Prevention of Arterial Reocclusion After Thrombolysis With Recombinant Lipoprotein–Associated Coagulation Inhibitor

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Background. This study was designed to determine whether arterial reocclusion after thrombolysis can be prevented by lipoprotein-associated coagulation inhibitor (LACI), a physiological inhibitor of tissue factor–induced coagulation mediated by the extrinsic pathway.

Methods and Results. Thrombosis was induced in femoral arteries of anesthetized dogs with the use of anodal current to elicit extensive vascular injury and formation of platelet-rich thrombi in one artery and with thrombogenic copper wire to elicit fibrin-rich thrombi without appreciable vascular injury in the contralateral artery. Recanalization of both vessels was induced with t-PA (1.7 mg/kg i.v. over 1 hour) and verified with Doppler flow probes. Reocclusion occurred within 2 hours in seven of seven arteries with electrical injury–induced thrombosis and in four of seven arteries with copper wire–induced thrombosis in the absence of LACI. In dogs given infusions of recombinant DNA–produced LACI (225 µg/kg over 15 minutes, followed by 4 µg/kg/min i.v.) after completion of the infusion of t-PA, no reocclusion occurred during the 2-hour interval of observation in any of the five arteries subjected to electrical injury (p < 0.001), and cyclic partial occlusions were nearly abolished (0.4 ± 0.4/hr in LACI-treated dogs compared with 13.7 ± 5.5/hr in saline-treated dogs, p < 0.0001). In contrast, reocclusion occurred in two of five arteries with indwelling copper wires, and cyclic partial occlusions were unaffected despite LACI. LACI prolonged the partial thromboplastin time modestly (1.7 ± 0.2 × baseline) but did not affect platelet counts or aggregation assessed ex vivo.

Conclusions. Inhibition of the extrinsic pathway of coagulation with LACI prevents thrombotic arterial reocclusion after thrombolysis in vessels subjected to extensive vascular injury. Our results demonstrate that activation of the extrinsic pathway plays a critical role in thrombotic reocclusion and that LACI provides a highly targeted approach to facilitate sustained recanalization without directly inhibiting platelets. (Circulation 1991;84:821–827)

Although coronary artery thrombolysis salvages ischemic myocardium and improves survival, early thrombotic reocclusion can limit its efficacy.1–3 Mechanisms responsible for reocclusion are complex and involve accumulation of platelets at the site of initial thrombosis and accretion of fibrin, reflecting activation of the coagulation system. We and others4–7 have shown that inhibition of platelet aggregation attenuates without necessarily abolishing reocclusion. Unfortunately, required doses of some platelet antagonists can markedly alter hemostasis and predispose to bleeding.8 We have hypothesized that early reocclusion reflects increased thrombin activity, attributable in part to procoagulant effects of plasmin induced by fibrinolysis with plasminogen activators,9–11 and that activation of platelets may be attributable largely to the increased thrombin activity elicited by fibrinolysis and by procoagulant molecules released from thrombi undergoing lysis.12–14 In support of this hypothesis we have found that inhibition of thrombin activity with recombinant desulphatohirudin accelerates thrombolysis and prevents reocclusion with platelet-rich thrombi in canine coronary arteries while exerting only modest effects on hemostasis.5

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Increased thrombin activity associated with thrombolysis can result through activation of either factor XII (intrinsic pathway), factor VII (extrinsic pathway), or both. The extrinsic pathway is activated when tissue factor, located in the subendothelium or in atherosclerotic plaque, binds to factor VII or VIIa, with consequent activation of factors IX and X by the complex. Thrombolysis may activate this pathway preferentially because of reexposure of subendothelium or ruptured plaque to the circulating procoagulant factor VII, thereby contributing paradoxically to early reocclusion.

Circulating lipoprotein-associated coagulation inhibitor (LACI) inhibits activation of the extrinsic pathway by formation of an inert tissue factor/factor VIIa/factor Xa/Ca\(^+\)/LACI complex and thus may attenuate activation of thrombin during thrombolysis. However, the physiological concentration of LACI may not be sufficient to prevent activation of the extrinsic pathway locally in response to thrombolysis. The present study was performed to evaluate the role of the extrinsic pathway in reocclusion after thrombolysis by determining whether direct pharmacologic inhibition of the extrinsic pathway with recombinant LACI attenuates thrombotic reocclusion after thrombolysis with tissue-type plasminogen activator (t-PA). Effects of LACI were evaluated with the combined use of two disparate femoral arterial thrombosis preparations we have characterized previously that were developed to yield thrombi of distinct morphology induced simultaneously in the same animal. One entails extensive electrical injury of the arterial wall to yield platelet-rich thrombi typical of thrombosis initiated by exposure of tissue factor in the subendothelium. The other entails thrombosis induced with a coil of copper wire inserted into the lumen to yield fibrin-rich thrombi typical of activation of the coagulation system regardless of the presence of tissue factor. Although studies could be performed in only 15 dogs because of the limited amount of recombinant DNA-produced LACI that can be synthesized presently, the inhibitory effect of LACI on reocclusion was sufficiently striking and consistent to permit definitive conclusions.

