The Thrombin Mutant W215A/E217A Shows Safe and Potent Anticoagulant and Antithrombotic Effects in Vivo*

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Administration of the thrombin mutant W215A/E217A (WE), rationally designed for selective activation of the anticoagulant protein C, elicits safe and potent anticoagulant and antithrombotic effects in a baboon model of platelet-dependent thrombosis. The lowest dose of WE tested (0.011 mg/kg bolus) reduced platelet thrombus accumulation by 80% and was at least as effective as the direct administration of 40-fold more (0.45 mg/kg bolus) activated protein C. WE-treated animals showed no detectable hemorrhage or organ failure. No procoagulant activity could be detected for up to 1 week in baboon plasma obtained following WE administration. These results show that engineered thrombin derivatives that selectively activate protein C may represent useful therapeutic agents for the treatment of thrombotic disorders.

Thrombosis involves the localized accumulation of fibrin, platelets, and other blood elements, which may restrict blood flow to and from organs and tissues (1, 2). Thrombo-occlusive events with significant morbidity and mortality include pulmonary and peripheral thromboembolism, myocardial infarction, ischemic stroke, sepsis, and heparin-induced thrombocytopenia (1). Thrombin possesses intrinsic procoagulant (fibrinogen clotting and platelet aggregation) and anticoagulant (activation of protein C) activities (3). Continuous infusion of low dose wild-type human thrombin (WT)† has previously been shown to be a relatively safe antithrombotic agent in baboons (4) capable of binding to thrombomodulin and generating endogenous activated protein C (APC), a naturally circulating anticoagulant enzyme (5). However, infusion of WT produces some fibrin formation and platelet aggregation effects that were enhanced at higher doses or in regions where WT might be concentrated locally. To fully exploit thrombin as an anticoagulant, its ability to cleave fibrinogen and to activate platelets must be selectively compromised (6). Toward this end, several thrombin mutants have been engineered to significantly tip the balance between procoagulant and anticoagulant activities in favor of protein C activation (6–9). Although thrombin mutants have produced encouraging anticoagulant effects in vivo (7), no information regarding their antithrombotic potential has been available. Accordingly we tested in a baboon model the effectiveness and safety of the most potent anticoagulant thrombin produced to date, the mutant WE, which was rationally engineered to be practically devoid of activity toward fibrinogen and the platelet receptor PAR1 but to retain the ability of thrombin to activate protein C in the presence of thrombomodulin (9).

MATERIALS AND METHODS

WT and WE were expressed, purified, and characterized in detail as described previously (9). WT and WE were stored in frozen aliquots until use in thrombosis or coagulation experiments. To confirm that injection of WE would be at least as safe as injection of WT in baboons, we compared the procoagulant activities of the two thrombins in baboon plasma prepared by pooling citrated plasma samples from five animals. All primate experimental protocols were approved by the Institutional Animal Care and Use Committee, Emory University. Since plasma-derived and recombinant human APC have previously been shown to be comparably anticoagulant in human and baboon plasma and both were antithrombotic in baboons (10, 11), in this study we used an injectable formulation of lyophilized human plasma-derived APC (a gift from the American Red Cross) as positive control for WE. The anticoagulant activity of APC was tested prior to administration by measuring its effect on the activated partial thromboplastin time (APTT) of citrated plasma. At least nine consecutive experiments were performed in each study subject on separate days with at least daily intervals between experiments. The antithrombotic effects of both APC and WE were tested at three dose levels in three awake juvenile baboons weighing 9.4–10.8 kg. Intravenous bolus injections of 0.1, 0.2, or 0.45 mg/kg (1.8, 3.6, or 8 nmol/kg) APC or 0.011, 0.022, or 0.055 mg/kg (0.3, 0.6, or 1.5 nmol/kg) WE in 10 ml of sterile solution containing 2.5% dextrose and 0.45% saline were given to each study subject at time 0. The theoretical peak concentrations of the enzymes in circulating blood were in the range of 30–80 nM (1.7–7.5 μg/ml) for APC and 1.95–40 nM (0.18–3.67 μg/ml) for WE.

