caprylic acid precipitated sheep antibodies were subjected to affinity purification by passing the antibodies dialyzed against 20 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4 (HBS) over a 1.0-mL Sepharose CL-4B to which 1.0 mg of TAFI had been coupled. TAFI was coupled using the protocol described for immobilization of MoAbTAFI#16. Antibodies retained on the affinity column were eluted using Gentle Elution Buffer (Pierce). After extensive dialysis against PBS, affinity-purified antibodies were concentrated using an Amicon concentrator with a PY-10 filter (Amicon, Oakville, Ontario, Canada), diluted by the addition of an equal volume of glycerol, and stored at -20°C. HRP was conjugated to the polyclonal anti-TAFI IgG using a peroxidase coupling method. The HRP-coupled polyclonal anti-TAFI was precipitated by the addition of (NH4)2SO4 to 50% saturation and pelleted by centrifugation. The pellet was washed twice by resuspension in 50% saturated (NH4)2SO4/H2O followed by centrifugation. The final pellet was dissolved in a volume of 50% glycerol/H2O to yield a 1.0 mg/mL solution of HRP-conjugated polyclonal anti-TAFI, which was stored at -20°C.

Development of an enzyme-linked immunosorbent assay (ELISA) for quantification of TAFI. Wells of a 96-well microtiter plate, Corning (Corning, NY), were coated with MoAbTAFI#13 by incubating 10 µg/mL MoAbTAFI#13 in 50 mmol/L sodium carbonate, pH 9.6, for 2 hours at 22°C. Nonspecific sites were blocked with BSA. Samples were diluted in 0.1% BSA in HBS with 0.1% Tween 20 and 100 µL of each sample was applied to each well and incubated for 1.5 hours at 22°C. HRP-conjugated anti-TAFI was then incubated...
with each sample and specific binding was determined o-phenylene-
diamine dihydrochloride (Sigma). The color was allowed to develop for
10 minutes. The reaction was then quenched by the addition of
sulfuric acid to each well. Absorbance was measured at 490 nm and
considered by subtracting the absorbance at 650 nm using a Thermo-
max microtiter plate reader (Molecular Devices, Sunnyvale, CA).
Using this ELISA, the concentration of TAFI in a plasma pool (>60
donors) was determined to be 73 nmol/L, which compares favorably with
a concentration of 50 nmol/L that was calculated based on recovery of
activity during purification. Concentrations in plasma
obtained from 6 volunteers ranged from 50 to 175 nmol/L.

Western blotting. Samples were diluted in sample preparation
buffer (1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol
[MBE], and 10% glycerol with bromophenol blue) and subjected to
SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5%
to 15% gradient gel in a mini-slab gel electrophoresis apparatus
(Hoefer, Technical Marketing, Ottawa, Ontario, Canada) according to
the procedure of Neville. Protein was then transferred to Nitro-
cellulose (Schleicher & Schuell, Keene, NH) at 500 mA for 1 hour
using the electroblotting techniques described by Towbin et al. Nitro-
cellulose was blocked with 5% nonfat dry milk in 20 mmol/L
Tris, 0.15 mol/L NaCl, 0.05% Tween 20 at pH 7.4 (TBS-T). TAFI
antigen was probed for 1.0 hour with MoAbTAFI#13, which
was dissolved in TBS-T to a concentration of 5 μg/mL. MoAbTAFI#13
was probed for 1.0 hour with an HRP-conjugated antinouse
IgG (Southern Biotechnologies, Birmingham, AL) diluted in 5,000
in TBS-T. The secondary antibody was detected by enhanced chemi-
luminescence (Chemiluminescence Detection Kit; DuPont, Boston,
MA) using X-Omat film (Kodak Scientific Imaging) and an M35A
X-Omat processor (Eastman Kodak, New Haven, CT).

