Inhibition of Thrombus Formation by Activated Recombinant Protein C in a Primate Model of Arterial Thrombosis

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Activated protein C (APC) is an antithrombotic enzyme. The therapeutic potential of infused human recombinant APC (rAPC) was studied in a primate model of platelet-dependent thrombosis. Eight baboons with chronic femoral arteriovenous shunts received rAPC infusions for 1 hour. The shunts were extended with 5-cm long, 4-mm-i.d. thrombogenic Dacron graft segments for the time of infusion. The plasma level of the enzyme, the blood flow in the shunt, and the deposition of indium-111-labeled platelets and iodine-125 fibrinogen on the graft were measured. The influence of rAPC infused at doses of 0.25 and 1.0 mg/kg-hr was compared with the effects of control infusions of saline. Five of eight control grafts occluded within 60 minutes, whereas there was no change in the blood flow during rAPC infusion. Deposition of platelets was inhibited by 13±10% and by 42±13% (mean±SEM) after 30 minutes of infusion at the two doses, which gave rise to circulating rAPC plasma concentrations of 0.4 and 1.9 mg/l, respectively. Both doses significantly inhibited fibrin deposition in the graft. Circulating plasma markers of thrombus formation and of fibrinolysis did not increase significantly during rAPC infusion; measurements of bleeding time were also within normal limits. Thus, rAPC, like human plasma-derived APC, inhibited thrombus formation without impairing primary hemostasis. (Circulation 1990;82:578–585)

Activated protein C (APC) is an antithrombotic serine protease. It is generated from vitamin K-dependent plasma protein C (PC) by the catalytic complex of thrombin and thrombomodulin. APC circulates in blood at a plasma level of 4 mg/l. APC acts by inhibiting thrombin formation by means of enzymatic cleavage and destruction of coagulation factors Va and VIIIa, thus providing negative feedback regulation of coagulation. The physiological importance of APC is shown by clinical observations that 1) the incidence of heterozygous PC deficiency is higher in patients with thrombophilia (4%) than among healthy individuals (0.4%); 2) there is a positive correlation between thrombotic events and heterozygous PC deficiency in some thrombophilic families; 3) severe thrombophilia of homozygous protein C-deficient infants is successfully controlled with either repeated replacement of protein C or liver transplantation; and 4) isolated acquired PC deficiency may be associated with serious thrombotic complications.

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Experimental results obtained in various animal species indicate that purified plasma-derived APC is a potent and safe antithrombotic agent when used in pharmacological doses. The enzyme inhibits thrombus formation in microcirculatory, venous, and arterial thrombosis models.

In this study, we evaluated the effects of activated recombinant protein C (rAPC) in a nonhuman primate model of arterial thrombosis and found that infusion of rAPC inhibited platelet-dependent thrombus formation at plasma levels that did not impair primary hemostasis.

Methods

Experimental protocol

Eight young, healthy male baboons (Papio anubis), weighing 9–13 kg and bearing chronic femoral arterio-
venous shunts, were used in the experiments. All animal procedures, including preparation of the shunts, insertion of thrombogenic grafts, and detection of thrombus formation, were performed as described elsewhere. This model has been shown to provide reproducible results in repeated experiments in the same animal, that is, grafts may be evaluated at daily intervals for up to 4 days with equivalent results. All studies were approved by the institutional animal care and use committee in accordance with federal guidelines.

In brief, the baboons were injected with indium-111–labeled autologous platelets (1 mCi) before the control experiment and with iodine-125–labeled fibrinogen (FGN) (0.005 mCi) before each experiment. At the time designated 0 minutes, the shunts were extended with a 5-cm-long, 4-mm-i.d. Dacron graft segment. Thrombus formation in these grafts was observed both in real time and retrospectively. The real-time observations involved radioimaging of In-labeled platelet deposition with a scintillation camera and concurrent measurement of the shunt blood flow rate using a Doppler flowmeter. Increased platelet deposition and reduced blood flow were positive signs of thrombus formation. Because deposition of platelets from circulating blood is directly related to exposure time and circulating platelet count, the inhibition of platelet deposition was normalized with respect to control values as described elsewhere. The grafts were removed if occlusion occurred before 60 minutes from the beginning of the experiment and were washed and stored in 2.5% glutaraldehyde. I-labeled FGN radioactivity was measured in counts per minute (cpm) after allowing the graft to decay for at least 30 days. At 30 days, the remaining platelet-bound In radioactivity was 0.006% of the value at 0 minutes, compared with the 70% of In radioactivity that remained at 30 days. The remaining In radioactivity was subtracted from the total radioactivity and total fibrin deposition was calculated by dividing this value by the plasma radioactivity determined at the same time as the graft radioactivity (cpm/ml) and multiplying by the circulating FGN concentration (mg/ml).

