Prevention of Arterial Reocclusion After Thrombolysis With Activated Protein C
Comparison With Heparin in a Canine Model of Coronary Artery Thrombosis

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Background Reocclusion of recanalized coronary arteries often limits the efficacy of coronary thrombolytic therapy in patients with acute myocardial infarction. Activated protein C (APC) is an important regulatory enzyme in hemostasis. In view of the potential of human APC as an anticoagulant and profibrinolytic agent, the effect of APC on thrombolysis with recombinant tissue-type plasminogen activator (rTPA) was studied in a canine model of coronary artery thrombosis.

Methods and Results Continuous artery flow monitoring in the left anterior descending coronary artery of 30 anesthetized adult beagles was performed by a magnetic flowmeter. Localized thrombosis was produced in the left anterior descending coronary artery and administration of rTPA (alteplase, 0.45 mg/kg IV) was done for 30 minutes. The dogs were randomly assigned to receive one of the following intravenous adjunctive therapies: (1) control group (n=10): human albumin at a rate of 0.83 mL/min; (2) APC group (n=10): human plasma-derived APC (0.6 mg/kg) with human albumin as a vehicle at a rate of 0.83 mL/min; and (3) heparin group (n=10): heparin (200 U/kg) with saline at a rate of 0.83 mL/min. Each adjunctive therapy was started simultaneously with rTPA and lasted for 60 minutes. Coronary reocclusion occurred in all dogs of each adjunctive treatment group in 19.1±1.9 minutes (mean±SEM). In a 120-minute observation after the termination of rTPA, reocclusion developed in all the dogs in the control and heparin groups but in only 3 of the 10 dogs in the APC group (P<.002 versus control and heparin). Time from reocanlization to reocclusion (minutes, mean±SEM) was prolonged in the APC group (103.2±14.2) as compared with the control (10.2±2.3, P<.001) and heparin (30.3±11.8, P<.002) groups. Activated partial thromboplastin time was prolonged similarly in each group after thrombolytic therapy. On the other hand, bleeding time was prolonged in only the heparin group after the treatment. Serious hemorrhagic side effects were not observed in all three groups.

Conclusions APC prevents coronary artery reocclusion after recanalization with rTPA in a canine model of coronary artery thrombosis. This finding suggests that APC may be useful as an adjunctive treatment to enhance the effects of thrombolytic therapy in patients with acute myocardial infarction. (Circulation. 1994;90:427-432.)

Key Words • coronary thrombosis • activated protein • anticoagulants • fibrinolysis

Although enzymatic recanalization of an occluded coronary artery with recombinant tissue-type plasminogen activator (rTPA) or streptokinase has been shown to reduce mortality significantly in patients with acute transmural myocardial infarction,1-14 reocclusion of recanalized coronary arteries, which occurs in 10% to 20% of the patients after recanalization, limits its efficacy.5-11 Adjunctive therapy with currently available antiplatelet (aspirin)12 and anticoagulant (heparin)13 agents has shown some benefit in overcoming this problem. However, the fact that early reocclusion occurs despite the use of such agents indicates the necessity for more potent and specific pharmacological interventions.

Early reocclusion of recanalized coronary arteries may result, in part, from activation of platelets.14 Thrombin is believed to have a central role in arterial thrombus development and stabilization through the generation of fibrin, the activation of factor XIII, and, most important, as the primary mediator of platelet activation.15 Thus, pharmacological interventions that inhibit the action of thrombin have triggered extensive research to prevent thrombotic reocclusion after thrombolytic therapy.16-19

Protein C is one of the most important antithrombotic components.20,21 After activation by thrombin-thrombomodulin complex on the endothelial cells, activated protein C (APC) inactivates factors VIIIa and Va and leads to inhibition of thrombin formation.22 APC also has been shown to enhance clot lysis by inhibiting the activity of plasminogen activator inhibitor-1 (PAI-1) in endothelial cell cultures23,24 and in clot lysis assays.25,26 These properties show that APC may be useful for the treatment of thrombotic disorders. In fact, there are several reports that APC has favorable effects on thrombosis in animal experimental models27-28 and in patients with disseminated intravascular coagulation.30

In the present study, we have evaluated the effects of APC on the efficacy of rTPA-mediated thrombolysis and subsequent acute reocclusion in a canine model of coronary artery thrombosis.

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Methods

This study was approved by the animal studies committee of our institute and conforms with the position of the American Heart Association on research animal use.31

Activated Protein C

Protein C was purified from freshly prepared citrated human plasma according to the method described by Comp et al.32 After activation of protein C by human thrombin, APC was purified according to the method described by Taylor et al.27 Purified APC displayed a single peak on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified preparation of APC was pasteurized by heating at 65°C for 96 hours in dry state. No significant change was observed in the crossed immunoelectrophoresis, circular dichroism spectra, ultraviolet absorption spectrum, and fluorescence spectrum after heat treatment.