Ten minutes after the WE or APC bolus, a thrombogenic device was inserted into a chronic exteriorized arteriovenous (AV) shunt. Thrombosis was assessed by gamma camera imaging of 111indium-labeled platelets and monitored for deposition at described previously (4) with minor modifications as follows. The thrombogenic device was a 120-cm-long, 3-mm-internal-diameter silicon rubber shunt containing a highly thrombogenic 2-cm-long, 4-mm-internal-diameter knitted Dacron vascular graft that served to initiate and localize thrombus formation. The blood flow rate through the shunt was maintained at 40–50 ml/min by clamping the proximal section of the shunt. The radioactivity of a 45-cm-long middle section of the device also containing the short Dacron segment in a central position was measured continuously by γ camera imaging with data acquisition at consecutive intervals between 10 and 70 min. Since no measurable thrombus formed during the first 5 min of blood exposure, the first 5-min image was taken as background. Radioactivity above background in subsequent measurements indicated local deposition of radiolabeled platelets and the presence of platelet-rich thrombi. The number of deposited platelets was calculated from the radioactivities of the device, the radioactivity of a peripheral 3-ml blood sample, and the platelet count (4). The device was removed 70 min after APC or WE dosing, and the Dacron segment was saved for...
determination of fibrin deposition by 125\textit{i}odo-fibrinogen/fibrin counting after allowing at least 30 days for 111\textit{i}ndium decay as described previously (4). Each thrombosis study, blood flow was restored by reconnecting the segments of the chronic shunt using 2-0-6 cm-long Teflon tubing connectors (3.0-mm inner diameter). Up to three control studies without antithrombotic treatment were also performed in each animal. The antithrombotic effect of APC or WE treatment was assessed as the reduction in deposited platelets and/or fibrinogen versus untreated controls over the blood exposure period. Platelet deposition results were evaluated using regression analysis with a single dependent variable (platelet deposition) and time, animal, therapy, and dosage as independent predictors. The regression analysis was also used to evaluate dose-response relationships.

The antihemostatic effects of the antithrombotic enzymes were assessed following injection of 0.1, 0.2, or 0.45 mg/kg (1.8, 3.6, or 8 nmol/kg) APC or 0.011, 0.022, 0.055, 0.11, or 0.22 mg/kg (0.3, 0.6, 1.5, 3, or 6 nmol/kg) WE. Blood was drawn from the AV shunt or by standard venipuncture in animals without shunts (i.e., the high dose WE studies). The total volume of blood drawn for all in vitro measurements was less than 10 ml/day in each study subject. Blood samples (0.45 or 0.9 ml) were drawn into 3.2% trisodium citrate at regular intervals for at least 100 min after dosing for immediate assessment of APTT by using a coagulometer (Bayer, Inc.). Because APC is progressively inhibited in plasma (data not shown). It was thus determined that either protein C activator Protac in samples drawn before and 100 min after WE or APC administration as described previously (4, 13) with the following modifications. All citrated baboon plasma samples for protein C testing were incubated at room temperature for 48 h prior to performing the protein C test to allow for normalization of APTT values. In each case, normalization of APTT to within 10 s of the APTT value in the corresponding base-line sample was confirmed prior to protein C testing. Pooled normal baboon plasma, also incubated for 48 h, was diluted 1:1 in protein C-depleted human plasma (George King Bio-Medical, Overland Park, KS) and then in serial dilutions to generate a protein C activity standard curve. The baboon samples were diluted 1:3 with protein C-depleted human plasma. Lysophospholipid Protac C vials were reconstituted into a 3-ml volume as recommended (American Diagnostica, Greenwich, CT), and 40 \mu l of the activator was incubated with 20 \mu l of the 1:3 diluted baboon sample at 37 °C for 1 min before adding a 30-\mu l aliquot of the mixture to the APTT card. The protein C activity of the sample was expressed as a percentage of the normal value using the standard curve generated using pooled baboon plasma.