The animals were physically examined and carefully observed for signs of spontaneous bleeding, including determinations of hematocrit and hemoglobin before and after each experiment. Standardized template bleeding times were performed before and during rAPC infusion to judge the effect of APC on primary hemostasis.

Simultaneously with the initiation of blood flow, rAPC was infused proximal to the graft at rates of either 0.25 or 1.0 mg/kg-hr, as described in earlier experiments. One third of the enzyme dose was given as a bolus and the remaining two thirds were given by continuous infusion. On the first day of the study, each animal had a control infusion of saline, and on each of two subsequent days, each received one lower and one higher dose of rAPC.

The rAPC was activated from recombinant protein C (rPC) using immobilized thrombomodulin-thrombin. The preparation, purification, and characterization of rAPC has been described elsewhere. The specific activity of APC was determined in a clotting assay. Briefly, the assay was based on the ability of APC to prolong the activated partial thromboplastin time (APTT). Normal human plasma (NHP) or rAPC was diluted and incubated in Protac®, American Diagnostica Inc., Greenwich, Conn., a snake-venom activator of PC. PC-depleted human plasma and APTT reagent were then added and the mixture was clotted by recalcification. The specific activity of APC was determined by comparison of results using standard dilutions of the NHP and of the enzyme.

The anticoagulant activity of the circulating rAPC was determined from citrated plasma samples in an APTT assay using a fibrometer. The assay was performed within 5 minutes of blood sampling to minimize in vitro inhibition of the enzyme by its physiological plasma protease inhibitors. Standard dilutions of rAPC were made in vitro using a preinfusion plasma sample from each animal, with the results given as a standard reference curve for each animal. Circulating levels of procoagulant factors VIII and V (FVIII and FV) were measured with factor deficient human plasma (George King Biomedical, Inc., Overland Park, Kan.) after inhibition of the anticoagulant rAPC in the samples. The rAPC in 10 μl citrated baboon plasma was inhibited by addition of 20 μl immunoaffinity-purified polyclonal sheep anti-human PC antibodies (αPC-pab, 3 μM final). The αPC-pab was purified from sera of immunized sheep (kindly provided by Dr. H.P. Schwarz, Vienna, Austria). The specific IgG from the IgG fraction was absorbed to a Sepharose column containing immobilized human PC, eluted with 4 M guanidine, and dialyzed into Tris-buffered saline. After incubation of the samples with αPC-pab for 460 seconds at 37°C in Tris-buffered saline (pH 7.4) containing 0.5% casein in a final volume of 40 μl, 100 μl APTT reagent and factor-deficient human plasma were added and incubated for 200 seconds, and the clotting time was determined after recalcification with 100 μl CaCl₂ (25 mM). Pooled normal baboon plasma (NBP, 10 μl) incubated with rAPC (10 μl, 60 nM) and αPC-pab (20 μl) was used as a reference standard. The αPC-pab abolished the anticoagulant activity of 60 nM APC added to NBP or to the 0-minute baboon samples. The APTT was longer than 2 minutes with rAPC and without αPC-pab, 52.3±3.8 seconds (n=9) with both rAPC and αPC-pab, and 50.5±4.3 seconds (n=7) without APC and with αPC-pab. The FV and FVIII levels were determined as percentages of control values using the NBP standard curve.

The amidolytic activity of the circulating rAPC and the circulating levels of the enzyme were determined in a solid phase assay as described. Briefly, the enzyme in plasma was protected from its physiological inhibitors with citrate/benzamidine acting as anti-
coagulants. It was separated from plasma using an immobilized monoclonal anti-human PC light-chain antibody (designated C3), and the enzymatic activity bound to the immobilized antibody was measured using a chromogenic substrate.