Canine Coronary Artery Thrombosis Model With Superimposed Endothelial Cell Damage and High-Grade Stenosis

The experimental model was used according to the method described by Gold et al.16,33,34 with some modification. Adult beagles (weight, 9.0 to 11.0 kg) were anesthetized with pentobarbital (30 mg/kg body wt IV) and additional doses as required. The dogs were intubated and placed on a respirator with a tidal volume of 10 to 15 mL/kg. The left femoral artery was cannulated for continuous blood pressure recording. The left femoral and left jugular veins were also cannulated for IV administration of drugs and blood sampling. Lidocaine (50-mg bolus followed by a constant infusion of 0.1 mg/kg per minute IV) was given for prophylaxis of arrhythmias. Thoracotomy was performed through the left fifth intercostal space. The pericardium was opened and suspended to create a pericardial cradle. The left anterior descending coronary artery (LAD) was dissected out of the epicardium, and a 2.5-cm segment was isolated distal to the first diagonal branch. A 0.7-mm internal diameter catheter was inserted into a side branch of the isolated LAD segment, and a magnetic flow probe (model FH20ST, Nihon Kohden Corp) was placed on the proximal portion of the artery for continuous blood flow monitoring.

Heparin (heparin sodium injection, Green Cross Corp) 200 U/kg was then given intravenously as a bolus. A mechanical vascular occluder (renal artery clamp, model 1934, MT Giken Co, Ltd) was progressively constricted around the LAD just distal to the proposed site of thrombus formation to limit the blood flow to 40±10% of the baseline value.

The isolated LAD segment was traumatized by four consecutive external compressions with blunt forceps during 3 to 5 seconds to damage the endothelium and promote thrombus adherence. Snare occlusions were made distal to the flow probe and proximal to the constriction site. Thrombin (0.2 mL of 100 U/mL CaCl2 solution; Bolheal, Fujisawa Pharmaceutical Co, Ltd) mixed with 0.6 mL of blood was injected through the side branch catheter into the emptied coronary artery segment. After 5 minutes, the proximal snare was released, and 2 minutes later, the distal tourniquet was released.

Experimental Protocol

Fig 1 depicts the general experimental protocol used in the present study. Thirty dogs were randomly assigned to one of the three adjunctive treatment groups listed in Fig 1. In all the treatment groups, rTPA (alteplase, GRTPA, Tanabe Seiyaku Co, Ltd) was administered at a total dose of 0.45 mg/kg IV over 30 minutes. Adjunctive treatments were initiated simultaneously with rTPA and maintained for 60 minutes (ie, terminating 30 minutes after the completion of rTPA therapy). A 120-minute observation period followed the termination of rTPA therapy. Specific adjunctive treatments for the individual treatment were infusion of human albumin at a rate of 0.83 mL/min IV (control group, n=10), administration of APC at a total dose of 0.6 mg/kg IV with human albumin as a vehicle at a rate of 0.83 mL/min (APC group, n=10), and administration of heparin at a total dose of 200 U/kg IV with saline at a rate of 0.83 mL/min (heparin group, n=10).

The APC dose was determined from the results of a preliminary dose-ranging trial that showed that reocclusion occurred in all 5 dogs treated with APC 0.1 mg/kg (P<0.02 versus APC 0.6 mg/kg) and in 5 of 7 dogs treated with APC 0.3 mg/kg (Fig 2). APC 0.9 mg/kg was not appropriate because of marked prolongation of the activated partial thromboplastin time (aPTT) and bleeding from surgical incisions. Therefore, we determined the dose of APC in this study as 0.6 mg/kg.

Criteria for Recanalization and Reocclusion

Thrombotic occlusion of the LAD was judged complete when coronary blood flow was decreased to and stabilized at zero. Infusion regimen was initiated 30 minutes after the LAD occlusion. Recanalization was defined when the flow returned to 25% or more of poststenotic value. Recanalization time was defined as the time from the start of the thrombolytic therapy to recanalization, which was documented by the return of the blood flow as described above. Reocclusion was defined when

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**Fig 1.** Schematic representation of the time course of the experiments. Coronary thrombolytic therapy with r-PA (tPA, alteplase) was done in all dogs of each adjunctive treatment group. APC indicates activated protein C; r-PA, recombinant tissue-type plasminogen activator.