**RESULTS**

Prior to thrombin administration in vivo, APC was shown to anticoagulate baboon and human plasma, producing comparable prolongation of the APTT in both cases (data not shown). WE was at least 6000-fold less procoagulant than WT in baboon plasma (data not shown). It was thus determined that either agent could be safely administered to baboons. The antithrombotic and antihemostatic effects of escalating doses of WE and exogenous APC were determined in baboons. Thrombosis was assessed by \gamma camera imaging of 111\textit{i}ndium-labeled platelet

**TABLE I**

| Change in platelet count, fibrinogen level, protein C level, and bleeding time following no treatment (control) or treatment with three different doses of APC and five different doses of WE in baboons | Agent (dose in mg/kg) |
|---|---|---|---|---|---|---|---|---|---|
| | WE (0.22) | WE (0.11) | WE (0.055) | WE (0.022) | WE (0.011) | APC (0.45) | APC (0.2) | APC (0.1) | Control (0) |
| Thrombus (+, yes; −, no) | − | − | + | + | + | + | + | + | + |
| Change in platelet count (%)* | ND* | ND* | −1.6 ± 3.6 | 1.2 ± 4.2 | 3.1 ± 3.5 | −3.3 ± 1.8 | 1.6 ± 2.6 | −6.5 ± 4.8 | −13.3 ± 1.3 |
| Change in fibrinogen level (%)* | ND* | ND* | 0.63 ± 2.9 | 0.90 ± 2.7 | 0.46 ± 1.8 | 0.25 ± 0.2 | 0.48 ± 0.2 | 0.96 ± 0.001 |
| Change in protein C level (%)* | ND* | ND* | 3.2 ± 2.9 | −0.5 ± 2.7 | −1.8 ± 1.6 | 2.7 ± 0.2 | −2.2 ± 2.7 | −5.9 ± 2.9 | −10.5 ± 1.3 |
| Change in bleeding time (0 vs. 100 min) (s); mean ± s.E.M. | ND* | ND* | 0.38 ± 0.001 | 0.93 ± 0.02 | 0.31 ± 0.007 | 0.057 ± 0.07 | 0.507 ± 0.163 | 0.012 |
| Change in bleeding time (0 vs. 40 min) (s); mean ± s.E.M. | 0.014 | 0.020 | 0.029 | 0.025 | 0.002 | 0.072 | NA** | 0.761 | 0.789 |
| Change in bleeding time (0 vs. 40 min) (s); mean ± s.E.M. | 0.068 | 0.099 | 0.350 | 0.666 | 0.397 | 0.047 | 0.346 | 0.707 | 0.807 |

* Given as the average percent changes in three study subjects; duplicate original measurements where applicable.
** Not determined because these samples were not collected.
** Probability of difference (p value) between measured values "pre" and "post" treatment was determined using the t test (paired, two-tailed).
*** Not applicable.
deposition following placement of a thrombogenic Dacron graft segment into a femoral AV shunt. In the three control experiments, total thrombus platelet deposition averaged 17.9 ± 5.7 × 10⁶ platelets after 60 min of blood exposure. Both WE and high dose APC inhibited thrombosis as shown by the decrease in platelet deposition versus the results obtained in the corresponding untreated control animals (Fig. 1). At 70 min, the highest dose of WE, 0.055 mg/kg, reduced platelet deposition by 82%, and this reduction was statistically significant (p < 0.001). The lower doses of WE, 0.022 and 0.011 mg/kg, reduced platelet thrombus formation by 77% (p < 0.001) and 80% (p < 0.01), respectively. Similarly, as shown in Fig. 1, the highest dose of APC, 0.45 mg/kg, reduced thrombus formation by 71% (p < 0.001), while the lower doses of APC, 2.0 and 1.0 mg/kg, were less effective and reduced platelet deposition by 32% (p = 0.078) and 49% (p < 0.01), respectively. The differences between the lower doses of either WE or APC were not statistically significant for dose response. Concordant with these findings, platelet count and fibrinogen levels decreased moderately due to thrombus formation in untreated controls but not in WE-treated or APC-treated animals (Table I).

At the end of the thrombosis experiments, fibrin deposition averaged 1.9 ± 0.07 mg in the control studies (Fig. 2). This value was not significantly reduced by infusion of APC at doses of 0.45 mg/kg (1.8 ± 0.3 mg, p > 0.5), 0.2 mg/kg (1.7 ± 0.2 mg, p > 0.5), or 0.1 mg/kg (2.0 ± 0.3 mg, p > 0.5), or 0.1 mg/kg (2.0 ± 0.3 mg, p > 0.5). In contrast, as shown in Fig. 2, WE reduced fibrin accumulation at doses of 0.055 mg/kg (0.9 ± 0.1 mg, p < 0.05), 0.022 mg/kg (1.3 ± 0.2 mg, p < 0.05), or 0.011 mg/kg (1.2 ± 0.1 mg, p < 0.05).