The effect of rAPC on in vitro clot lysis was determined by measuring radiolabeled FGN and fibrin degradation products released into the sera of whole blood clots formed ex vivo. Immediately after drawing 0- and 45-minute arterial blood samples (0.5 ml) from baboons receiving rAPC infusion, the blood was mixed and clotted in glass tubes containing 10 µl human thrombin (1 IU/ml final concentration) with or without 10 µl tissue-type plasminogen activator (t-PA; 15 ng/ml final concentration; t-PA kindly provided by Dr. D. Collen, University of Leuven, Belgium). After incubation for 20 hours at 37°C to allow coagulation and fibrinolysis to proceed, the clots were centrifuged (3,000g for 10 minutes). One hundred microliters of supernatant (sera) was separated and both the sera and the pellet (clot plus residual sera) were stored for 30 days, allowing 99.99% of the pellet to decay before determination of 125I radioactivity in a gamma counter (model 4/600, Micromedic Systems, Harrsham, Pa.). An increase in the percentage of nonsedimenting radioactivity (in sera) compared with total radioactivity (in whole blood sample) following rAPC infusion (45-minute sample compared with the 0-minute sample) was an indicator of the enhancement of whole blood clot lysis due to rAPC.

Indirect markers of thrombus formation (e.g., FGN, fibrinopeptide A [FPA], β-thromboglobulin [βTG], platelet factor 4 [PF4], and D-dimer levels in plasma samples) as well as measurements of bleeding time, circulating platelet count (PLC), hemoglobin concentration (HB), and hematocrit (HT) were determined as described elsewhere.16,17,19

Statistical analyses of the results were performed using either the Mann-Whitney U test without assumptions about the normality of the population distribution or by analysis of variance (Studentized range test) when more than two means were compared,20 unless otherwise stated. Values are given as mean ± SEM unless otherwise stated.

Results

Circulating Levels of Activated Recombinant Protein C

To measure the rAPC activity, blood samples were drawn at 0, 15, 60, 75, and 90 minutes after initiation of blood flow through the Dacron graft. The infusion of rAPC started at 0 minutes and was terminated at 60 minutes. Figure 1 (panels A and B) illustrates the plasma levels of the enzyme calculated from the results of the clotting and amidolytic assays when rAPC was infused at two different doses. The level of circulating endogenous APC that might have been generated during control experiments was below the detection limit of both assays. In the lower-dose experiments (Figure 1A) the two activity assays gave similar results. rAPC activity in plasma was 0.42±0.05 mg/l (amidolytic assay) or 0.39±0.06 mg/l (anticoagulant assay) at 15 minutes and 0.34±0.04 mg/l (amidolytic) or 0.49±0.17 mg/l (anticoagulant) after 60 minutes of infusion. Circulating rAPC activity decreased to one half of the 60-minute value (amidolytic) by 8–10 minutes after termination of the infusion. During infusion of 1.0 mg/kg-hr rAPC (Figure 1B) the APTT values increased from 38 seconds to more than 2 minutes. Therefore, the plasma levels of rAPC were calculated from the amidolytic activity only. Circulating rAPC levels of 2.0±0.2 mg/l at 15 minutes and of 1.9±0.2 mg/l at 60 minutes were measured. The enzymatic activity of circulating rAPC decreased by 50% in 12 minutes after the infusion was terminated at 60 minutes. The difference between the rAPC levels of the samples from the higher- and lower-dose infusion experiments was statistically significant (p<0.01, n=8 in each group).

Antithrombotic Effects

Graft patency. Blood flow rates averaged 195±46 ml/min (26 cm/sec) through the 4-mm-i.d. graft segment. This rapid arterial flow resulted in the forma-
tion of platelet-rich thrombi, as shown previously by
scanning electron microscopy. This process gener-
ally results in graft occlusion. Figure 2 summarizes
the measurements of graft patency. Five of eight
control grafts occluded within 60 minutes when no
rAPC was infused. In 16 infusions of either the lower
or the higher dose of rAPC, none of the grafts
occluded, and blood flow rates did not change over
the 60 minute study interval.