Preparation of plasma for lysis assays. Pooled (3 to 4 donors)
fresh frozen human plasma was purchased from Haematologic Tech-
nologies Inc (Burlington, VT) and dialyzed against 4 L of HBS, at
4°C with 4 changes overnight. The dialysate, termed normal human
plasma (NHP), was stored at −70°C in 2.0 mL aliquots. TAFI-
deficient plasma (Tdp) was produced by passing an aliquot, maxi-
mally 50 mL, of NHP over a 2.0-mL anti-TAFI affinity column
(MoAbTAFI#16-Sepharose 4B) equilibrated in HBS. Depletion of
TAFI was verified by Western blot analysis of a sample from each
individual fraction (data not shown). Only the flow-through fractions
depleted of TAFI with an equal to the NHP loaded onto the
individual fraction (data not shown). Only the flow-through fractions
tained of TAFI by ELISA. Figure 1 shows the signal associated with a serial dilution of either a preclon-
us amount of postcolumn sample or detected using the ELISA described
above. Specificity of MoAbTAFI#13 is shown by Western blot analysis,
as shown in the inset. Chemiluminesence using MoAbTAFI#13
and HRP-conjugated antinouse IgG distinguished a band in NHP
that exhibited an identical electrophoretic mobility to purified TAFI
(compare lanes 1 and 3). However, this band is not present in plasma
that had been passed over immobilized MoAbTAFI#16 (lane 2).
These data indicate that the affinity column comprising MoAb-
TAFI#16 is able to deplete TAFI from plasma. Furthermore, quanti-
tation of TAFI by ELISA indicates that a single pass of plasma over
the affinity column resulted in the removal of 99.9% of TAFI from
plasma.

Lysis assay. The assay was performed by initially diluting the
plasma, either NHP or Tdp, with HBS such that the A590 was 16,
typically 1 part plasma to 2 parts buffer. and PCPs vesicles were
added to a concentration of 10 μmol/L. Closts (100 μL) were formed
by the addition of 91 μL of diluted plasma to wells of a microtiter
plate containing separated 2-μL aliquots of thrombin/Ca²⁺ (final
concentrations of 6.0 mmol/L and 5 mmol/L, respectively) and tPA
(final concentration, 294 pmol/L). The remaining 5.0 μL volume
was composed of buffer for control experiments or buffer with APC,
MoAbTAFI#16, GEMSA, or PTI at various concentrations, de-
pending on the requirements of the experiment. When purified TAFI
was added back to Tdp, it was added directly to the diluted plasma.
After adding the plasma to the microtiter wells, the plate was trans-
fated to a Thermomax microtiter plate reader (Molecular Devices).
Temperature was maintained at 37°C and the turbidity was monitored
at 405 nm and 2.5-minute intervals. Lysis profiles were generated
by plotting A405 as a function of time. Clotting occurring within the
first 5 minutes is represented as an increase in turbidity, whereas
fibrin dissolution correlates with a reduction in turbidity. Lysis time
is defined as the time required to achieve the transition midpoint
in the reduction from the maximum turbidity to baseline values.

Chromogenic assay for TAFIa. Hydrolysis of the chromogenic
substrate, hipparyl arginine, was used to determine whether MoAb-
TAFI#16 inhibits TAFIa. In a 25-μL reaction volume, TAFI (0.8
μmol/L, final concentration) was incubated with thrombomodulin (40
μmol/L, final concentration), Ca²⁺ (5.0 mmol/L, final concentration),
and thrombin (8.0 mmol/L, final concentration) in the presence and
absence of MoAbTAFI#16 (12.5 μmol/L) for 10 minutes at 22°C.
Thrombin was then inhibited by the addition of Phe-Pro-Arg chlo-
romethyl ketone (FPA-CK; 50 μmol/L final concentration). A nega-
tive control was produced by adding FPA-CK before initiation of
the reaction by the addition of TAFI. The effect of MoAbTAFI#16
on TAFIa was determined by the addition of the antibody subsequent
to the activation of TAFI. The volume was corrected in each of the
other 3 reactions by the addition of a volume of buffer equal to that
to the added MoAbTAFI#16. A 25-μL aliquot was removed from
each of the 4 reactions (30 μL final concentration) and added to 975
μL of hippuryl arginine (410 μmol/L) in HBS with 0.01% Tween
80. The time course of hippuric acid formation at 22°C was followed
by monitoring absorbance at 254 nm and 0.5-minute intervals using a
Beckman DU-65 spectrophotometer (Fullerton, CA).