Methods

Animal Preparations

Procedures involving animals were conducted according to the guiding principles of the American Physiological Society and were approved by the Animal Studies Committee at Washington University. After mongrel dogs (4–10 kg) were anesthetized with pentobarbital (30 mg/kg i.v.), their lungs were ventilated mechanically through a cuffed endotracheal tube, and catheters were placed in both external jugular veins for infusion of agents and withdrawal of blood samples. Both femoral arteries were exposed distal to the saphenous branch, and smaller side branches were ligated. Doppler flow probes were placed proximal to the sites later used for induction of thrombosis.

Induction of Thrombosis

Thrombosis was induced in one femoral artery by applying anodal current through a transluminal electrode, which resulted in vascular injury as described by Romson et al. and induced in the contralateral artery with an indwelling coil of copper wire as modified from the technique of Blair et al. Briefly, for electrically induced thrombosis, a 23-gauge needle electrode was inserted obliquely into the lumen of one artery distal to the Doppler probe and stabilized with sutures through extravascular tissue on either side of the vessel. Electrical stimulation was initiated by connecting the electrode in series with the positive terminal of a 9-V battery, an ammeter, and a 50-kΩ potentiometer. A ground wire was sutured to subcutaneous tissue to complete the circuit. Current (300 μA) was applied to the electrode until complete thrombotic occlusion had occurred, verified as zero flow velocity on the Doppler tracing. Before occlusion was complete, a pattern of cyclic flow variations, consisting of a gradual decrease in flow velocity followed by an abrupt increase, was evident on the Doppler tracing, similar to those noted by others and attributed to intermittent accumulation and dislodgement of platelet thrombi. When cyclic flow variations became evident, the sharpened end of a coil of thrombogenic copper wire was pushed through the wall of the contralateral femoral artery distal to the Doppler probe and rotated until approximately three complete turns of the coil were within the lumen. Because thrombosis occurs more rapidly after implantation of a copper coil compared with electrical injury, delayed placement of the coil produced comparable intervals between the onset of complete occlusion and administration of the plasminogen activator for both arteries.

Experimental Protocol

Thirty minutes after complete thrombotic occlusion had occurred in both femoral arteries, human recombinant t-PA (Activase, Genentech, South San Francisco, Calif.) was infused intravenously at a total dose of 1.7 mg/kg (1.2×10⁶ IU/mg) over 60 minutes with 10% of the dose given as a bolus in the first 2 minutes. Peak t-PA antigen level in plasma averaged 1,379 ng/ml (n=4). Successful recanalization was defined prospectively as a return of average flow velocity to at least 50% of the baseline value.

Dogs were randomly selected before the study to be given intravenous infusions of either 0.9% saline (controls) or recombinant LACI (provided by Monsanto, St. Louis, Mo.) (225 μg/kg over 15 minutes followed by 4 μg/kg/min) beginning immediately after the completion of the infusion of t-PA and continuing for 2 hours. During this interval, average and pulsatile femoral flow velocities were monitored continuously, and the occurrence of cyclic flow variations and persistent reocclusion, defined as zero flow velocity persisting for at least 1 minute, was recorded. At the end of each study, the arterial
TABLE 1. Characteristics of Femoral Arterial Occlusion, Recanalization, and Reocclusion After Induction of Thrombosis by Electrical Injury to the Vessel Wall

<table>
<thead>
<tr>
<th>Agent</th>
<th>Oclusion</th>
<th>Recanalization</th>
<th>Reocclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time of onset after initiation of current (min)</td>
<td>Interval before onset of infusion of t-PA (min)</td>
<td>Time of maximal flow after onset of infusion of t-PA (min)</td>
</tr>
<tr>
<td>Saline (control) (n=7)</td>
<td>103.1±24.9</td>
<td>38.3±16.4</td>
<td>58.8±9.4</td>
</tr>
<tr>
<td>LACI (n=5)</td>
<td>94.4±38.9</td>
<td>46.8±11.8</td>
<td>53.2±3.7</td>
</tr>
</tbody>
</table>

LACI, lipoprotein-associated coagulation inhibitor.
*p<0.001, †p<0.001 vs. saline.