Platelet deposition. Platelet deposition measure-
ments determined from real-time imaging are shown
in Figure 3 (panel A). The steady increase in platelet
deposition observed with all control grafts was inter-
rupted by thrombotic occlusion in five grafts. The
three control grafts which were patent at 60 minutes
contained an average of 13.5±1.2×10⁹ deposited
platelets. All eight grafts in the experiments with the
lower dose of rAPC were patent at 60 minutes and
contained 10.2±1.5×10⁹ deposited platelets. Platelet
thrombus accumulation was further reduced to
4.1±0.6×10⁹ platelets when 1.0 mg/kg-hr rAPC was
infused, a value that was significantly less than that
seen after the infusion of the 0.25 mg/kg-hr dose
(p<0.02). At 30 minutes, platelet deposition was
inhibited, compared to control values, by 12.8±9.6%
(n=7, p>0.05) when rAPC was infused at the lower
dose and by 42.3±13.2% (n=7, p<0.01) when the
higher dose was given. Inhibition of platelet deposition
was not calculated in one baboon because the
control graft occluded at 25 minutes.

Fibrin deposition. The effect of rAPC infusion on
total fibrin deposition was determined in six animals.
Figure 3B summarizes the results calculated from the
measurements of ¹²⁵I-related graft radioactivity. The
average termination time of those experiments was at
43 minutes due to thrombotic occlusions in four of
the six control studies. A mean of 7.09±0.78 mg fibrin
accumulated in the six control grafts. When 0.25
mg/kg-hr rAPC was infused into six animals, fibrin
deposition averaged 4.05±0.50 mg after 60 minutes.
After infusion of 1.0 mg/kg-hr rAPC for 60 minutes,
2.8±0.44 mg fibrin was contained in the grafts.
Analysis of variance (one-way, p<0.05) and rank test
(two-tailed, p<0.01) indicated that the three samples
were significantly different from each other. No valid
comparison could be made between the control and
rAPC treatment experiments because of the early
occlusions in controls.

Circulating markers of thrombus formation and
thrombolysis. Table 1 summarizes the results of deter-
minations of plasma levels of FPA, βTG, and PF4.
Levels of circulating markers of fibrin formation
(FPA) and platelet activation (βTG, PF4) were
significantly increased at the end of the control
experiments. Infusion of 0.25 mg/kg-hr rAPC pre-
vented the increase in FPA levels but only modestly
reduced the elevation in levels of PF4 and βTG; that
is, the lower dose of rAPC infusion did not prevent
the increase in circulating markers of platelet activa-
tion. However, after infusion of 1.0 mg/kg-hr rAPC
no significant elevation of FPA, PF4, or βTG levels
could be detected. D-Dimer levels were not elevated
significantly after 45 minutes of saline infusion in
controls and did not change after rAPC infusion
compared with the control or preinfusion values. The
Table 1. Changes in Circulating Markers of Thrombosis in Baboons Infused With Activated Recombinant Protein C

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sample</th>
<th>0</th>
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<th>1.0</th>
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<tr>
<td>PLT (x10^9/l)</td>
<td>Pre</td>
<td>310±40</td>
<td>380±55</td>
<td>360±45</td>
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<tr>
<td></td>
<td>Post</td>
<td>240±33</td>
<td>365±43</td>
<td>370±50</td>
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<td>p&lt;0.05</td>
<td>p&gt;0.05</td>
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<td></td>
<td></td>
<td>p&lt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>PF4 (µg/l)</td>
<td>Pre</td>
<td>5.1±3.2</td>
<td>8.3±3.5</td>
<td>6.9±2.8</td>
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<tr>
<td></td>
<td>Post</td>
<td>22.0±15.6</td>
<td>14.8±5.5</td>
<td>9.3±1.6</td>
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<tr>
<td></td>
<td></td>
<td>p&lt;0.02</td>
<td>p&lt;0.05</td>
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<td>p&lt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
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<tr>
<td>βTG (µg/l)</td>
<td>Pre</td>
<td>4.0±2.7</td>
<td>4.6±2.4</td>
<td>4.6±5.9</td>
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<tr>
<td></td>
<td>Post</td>
<td>27.3±8.6</td>
<td>12.5±5.4</td>
<td>6.6±3.0</td>
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<tr>
<td></td>
<td></td>
<td>p&lt;0.02</td>
<td>p&lt;0.05</td>
<td>p&gt;0.05</td>
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<tr>
<td>FGN (g/l)</td>
<td>Pre</td>
<td>3.9±1.1</td>
<td>2.7±0.3</td>
<td>3.3±0.7</td>
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<tr>
<td></td>
<td>Post</td>
<td>3.7±1.5</td>
<td>2.6±0.4</td>
<td>3.3±0.6</td>
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<tr>
<td></td>
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<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>FPA (pM)</td>
<td>Pre</td>
<td>3.9±0.9</td>
<td>4.5±1.7</td>
<td>4.5±2.6</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>18.0±6.0</td>
<td>4.6±1.4</td>
<td>3.9±1.8</td>
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<td>p&lt;0.05</td>
<td>p&gt;0.05</td>
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<tr>
<td>D-Dimer (ng/l)</td>
<td>Pre</td>
<td>320±130</td>
<td>260±150</td>
<td>450±240</td>
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<td></td>
<td>Post</td>
<td>430±140</td>
<td>270±160</td>
<td>420±290</td>
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<td></td>
<td>p&gt;0.05</td>
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rAPC, activated recombinant protein C; PLT, circulating platelet count; PF4, platelet factor 4; βTG, β-thromboglobulin; FGN, fibrinogen; FPA, fibrinopeptide A.