RESULTS

APC shortens lysis time of clots formed from normal hu-
man plasma. A turbidometric lysis assay was used to show
the effect of APC on tPA-induced lysis of clots formed from
normal human plasma. NHP dialyzed and diluted one-third
with HBS was allowed to clot in the presence of thrombin,
Ca²⁺, PCPs vesicles, tPA, and various concentrations of
APC (0 to 30 nmol/L). Clots were maintained at 37°C and
turbidity (absorbance at 405 nm) was monitored. Lysis profi-
les are represented as plots of turbidity as a function of
time. Lysis time, defined as the time required to attain the
transition midpoint of the lysis profiles, was determined for
each lysis profile. The inverse dependence of lysis time on
APC concentration confirms the profibrinolytic effect of
APC (Fig 2). Lysis time decreased from 132.5 minutes in the
absence of APC to 45 minutes in the presence of saturating
concentrations of APC. However, APC could not overcome
the prolongation of lysis by TAFIa. In this experiment, APC
exhibits an EC₅₀ = 4 nmol/L and achieves saturation at less
than 12.5 nmol/L. Furthermore, APC appears to inhibit the
activation of TAFI and not that of TAFIa.

MoAbTAFI#16 inhibits the activation of TAFI catalyzed by a
catalyzed by a complex comprising thrombin and thrombomodulin.
The ability of MoAbTAFI#16, an MoAb raised against puri-
ified human TAFI, to inhibit activation of TAFI by thrombin
in complex with thrombomodulin was assessed using a two-
stage assay. TAFIa activity generated for each set of condi-
tions was monitored using the chromogenic substrate hip-
puryl arginine (Fig 3). No TAFIa activity was generated when either MoAbTAFI#16 or FPA-CK, a specific inhibitor of thrombin, was present during the incubation of TAFI with thrombin and thrombomodulin. However, the addition of MoAbTAFI#16 subsequent to activation was without effect on TAFIa activity, because the rate of hippuryl arginine hydrolysis was identical to the control rate. These data indicate that MoAbTAFI#16 does not inhibit TAFIa, but does prevent the activation of TAFI.

The profibrinolytic effect of APC is abrogated by MoAbTAFI#16. The effect of MoAbTAFI#16 on tPA-induced lysis of clots formed from normal human plasma was investi-
gated because it was able to inhibit the activation of TAFI in a purified system. Lysis times were determined from the turbidometric lysis profiles produced in the presence of various concentrations of MoAbTAFI#16 and are shown in Fig 4. In the absence of the antibody, the lysis time was approximately 145 minutes. However, the lysis time decreased saturably with increasing concentrations of MoAbTAFI#16, exhibiting a minimum lysis time of 45 minutes in the presence of 80 nmoVL antibody. In the presence of 50 nmoVL APC, lysis time was also 45 minutes; however, the addition of 80 nmoVL MoAbTAFI#16 was unable to shorten lysis time any further. These data suggest that both APC and MoAbTAFI#16 promote fibrinolysis through a common pathway.

TAFI is required to observe the profibrinolytic effect of APC in clots formed from plasma. Although we were able to show that 80 nmoVL MoAbTAFI#16 both mimicked the apparent profibrinolytic effect of APC and abrogated the profibrinolytic effect of APC in clots formed from NHP, we wished to determine the lysis time-dependence on the concentration of TAFI in both the presence and absence of APC. Therefore, the effect of APC on the lysis time of clots formed from TdP or TdP subsequently reconstituted with various concentrations of purified TAFI was determined (Fig 5). The lysis time in the presence and absence of 50 nmoVL APC was approximately 50 minutes, indicating that both APC and TAFI have no effect on lysis time of clots produced from TdP and that the lysis time of TdP is comparable to the lysis time of clots formed from NHP in the presence of saturating concentrations of APC (Fig 1). In the absence of APC, lysis time of clots formed from TdP reconstituted with TAFI (0 to 60 nmoVL) exhibited a concentration-dependent prolongation from 50 to 180 minutes with an EC50 = 10 nmoVL TAFI. However, a TAFI-dependent increase in clot lysis time was completely inhibited by 50 nmoVL APC, because the lysis time in the presence of APC remained invariant at 50 minutes over the range of TAFI concentrations studied. These data indicate that the profibrinolytic effect of APC is not apparent in TdP; however, it can be reconstituted in TdP by the addition of purified TAFI. Therefore, the profibrinolytic effect of APC in clots formed from plasma, as quantified using a turbidometric lysis assay, is due solely to a mechanism involving TAFI. The data also indicate that as little as 3 nmoVL TAFI is required to observe an APC-dependent profibrinolytic effect. Furthermore, the TAFI-dependent prolongation of lysis time approaches saturation with 60 nmoVL TAFI, which is within the physiologic range of concentrations determined both immunologically (see the Materials and Methods) and is similar to the concentration calculated based on recovery of activity during purification.