**Fig 2.** Graph illustrates frequency of reocclusion after recanalization of different doses of activated protein C (APC) as an adjunctive therapy. Dogs were treated with APC of three different doses (0.1, 0.3, and 0.6 mg/kg) and alteplase.
the flow decreased to less than 25% of poststenotic value. Reocclusion time was defined as the interval between documented recanalization and reocclusion. Dogs without recanalization 120 minutes after rTPA termination were considered not to have had reocclusion, and the reocclusion time was counted as the time from recanalization to the end of observation.

**Hematological Studies: Ex Vivo Clotting Assay and Bleeding Time Determination**

Effects of the treatments on the intrinsic coagulation cascade were assessed by determination of aPTT. Venous blood (4.5 mL) was drawn into a syringe containing 0.5 mL of 3.8% trisodium citrate solution. The blood was centrifuged for 15 minutes at 3000 rpm. The plasma was removed and stored at -80°C for later assay. The aPTTs were determined with an automated clot timer and commercially available reagents. Buccal mucosal template bleeding times were measured by the method of Jergens et al with the use of a Simplate bleeding time device (Organon Teknika Corporation). Venous blood sampling for the aPTT and measurement of template bleeding time were done before the bolus administration of heparin, and immediately after and 120 minutes after rTPA completion.

**Pathological Examination**

At the end of the experiment, the dogs were killed with an overdose of pentobarbital. To examine whether the evidence of severe internal bleeding exists, the cerebrospinal fluid was drawn and the brain was dissected to detect massive bleeding. The thrombosed segment of the LAD was removed intact, embedded in paraffin blocks, and sectioned longitudinally. Longitudinal sections were stained with hematoxylin-eosin and examined microscopically.

**Statistical Analysis**

Values are reported as mean±SEM values. Fisher's exact test was used to compare the incidence of recanalization and reocclusion among the groups. Reocclusion time was analyzed by a Kruskal-Wallis one-way ANOVA followed by Mann-Whitney test for significant differences between individual pairs because of a nonparametric distribution of the data. Except for reocclusion time, among-group comparisons were conducted with a one-way ANOVA followed by Fisher's protected least significant difference test for multiple comparisons. Within-group comparisons at multiple time points were conducted with a one-way ANOVA with repeated measures.

**Results**

**Coronary Occlusive Thrombus**

All dogs experienced complete coronary occlusion of the LAD. The range of times required for thrombotic occlusion was 7 to 35 minutes. Times required for thrombotic occlusion in each group (minutes) were 20.3±3.5 in the control group, 13.3±1.9 in the heparin group, and 13.2±2.3 in the APC group. There were no significant differences among the three groups.

**Recanalization**

Coronary recanalization occurred in all dogs of each adjunctive treatment group. There were no significant differences in recanalization time (minutes) among the three groups (17.8±3.4 in the control group, 18.5±3.6 in the APC group, and 21.0±3.4 in the heparin group).

**Reocclusion**

In a 120-minute observation after the termination of rTPA infusion, coronary reocclusion after recanalization developed in all the dogs of the control and heparin groups. On the other hand, reocclusion occurred in only 3 of the 10 dogs treated with APC. The reocclusion rate of the APC group was significantly lower than those of the control and heparin groups (P<.002) (Fig 3, top).

Coronary reocclusion occurred 10.2±2.3 and 30.3±11.8 minutes after the recanalization in the control and heparin groups, respectively. There was no significant difference in reocclusion time between the two groups, whereas the reocclusion time was dramatically prolonged in the APC group (103.2±14.2 minutes) compared with those of the control (P<.001) and heparin (P<.002) groups (Fig 3, bottom).

**Hemostatic Parameters**

The effects of the adjunctive treatments on aPTT and buccal mucosal bleeding time are shown in the Table. aPTT was prolonged immediately and 2 hours after the completion of rTPA administration similarly in all three groups (after rTPA versus before rTPA, P<.01; after rTPA 2 hours versus before rTPA, P<.05). Two hours after rTPA, aPTT's in the heparin group tended to be longer compared with those in the other two groups. Bleeding time was prolonged in the heparin group (after rTPA versus before rTPA and after rTPA 2
### Changes of APTT and Bleeding Time in Each Adjunctive Treatment Group

<table>
<thead>
<tr>
<th>Adjunctive Treatment Groups</th>
<th>Pre rTPA, s</th>
<th>Post rTPA, s</th>
<th>Post rTPA, 2 h</th>
<th>Pre rTPA, min</th>
<th>Post rTPA, min</th>
<th>Post rTPA, 2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>17.6±2.1</td>
<td>&gt;200*</td>
<td>21.8±1.2†</td>
<td>3.4±0.6</td>
<td>4.4±0.4</td>
<td>3.5±0.2</td>
</tr>
<tr>
<td>APC (n=10)</td>
<td>15.8±1.2</td>
<td>&gt;200*</td>
<td>23.2±1.1†</td>
<td>3.6±0.3</td>
<td>4.4±0.5</td>
<td>4.2±0.6</td>
</tr>
<tr>
<td>Heparin (n=10)</td>
<td>15.3±0.5</td>
<td>&gt;200*</td>
<td>35.5±8.6†</td>
<td>4.0±0.4</td>
<td>7.2±0.9†</td>
<td>3.8±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM except post rTPA of aPTT.

aPTT indicates activated partial thromboplastin time; rTPA, recombinant tissue-type plasminogen activator; and APC, activated protein C.