Values are given as mean±SEM of results determined from plasma or blood samples taken at 0 minutes (pre) and after 45 minutes of rAPC infusion, or earlier if occlusion had occurred before 45 minutes, as in some controls (post). The probability values (p) for the difference between the pre and post data are given for each marker within each column below the mean values for pre and post results. Comparisons (p) of the peak values for each marker at 0.25 or 1.0 mg/kg-hr (second and third columns) to the peak value in the control study with no rAPC (first column) are displayed on horizontal lines.

In Vitro Parameters of Hemostasis

Bleeding time. Preinfusion bleeding times averaged 4.5±1.2 minutes (n=16). Plasma levels of circulating rAPC and measurements of bleeding time (marker of primary hemostasis) were not significantly correlated, as neither the lower nor the higher dose of rAPC prolonged the bleeding time significantly (5.2±1.3 minutes and 5.1±1.5 minutes, respectively [n=8]). No rebleeding was observed within 30–40 minutes from the standardized cutaneous cuts made between 15 and 30 minutes after initiation of rAPC infusion. There was no sign of extravasation of blood during rAPC infusion; that is, no ecchymoses or hematomas appeared on visible areas of the body and there was no oozing from small cuts. A minimal but significant drop in PLC was observed shortly after completion of control experiments; however, there was no change in PLC when rAPC was infused during perfusion of the graft. There was no significant acute fibrinogen consumption during the perfusion of the grafts (Table 1). HB and HT values did not change significantly in the control and rAPC infusion experiments. The animals' pulse rates and the blood flow in the grafts remained stable.

In Vivo Parameters of Hemostasis

Clotting time. Infusion of rAPC caused a prolongation of the clotting time, a marker of secondary hemostasis. The APTT increased from 36±1 seconds to 75±5 seconds in the lower-dose experiments and from 36±2 seconds to 160±18 seconds in the higher-dose experiments after 15 minutes of rAPC infusion. The corresponding values at the end of the 60-minute infusions were 85±7 seconds and more than 180 seconds. FV activity was decreased by 2.4±13.4% at the end of the experiments with the lower dose of rAPC and by 14.6±6.1% at the end of the experiments with the higher dose. The corresponding values for FVIII activity were decreased by 12.4±11.1% and 11.5±14.5%, respectively. The decrease in the procoagulant activity of circulating FV and FVIII was not statistically significant (p>0.05 for all measurements).