Comparison of APC to inhibitors of carboxypeptidase B-like proteases (GEMSA and PTI) and MoAbTAFI#16 in their ability to enhance lysis time of clots formed from human plasma. The antifibrinolytic effect of TAFI can be overcome in at least three ways: by preventing the formation of its activator, thrombin; by preventing the activation of TAFI; and by inhibiting TAFIa. Therefore, the efficacy of APC, an inhibitor of thrombin formation; MoAbTAFI#16, an inhibitor of TAFI activation; and GEMSA and PTI, inhibitors of carboxypeptidase B-like enzymes, to inhibit the TAFI-dependent antifibrinolytic effect on lysis time was investigated. The TAFI-dependent profibrinolytic effect of each compound was evaluated by determination of lysis time of clots formed from both NHP and TdP in the presence of various concentrations of each inhibitor. APC, GEMSA, and MoAbTAFI#16 at every concentration studied did not affect lysis time of clots formed from TdP. In general, the lysis time remained fairly constant at approximately 50 minutes, indicating that none of the inhibitors used exhibited TAFI-dependent profibrinolytic effect. Therefore, the addition of APC to TdP both prolongs lysis time and reconstitutes the profibrinolytic effect of APC. Plasma depleted of TAFI using immobilized MoAbTAFI#16 was diluted one-third with HBS and supplemented with 10 nmoVL PCPS vesicles. Clots were formed in the presence of Ca2+ (5.0 nmoVL/L), Ile (6.0 nmoVL/L), and tPA (294 pmol/L). Lysis times of clots prepared from TdP reconstituted with TAFI (0 to 60 nmoVL) were determined in both the absence (○) and presence (■) of 50 nmoVL APC and are plotted as a function of TAFI concentration. These data indicate that the profibrinolytic effect of APC in NHP is TAFI-dependent.
independent effects on lysis time. However, the data shown in Fig 6 indicate that, in clots formed from NHP, each inhibitor was able to decrease lysis time from 140 to 50 minutes in a concentration-dependent manner. The lysis time at saturating concentrations of each inhibitor exhibited a minimum lysis time (average, 50 minutes) that is identical to the lysis time of clots formed from TdP for every concentration of each inhibitor. Although the magnitude of the profibrinolytic effect was 90 minutes for each inhibitor, the ECSO associated with each one varied greatly. APC was most potent (EC50 = 5 nmol/L), followed by MoAbTAFI16 (EC50 = 15 nmol/L), PTI (EC50 = 50 nmol/L), and finally GEMSA (EC50 = 90 μmol/L). These data indicate that a shortening of lysis time can be achieved at various stages in the activation of TAFI.

**DISCUSSION**

In an attempt to understand the influence of APC on fibrinolysis, numerous studies have been performed. These studies have shown a profibrinolytic effect of APC both in vitro, in systems ranging from whole blood to purified blood components, PTI and GEMSA. The lysis time of clots formed from TdP, to represent nonspecific effects on fibrinolysis, was not affected by any of the profibrinolytic reagents tested. Clots were formed from NHP in the presence of various concentrations of profibrinolytic reagent and Ca²⁺ (5 mmol/L), thrombin (6 nmol/L), and tPA (294 pmol/L). Lysis times were determined for clots produced in the presence of various concentrations of APC (●), MoAbTAFI16 (▲), PTI (□), and GEMSA (▲) and are plotted as a function of inhibitor concentration. A shortening of lysis time in the absence of TAFI could not be shown for any component; however, all compounds saturate shortens lysis time of clots formed from NHP by 90 minutes. The data indicate that APC most potently enhances fibrinolysis and is followed by MoAbTAFI16, PTI, and GEMSA, respectively.