Assays of Blood Samples

Blood samples for hematologic studies were collected 15 minutes before the infusion of t-PA and 90 minutes after its completion. Aggregation of platelets in platelet-rich plasma was characterized as described previously with the use of bovine tendon collagen (Helena Laboratories, Beaumont, Tex.) as the agonist. The minimal (threshold) concentration of collagen eliciting a sustained increase in light transmission of greater than 50% was identified by serial assays with samples supplemented to contain final concentrations of collagen of 0.7, 1.4, 2.8, 5.6, and 11.1 μg/ml. Platelet counts in whole blood were determined by hemocytometry. Activated partial thromboplastin times (PTT) and prothrombin times (PT) were measured conventionally with the use of a coagulation timer (Coag-A-Mate SC, Organon Teknika, Durham, N.C.). Hematocrit was measured with a conductivity analyzer (Nova Biomedical, Waltham, Mass.).

The concentration of LACI in plasma was measured by a particle concentration fluorescence immunoassay as follows. Plasma samples were diluted 20-fold in Tris-buffered saline containing 0.5 M benzamidine-HCl, 0.1% bovine serum albumin, and 1% Lubrol. Diluted samples (50 μl) and standards prepared identically with use of purified human LACI were incubated for 40 minutes at room temperature with 20 μl of a 0.25% suspension of rabbit anti-human LACI IgG (polyclonal) bound to polystyrene particles (Flouricon 0.86 μm, Baxter Healthcare Corp., PANDEX Div., Mundelein, Ill.). Fluorescein 5-isothiocyanate (Isomer 1; Sigma)–labeled rabbit anti-human LACI antibodies were added (30 μl of 10 μg/ml solution), and the incubation was continued for an additional 30 minutes. LACI concentration was measured by comparison of fluorescence in standards and samples detected with a PANDEX analyzer (Baxter).

Statistical Analysis

Results are mean±SD. Fisher's exact test was used for comparison of the incidence of reocclusion in dogs given LACI or saline. Between-group differences for all other variables were compared by analysis of variance. A value of p<0.05 was considered significant.

Results

Thrombolysis and Reocclusion After Thrombosis Induced by Electrical Injury

Among the 15 vessels in which stable thrombotic occlusion was induced by electrical injury, three (20%) failed to recanalize after infusion of t-PA. In the remaining 12 vessels in which recanalization was successful, the time of occurrence of initial complete occlusion, of recanalization, and the extent of recanalization induced by t-PA did not differ significantly in animals subsequently given saline (controls) or LACI (Table 1). Recanalization of successfully recanalized arteries occurred within 2 hours in all seven dogs given saline. It did not occur in any of the five dogs given LACI (Table 1). In addition, cyclic flow variations were virtually abolished by LACI (Table 1, Figure 1).

![Figure 1. Cyclic flow variations after thrombolysis in vessels in which thrombosis was induced by electrical injury of the vessel (left panel) or with an implanted copper wire (right panel). Horizontal lines indicate the means for each treatment group. LACI, lipoprotein-associated coagulation inhibitor.](http://circ.ahajournals.org/)

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Microscopic analysis of arteries in which thrombosis was induced by electrical current revealed extensive transmural damage (Figures 2A and 2B). The endothelium and internal elastic lamina appeared to be decimated and smooth muscle cells in the media appeared to be contracted and necrotic.

Thrombolysis and Reocclusion After Thrombosis Induced With a Copper Wire

Among the 14 vessels in which stable thrombotic occlusion followed implantation of the copper wire, two (14%) failed to recanalize after infusion of t-PA. In the remaining vessels in which recanalization was successful, the time of occurrence and extent of recanalization did not differ significantly in dogs subsequently given saline (controls) or LACI (Table 2). Reocclusion occurred within 2 hours in four of seven dogs given saline, and frequent cyclic flow variations were evident in those that did not exhibit persistent reocclusion. In contrast to the results in vessels with electrically induced vascular injury, LACI did not significantly decrease the incidence of reocclusion or the frequency of cyclic flow variations after thrombolysis in vessels in which thrombosis had been induced with a wire (Table 2, Figure 1). Microscopic analysis revealed relatively little damage to the vascular wall in vessels exposed to the copper wire (Figures 2C and 2D).

Results of Hematologic Assays and Concentrations of LACI in Plasma

Infusion of LACI after t-PA did not affect hematocrit, platelet counts in whole blood, or the response of platelets to collagen-induced aggregation in platelet-rich plasma (Table 3). Baseline values for PTT and PT were 8.5 and 8.3 seconds, respectively. LACI prolonged the PTT to $1.7 \pm 0.2$ times baseline. The

**Figure 2.** Micrographs of femoral arteries after induction of thrombosis by direct current applied through an indwelling electrode (panels A and B) and by implantation of a coil of copper wire (panels C and D). The electrode and copper wire have been removed to permit processing of the tissue, but their original location with respect to the wall of the vessel is evident (arrows). At higher magnification, extensive damage to the intima and inner media is observed in the vessel wall injured electrically (panel B), but virtually no damage is seen in the vessel wall adjacent to copper wire (panel D).
PT was less affected and prolonged to only 1.2±0.2 times baseline.