Profibrinolytic effect. t-PA at a low concentration of 15 ng/ml incorporated into whole blood clots in vitro induced a nonsignificant increase in fibrinolysis as measured by detection of 125I-FGN-related radioactivity in the sera after 20 hours of incubation (Table 2; preinfusion samples). Infusion of 0.25 mg/kg-hr rAPC had no effect on the in vitro fibrinolysis under these assay conditions. No increase in fibrinolysis following rAPC infusion could be detected when the clot did not...
TABLE 2. Effect of Infused Activated Recombinant Protein C on Whole Blood Clot Lysis Induced by Tissue-Type Plasminogen Activator

<table>
<thead>
<tr>
<th>Dose of rAPC (mg/kg-hr)</th>
<th>Radioactivity in sera (%) of whole blood</th>
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<tr>
<td>0.25</td>
<td></td>
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<tr>
<td>t-PA added</td>
<td></td>
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<tr>
<td>None</td>
<td>Pre, 40±17</td>
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<tr>
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<td>Post, 35±13</td>
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<tr>
<td>15 ng/ml</td>
<td>Pre, 43±18</td>
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<td></td>
<td>Post, 42±16</td>
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<td>p&lt;0.05</td>
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</tbody>
</table>

rAPC, activated recombinant protein C; t-PA, tissue-type plasminogen activator.

Values represent soluble 125I-fibrin radioactivity in sera as a percentage of the radioactivity in the whole blood sample after 24-hour incubation. n=4 for each group. Blood was drawn from baboons at 0 minutes (pre) and at 45 minutes (post) after initiation of rAPC infusion and clotted using 1 unit/ml thrombin±15 ng/ml t-PA (final concentrations). Pre and post results were compared statistically to give indicated p values.

contain exogenous t-PA. However, when the clot was prepared from blood samples taken after infusion of 1.0 mg/kg-hr rAPC and the tubes also contained t-PA, there was a significant increase in the clot supernatant radioactivity (from 40±11% in the 0-minute sample to 75±18% at 45 minutes, p<0.02), suggesting that some enhanced clot lysis occurred.

Discussion

Because plasma-derived APC was previously shown to be a potent antithrombotic agent at plasma levels that did not compromise primary hemostasis, the antithrombotic activity of rAPC was studied with the same experimental model of platelet-dependent arterial thrombosis. Infusion of rAPC gave plasma levels of rAPC similar to those of the plasma-derived material seen in our previous experiments. The circulating half-life of rAPC after continuous infusion was stopped was somewhat shorter (8–12 minutes) than that of plasma-derived APC (12–15 minutes). This finding may indicate minor differences in the inhibition or clearance of plasma-derived APC compared with the genetically engineered material.

The APTT was a useful indicator of the circulating level of the enzyme when measured immediately, that is, within 2–5 minutes after blood drawing; however, the accuracy of the assay was limited at higher rAPC plasma levels due to prolongation of the clotting time to more than 2 minutes. Another limitation of the clotting assay for APC activity was its lack of specificity. The APC amidolytic assay was very specific and more sensitive than the APTT by at least one order of magnitude. Thus, the APTT assay was used as a complementary assay for determination of the level of the circulating enzyme. Because the APTT could be measured quickly, it proved reliable and useful for rapid determinations of circulating rAPC at plasma levels lower than 1 mg/l.

The observation that none of the grafts occluded during rAPC infusion provided evidence that the human recombinant enzyme was antithrombotic. Because plasma levels of 0.4 mg/l rAPC prevented thrombotic occlusion, the qualitative method of simply measuring blood flow was of no use for quantitating the antithrombotic effect of rAPC. Using this qualitative method, rAPC was previously shown by others to inhibit the thrombotic occlusion of experimental arteriovenous shunts in baboons.

Information regarding dose-response effects was obtained by dynamic measurements of platelet and fibrin thrombus formation. Inhibition of platelet deposition was evident at 30 minutes in experiments with the higher dose of rAPC. However, the antithrombotic effect of rAPC in the lower-dose rAPC infusion experiments was not evident at 30 minutes. The inhibition of platelet deposition could not be determined in experiments carried out to 60 minutes due to progressive occlusion of five control grafts. The dose-dependency results at 30 minutes were somewhat different from our previous experience with plasma-derived APC, in which APC had a more pronounced inhibitory effect on platelet deposition (43% and 76% inhibition for the 0.25 and 1.1 mg/kg-hr APC infusion experiments, respectively). This difference between rAPC and plasma-derived APC was statistically significant (p<0.03 for the lower dose and p<0.05 for the higher dose). Because rAPC was at least as effective an anticoagulant as plasma-derived APC in the APTT assay, no obvious explanation exists for this difference in antithrombotic effects.