Fig 6. The effect of APC on fibrinolysis was compared with that of MoAbTAFI16 and two inhibitors of carboxypeptidase B-like proteases, PTI and GEMSA. The lysis time of clots formed from TdP, to represent nonspecific effects on fibrinolysis, was not affected by any of the profibrinolytic reagents tested. Clots were formed from NHP in the presence of various concentrations of profibrinolytic reagent and Ca²⁺ (5 mmol/L), thrombin (6 nmol/L), and tPA (294 pmol/L). Lysis times were determined for clots produced in the presence of various concentrations of APC (●), MoAbTAFI16 (▲), PTI (□), and GEMSA (▲) and are plotted as a function of inhibitor concentration. A shortening of lysis time in the absence of TAFI could not be shown for any component; however, all compounds saturate shortens lysis time of clots formed from NHP by 90 minutes. The data indicate that APC most potently enhances fibrinolysis and is followed by MoAbTAFI16, PTI, and GEMSA, respectively.

of inactivation of PAI-1 by APC do not support the conclusion that APC would effectively compete for PAI-1 in the presence of tPA. The second reason is that complexes of APC and PAI-1 can be shown in the absence of an effect on fibrinolysis in systems comprising whole blood and APC from heterologous species. We were able to show that the profibrinolytic effect of APC in clots formed from human plasma is due directly and specifically to its ability to inhibit prothrombin activation. These data rationalize the observations of others who have shown that APC promotes lysis in normal plasma but not factor X- or VIII-deficient plasma, that other anticoagulants are also able to potentiate fibrinolysis, and that APC from a specific species is profibrinolytic only in those species in which it is also an anticoagulant. Inclusion of TAFI in a system composed of purified components, including a negatively charged surface, Ca²⁺, factors Va and Xa, and prothrombin was able to reproduce an APC-dependent profibrinolytic effect. However, those studies did not show that the mechanism of APC-dependent profibrinolytic effect in clots formed from purified components was identical to that which is observed in plasma.

This study shows that the effect of APC on fibrinolysis of clots formed from plasma is exclusively dependent on TAFI. Although APC can shorten lysis time in a saturable manner (Fig 2), it is unable to shorten lysis time in the presence of an antibody raised against TAFI, MoAbTAFI16 (Fig 4). Because APC had no effect on lysis time in the presence of MoAbTAFI16, it is unlikely that the thrombin generated during the lysis assay in the absence of APC had any secondary effect, such as changing clot structure, that could affect lysis time. MoAbTAFI16 is able to shorten lysis time in a saturable manner and to an extent similar to that of APC. The identical lysis times achieved in the presence of saturating amounts of either APC or MoAbTAFI16 suggest a common mechanism involving inhibition of the activation of TAFI. APC indirectly inhibits activation of TAFI by preventing the formation of thrombin and the data in Fig 3 show that MoAbTAFI16 directly inhibits activation of TAFI by interacting with TAFI. The interaction of the antibody with TAFI most likely precludes the interaction of TAFI with thrombin. This mechanism is suggested because the model for activation of TAFI by the complex of thrombin and thrombomodulin includes only the binary interactions involving thrombin and TAFI or thrombin and thrombomodulin but not between thrombomodulin and TAFI.

The requirement for TAFI in the profibrinolytic effect of APC in plasma was verified further by immunodepleting TAFI from plasma and showing that APC does not influence the lysis of clots formed from this plasma. Western blotting analyses and an ELISA developed during this study were used to show that the plasma was indeed depleted of TAFI (Fig 1). Although APC was without effect on lysis of clots formed from this plasma, supplementation of the plasma with purified TAFI both prolonged lysis time of the TdP in the absence of APC and restored the APC-dependent profibrinolytic effect (Fig 5). Saturation with respect to prolongation of lysis time occurred at a physiologically relevant concentration of TAFI (60 nmol/L).
The profibrinolytic effect of APC was compared with that of the specific inhibitor of TAFI activation, MoAbTAFI#16, and the general inhibitors of the carboxypeptidase B-like family, GEMSA and PTI (Fig 6). The magnitude of the effect of each reagent was identical, although they differed with respect to their potency (APC>MoAbTAFI#16>PTI->GEMSA). Cumulatively, these data indicate that TAFI plays a major antifibrinolytic role in clots formed from plasma under conditions in which substantial prothrombin activation occurs. In this system, APC potentiates fibrinolysis but only in a TAFI-dependent manner. Therefore, we propose that APC appears profibrinolytic primarily by inhibiting the production of thrombin, thereby preventing the activation of TAFI and its subsequent inhibition of fibrinolysis. A model is represented in Fig 7.