Concentrations of LACI in plasma at baseline were ≤20 ng/ml. Ninety minutes after the onset of infusion of LACI, the plasma concentration averaged 1,157 ng/ml (range, 963–1,465 ng/ml, n=3).

**Discussion**

Generation of thrombin accompanying fibrinolysis has been implicated as a factor that can attenuate prompt, sustained recanalization of thrombolytically occluded coronary arteries.5,9,25–27 Thrombin induces platelet aggregation and degranulation; converts fibrinogen to fibrin; activates factors V, VIII, and XIII; and induces expression of tissue factor activity in endothelial cells14,28,29; all of these may contribute to delayed lysis, rethrombosis, or both. Although thrombin can be formed by either intrinsic pathway activation mediated by activation of factor XII or extrinsic pathway activation that occurs when tissue factor is exposed to circulating blood, it is likely that extrinsic activation predominates under conditions of coronary arterial thrombosis induced by acute vascular injury.18 When extrinsic pathway activation occurs, factor Xa can activate more factor VII to VIIa, resulting in accelerated extrinsic pathway thrombosis. Furthermore, the tissue factor/VIIa complex can activate the intrinsic pathway by activating factor IX to form IXa.18

The results of the present study indicate that infusion of recombinant LACI, the physiological inhibitor of extrinsic pathway activation, after initially successful thrombolysis with t-PA prevents reocclusion and markedly inhibits cyclic flow variations in vessels manifesting injury induced by electrical current. It is less effective in preventing thrombotic occlusion attributable primarily to fibrin when local vascular damage is absent or modest. These results are consistent with the presence of tissue factor identified previously in the arterial media and adventitia16,17,30 and in the subendothelium15 that may become exposed when the luminal vascular wall is injured. Our results demonstrate for the first time that extrinsic pathway activation can play a critical role in thrombotic reocclusion after thrombolysis and that exogenous LACI can inhibit this pathway sufficiently to attenuate thrombotic reocclusion.

LACI inhibits coagulation mediated by the extrinsic pathway by forming an in situ complex with tissue factor and factors VII or VIIa and Xa, preventing factor VIIa–induced production of additional Xa and IXa.19,20 In addition, LACI binds to and directly inactivates factor Xa. The modest prolongation of the PTT we observed with infusions of LACI may be secondary to the binding of LACI to circulating Xa. Accordingly, it is possible that effects on reocclusion could be mediated, at least in part, by inactivation of Xa by LACI. However, the failure of LACI to prevent fibrin-rich reoccluding thrombi induced with a copper wire argues against this possibility.

**Clinical Implications**

Diverse approaches have been explored for prevention of reocclusion after thrombolysis, including prolonged infusions of thrombolytic agents,31,32 inhibition of platelet function,4–8,33–36 and inhibition of thrombin activity.5,25,37,38 Recently, large quantities of tissue factor have been demonstrated in atherosclerotic plaque,17 which may precipitate thrombosis as the plaque ruptures. Although electrothermal vascular injury used in this study likely exposed tissue factor in the vessel wall, the extent to which tissue factor contributed to rethrombosis after thrombolysis in our preparation remains to be defined and may differ from that in human atherosclerotic vessels. Further studies, both in experimental animals and in patients, are needed to determine the relative role and optimal strategy for inhibition of tissue factor and extrinsic pathway activation during thrombolysis. Nevertheless, LACI completely prevented reocclusion mediated by vessel wall injury that yielded platelet-rich thrombi and did not appear to adversely affect circulating platelet count or function as measured by

**TABLE 3. Effect of LACI on Hematologic Variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before LACI</th>
<th>With infusion of LACI*</th>
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</thead>
<tbody>
<tr>
<td>Hematocrit (%)</td>
<td>34.4±4.1</td>
<td>31.6±4.2</td>
</tr>
<tr>
<td>Platelet count (×10⁹/mm³)</td>
<td>272.6±38.2</td>
<td>282.4±63.9</td>
</tr>
<tr>
<td>Median platelet aggregation threshold concentration of collagen (µg/ml)</td>
<td>5.6</td>
<td>5.6</td>
</tr>
</tbody>
</table>

LACI, lipoprotein-associated coagulation inhibitor.

*90 minutes after onset of infusion.
collagen-induced aggregation assayed ex vivo. Thus, LACI may prove safer than antiplatelet agents that elicit marked and prolonged effects on platelet counts, bleeding time, or both. Furthermore, it may be more efficient than inhibition of the thrombin once generated and may reduce amplification of thrombosis through thrombin-mediated pathways.

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