*P <.01 vs pre rTPA; tP <.05 vs pre rTPA; tP <.01 vs pre rTPA and post rTPA 2 h; and §P <.05 vs control and APC.

### Hemorrhagic Side Effects

None of the dogs had macroscopic bleeding in the cerebrum, cerebellum, and medulla oblongata. Also, the cerebrospinal fluid was clear in all dogs.

Bleeding from the surgical incision was slightly increased after the initiation of the thrombolytic therapy similarly in the three groups. However, significant changes of blood pressure and heart rate before and after thrombolytic therapy were not observed in each group.

### Histological Examination

In all specimens, light-microscopic examination revealed that segments with reocclusion after initial reflow contained platelet-rich occlusive thrombus (as represented in Fig 4). The thrombus was overlying a damaged intima without endothelium.

### Discussion

In the present study, we demonstrated that APC suppressed the thrombotic reocclusion after thrombolytic therapy. This means that administration of APC might be a useful adjunctive regimen to enhance the effect of thrombolytic therapy.

With the adjunctive use of APC, reocclusion rate was suppressed, and time to reocclusion after recanalization was prolonged. The beneficial effect of APC was attained without any hemorrhagic side effects. Moreover, hemostatic parameters such as aPTT and bleeding time in the APC group were in the same level as those in the control group. On the contrary, bleeding time was prolonged significantly after the infusion of rTPA in the heparin group. Furthermore, aPTT 2 hours after the infusion of rTPA was more than twice that before rTPA only in the heparin group. Because thrombin is a powerful platelet activator, heparin might interfere with the process of platelet activation and aggregation systemically through its antithrombin effect and prolonged bleeding time.

Thus, despite less or the same anticoagulant effect, APC is more effective than heparin to prevent thrombotic reocclusion after thrombolytic therapy. APC exhibits potent anticoagulant activity by inactivating blood coagulation factors VIIIa and Va in the presence of phospholipids (ie, plasma membranes of platelets and endothelial cells) and protein S, which binds APC to cell membranes.22,36,37 On the other hand, heparin–antithrombin III complex strongly inhibits the coagulation factors in the circulation.38 This difference of anticoagulant mechanism enables APC to inhibit reocclusion without any deterioration of hemostatic parameters.

In histological examination, reocclusive thrombus consisted of platelet-rich fibrin clot. This is in agreement with the results of previous studies.16,33 APC may have a preventive effect on rethrombosis by the inhibition of platelet activation locally through its anticoagulant effect.

APC also promotes the fibrinolytic activity by inactivating PAI-1.23-26 Because plasma PAI-1 activity is believed to almost disappear in the presence of unphysiologically high levels of plasma TPA during and after the administration of the drug as in the present study, APC is unlikely to contribute to the enhancement of fibrinolysis in this study. Thus, not profibrinolytic but anticoagulant effect of APC is believed to play a main role in preventing early reocclusion after thrombolysis in the canine model of the present study.

Increased PAI-1 activity is observed in patients with acute myocardial infarction before39 and after40 coronary thrombolytic therapy, and this is believed to contribute to the risk of reocclusion.40 Thus, the administration of APC, which inactivates increased PAI-1, may be promising as a useful regimen for the treatment not only during but also after thrombolytic therapy in patients with acute myocardial infarction.

In the present study, all dogs obtained coronary recanalization after the administration of rTPA even in the control group. These findings are in agreement with those of the previous study in which the same model was used.33 Because coronary recanalization was achieved in all dogs in this study, effects of the adjunctive treatments on recanalization were unclear. The potential effect of APC on recanalization may be elucidated by use of other experimental models in which recanalization rates are lower.18

Protein C is a natural protein that exists in human plasma. The administration of human APC in the clinical setting therefore is believed to be more favorable compared with that of other heterospecies-derived or synthesized unnatural proteins. In fact, when APC was administered in patients with disseminated intravascular coagulation and normal human subjects, no unfavorable side effects were observed.30,41

In conclusion, APC administered adjunctively with rTPA prevents acute reocclusion after successful thrombolysis in a canine model of coronary artery thrombosis. This finding strongly suggests that APC may be a useful adjunctive regimen for thrombolytic therapy in patients with acute myocardial infarction.

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