Fig 7. A schematic representation describing the profibrinolytic effect of APC. The top panel indicates that, in the absence of APC, activation of prothrombin continues unabated, producing high concentrations of thrombin that are required to activate TAFI in the absence of thrombomodulin. Upon activation, TAFI is able to down-regulate fibrinolysis, potentially, by proteolytic inactivation of the cofactor for tPA. However, in the presence of APC (bottom panel), high levels of thrombin are not attained. Activation of TAFI, therefore, is attenuated; subsequently, fibrinolysis proceeds without the inhibition induced by TAFI. II, prothrombin; IIa, thrombin; Fn, fibrin; Fgn, plasminogen; Pn, plasmin; Fgn, fibrinogen; Fn, fibrin; FDP, fibrin degradation products.

The profibrinolytic effect of APC was compared with that of the specific inhibitor of TAFI activation, MoAbTAFI#16, and the general inhibitors of the carboxypeptidase B-like family, GEMSA and PTI (Fig 6). The magnitude of the effect of each reagent was identical, although they differed with respect to their potency (APC>MoAbTAFI#16>PTI->GEMSA). Cumulatively, these data indicate that TAFI plays a major antifibrinolytic role in clots formed from plasma under conditions in which substantial prothrombin activation occurs. In this system, APC potentiates fibrinolysis but only in a TAFI-dependent manner. Therefore, we propose that APC appears profibrinolytic primarily by inhibiting the production of thrombin, thereby preventing the activation of TAFI and its subsequent inhibition of fibrinolysis. A model is represented in Fig 7.

It is apparent that coagulation, fibrinolysis, anticoagulants, and, by inference, antifibrinolytics are not likely to work independently, as exemplified by the presented data. In the system used, the initial clot size was constant. Furthermore, 100% of the fibrinogen in the plasma was converted to fibrin; thus, fibrin accretion could not have occurred. Therefore, neither variation in clot size nor fibrin accretion could have affected lysis time. Because the same plasma was used for all experiments, variations in lysis times resulting from differing levels of inhibitors of fibrinolysis, specifically PAI-1, also cannot account for the profibrinolytic effect of APC. Therefore, the data indicate that the state of the coagulation system during fibrinolysis can affect the fibrinolytic system. This is borne out by the observation that patients deficient in factor XI tend to bleed in areas of high fibrinolytic potential and, as speculated by von dem Borne et al,9 may be the result of insufficient thrombin generation to satisfactorily stabilize the clot. Inhibiting the coagulation cascade, therefore, could render a fibrin clot more susceptible to degradation as a result of a lack of activated TAFI. A physiologic example in which this may be of therapeutic benefit is in the treatment of a myocardial infarction due to thrombus. It is a current belief that treatment of myocardial infarction by tPA may be more efficacious by combination therapy.42 Inclusion of an anticoagulant might inhibit the activation of TAFI and subsequently destabilize the fibrin clot rather than, or in addition to, preventing further clot formation. If TAFI is found to affect thrombolysis in this situation, then inhibitors of activated TAFI should be considered as new avenues of thrombolytic therapy. As shown, using an in vitro clot lysis assay, both APC and inhibitors of either activated TAFI or TAFI activation enhance lysis and would reduce the amount of tPA required to achieve a lysis time equal to that which would be obtained in their absence. Removal of thrombi, under conditions in which inhibition of coagulation or induction of fibrinolysis is either impossible or undesired, may be accomplished by the prevention of TAFI activation or inhibition of activated TAFI, thereby allowing the endogenous fibrinolytic system to work more efficiently. Alternatively, specific activators of TAFI may augment replacement therapies or maintain hemostasis in individuals whose coagulation system is compromised. The effect of the TAFI pathway on the human fibrinolytic system has not been investigated in vivo. However, because an effect of APC on fibrinolysis has been shown both in vivo, in dog30 and cat models,31 and in vitro, in a human plasma model, TAFI likely will be found to play a physiologic role in the balance between coagulation and fibrinolysis in humans.

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