The fibrin content of the graft thrombus at the end of the experiment was negatively correlated with the plasma level of rAPC (r = −0.89); the mean fibrin deposition values after infusion of 0.25 and 1.0 mg/kg-hr rAPC for 60 minutes were lower than the control values. Although the two means of fibrin deposition values were slightly different in the lower and in the higher rAPC infusion dose experiments, suggesting dose-dependency for the antifibrin effect, they did not reflect the difference in the circulating rAPC levels. Because these results represent end point determinations, the time course of inhibition could not be quantitated as in the case of platelet deposition. Establishing the dose-dependency might require several dose range studies.

Plasma markers of fibrin formation (FPA), platelet activation (βTG and PF4), and fibrinolysis (d-dimer) remained at baseline levels after infusion of 1.0 mg/kg-hr rAPC. Because FPA, βTG, and PF4 levels increased during control experiments, these data further document the potent antithrombotic activity of rAPC in this model. Procoagulant FV and FVIII levels did not decrease significantly during control or rAPC infusion experiments, suggesting that circulating rAPC, at plasma levels of up to 2 μg/ml for 60
minutes, does not deplete FV or FVIII or produce an acquired coagulation factor deficiency.

As observed in studies with plasma-derived APC,25 rAPC had no effect on the bleeding time, even at a plasma level of 1.9 mg/l. This finding is remarkable because most agents that are antithrombotic in this thrombosis model also impair primary hemostasis, as reflected by prolonged bleeding times. These agents include a synthetic peptide-chloromethyl ketone inhibitor of thrombin (PPACK),17 hirudin,22 monoclonal antibodies against platelet glycoprotein IIb/IIIa,19 and some clinically used antiplatelet drugs.23

The mechanism of the antithrombotic effect of APC in the platelet-dependent thrombosis model is not completely clarified. APC has no documented antiplatelet activity in vitro; for example, it does not inhibit platelet aggregation or adhesion. Although APC acts as an anticoagulant by preventing thrombin generation, therapeutic doses of another antithrombin anticoagulant (heparin, 100 units/kg) do not inhibit platelet deposition.24 Hirudin, the most specific known inhibitor of thrombin was, however, shown to be a potent antithrombotic agent in the same model.22 Therefore, we think that the antifibrin and antiplatelet effects of APC are related to its inhibition of thrombin generation and that platelet deposition in this model of arterial thrombosis is a thrombin-dependent process.

The ex vivo experiments on the profibrinolytic effect of rAPC were performed to examine whether rAPC could enhance the profibrinolytic potential of t-PA in this primate, because enhancement of fibrinolysis could contribute to the antithrombotic effect of the enzyme. Incorporation of 1.9 mg/l circulating rAPC into ex vivo–formed blood clots promoted t-PA–induced fibrinolysis. Assuming that circulating rAPC was also incorporated into graft thrombi, enhancement of fibrinolysis might have occurred in vivo as well, in accord with the results of other investigators.25,26 However, clot lysis in vitro was not increased in the absence of exogenous t-PA, and the d-dimer levels during infusion of both plasma-derived APC15 and rAPC did not increase, failing to demonstrate induction of fibrinolysis as a result of APC infusion. These data, however, do not exclude the possibility of enhancement of fibrinolysis, because fibrin generation is inhibited in the presence of APC. Thus, the suggested in vivo prothrombolytic effect of APC, which has been based on in vitro or ex vivo studies, remains unproven.

In conclusion, rAPC, like plasma-derived APC, was found to be an antithrombotic agent in a nonhuman primate model of arterial thrombosis. The importance of this observation was enhanced by the fact that rAPC, like APC, did not impair primary hemostasis as reflected in bleeding time measurements at antithrombotic plasma levels, despite its profound effect on the coagulation time in vitro. Moreover, recent studies showed that infusion of urokinase combined with APC in our thrombosis model resulted in a net additive effect of the antithrombotic activities of each agent.27 Thus, infused rAPC is a candidate for immediate and transient inhibition of arterial thrombotic events, for example, clot formation during cardiac surgery or rethrombosis after thrombolitic therapy, percutaneous transluminal coronary angioplasty, or endarterectomy.

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References


**KEY WORDS** • platelets • grafts • fibrinolysis
Inhibition of thrombus formation by activated recombinant protein C in a primate model of arterial thrombosis.

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