Pretreatment template bleeding time averaged 4.6 ± 0.3 min. The highest dose of APC modestly increased the template bleeding time, but all bleeding times remained below 10 min in each group (Table I). Both WE and APC treatments compromised coagulation-dependent hemostasis at all doses administered as reflected by significant systemic anticoagulation within 10 min after dosing (Fig. 3). After the observation period of 100 min, APTT values had returned to pretreatment base-line levels in animals treated with APC (p > 0.8 for each dose group) but remained prolonged in animals treated with WE at doses of 0.022 mg/kg and greater (p < 0.03 for each dose group). The prolongation of APTT at 10 and 40 min after injection of WE was positively correlated with the dose administered (r² = 0.89 at the 10-min time point, and r² = 0.93 at the 40-min time point). The APTT prolongation after bolus APC was also dose-dependent.
DISCUSSION

Most agents that exhibit antithrombotic effects, including APC, compromise blood coagulation and platelet-dependent hemostasis to some degree. This was confirmed in the present study since both APC and WE prolonged the APTT. Since the prolonged APTT of samples taken from WE-treated and APC-treated animals was found to progressively and comparably decrease during incubation, the observed anticoagulant effects can be attributed to the presence of circulating APC. The systemic anticoagulant response to all doses of WE persisted longer than the response to exogenous APC in the present study, or the response to WT in a previous baboon study (4), suggesting more persistent maintenance of circulating APC levels by WE.

The laboratory diagnosis of ongoing thrombosis or disseminated intravascular coagulation is suggested by an acute decrease in circulating fibrinogen and/or platelets. Interestingly all doses of WE prevented circulating fibrinogen and platelet consumption, notwithstanding the procoagulant stimulus provided by placement of thrombogenic devices within an ex vivo circuit. Thus, unlike higher doses of WT (4), WE activates the endogenous protein C pathway without producing clinically relevant procoagulant (fibrinogen consumption) or prothrombotic (platelet consumption) effects. This finding confirms that the WE mutant exhibits minimal procoagulant activity in vivo.

The dynamics and time course of arterial-type thrombus formation in this standardized baboon model are similar to events following the rupture of a coronary plaque (14). A significant percentage of untreated graft devices occlude within 1–2 h, and the pharmacological inhibition of graft thrombus formation and occlusion, comparable to that produced in the present study with WE, usually requires relatively large doses of antithrombotic agents like antithrombins (14) and inhibitors of the platelet fibrinogen receptor (15), which may produce increased bleeding. It was therefore remarkable that administration of the lowest dose of WE resulted in a profound and persistent antithrombotic effect that was accompanied by only modest impairment of coagulation and hemostasis. WE produced a near maximum effect at all doses tested, and no apparent dose-response relationship was found over doses ranging from 0.011 to 0.055 mg/kg, suggesting that the minimum efficacious dose of WE could be much lower than the 0.011 mg/kg dose evaluated in the present thrombosis model. Importantly the lowest dose of WE (0.011 mg/kg) appeared to be equi-efficacious with the highest dose of APC (0.45 mg/kg) since at these doses both agents potently and comparably inhibited platelet deposition. Inhibition of fibrin deposition by APC could not be confirmed probably because anticoagulation by exogenous APC was less persistent than that of WE throughout the 60-min interval of fibrin formation.

Whereas administration of APC had no effect on plasma protein C activity, a significant fraction of circulating protein C was consumed following injection of high dose WE resulting in partial protein C depletion. Based on the observed dramatic anticoagulant effects of endogenous APC produced following high dose WE, we suggest that activation of the endogenous protein C pool has the capacity for sustained antithrombotic as well as anticoagulant activity. Indeed it is possible that a significant proportion of the circulating protein C pool could be chronically activated by pharmacological doses of WE provided there is a sufficient endogenous or exogenous supply of protein C and thrombomodulin. Thus, a stable protein C activator such as WE could eventually achieve sustained pharmacological activation of the protein C pool for the treatment of thrombotic disorders that respond to APC.

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