Lipoprotein-associated coagulation inhibitor produces feedback inhibition of tissue factor (tissue thromboplastin)-induced coagulation in the presence of factor X. Recombinant lipoprotein-associated coagulation inhibitor (rLACl) was tested for its ability to modify thromboplastin-induced intravascular coagulation in a rabbit model that allows monitoring of iodine-125 fibrin accumulation/disappearance in the lung and sampling of blood for the measurement of coagulation parameters. Infusion of thromboplastin into the rabbit caused a rapid increase of radioactivity over the lungs, possibly due to the accumulation of labeled fibrin in the lungs, followed by a rapid decline of radioactivity, suggestive of removal of fibrin from the lungs. Thromboplastin also caused a rapid decrease of systemic fibrinogen that was accompanied by a lengthening of the activated partial thromboplastin time and prothrombin time. The effect of coinfusion of rLACl with thromboplastin or bolus injection of rLACl before thromboplastin infusion was studied. At a high dose of rLACl (800 μg/kg body weight), the thromboplastin-induced radioactivity increase in the lungs and the systemic fibrinogen decrease were completely suppressed. The activated partial thromboplastin time and prothrombin time of the plasma samples lengthened, possibly due to the presence of thromboplastin in circulation. The thromboplastin-induced radioactivity increase over the lungs was not completely suppressed by lower doses of rLACl (135 to 270 μg/kg body weight), but these doses of rLACl prevented systemic fibrinogen decrease. At a bolus dose of 23 μg/kg body weight, rLACl provided 50% protection of the fibrinogen consumption (fibrinogen decreased to 82% compared with 65% in rabbits treated with thromboplastin alone). These results show that rLACl is effective in the inhibition of thromboplastin-induced coagulation in vivo. © 1990 by The American Society of Hematology.

MATERIALS AND METHODS

Rabbit brain thromboplastin was obtained from Ortho Diagnostic (Raritan, NJ). Iodine-125 human fibrinogen was purchased from Amersham (Arlington Heights, IL). Ketamin, Innovar-Vet, and pentobarbital were purchased from Allmed Vet Supply (Fenton, MO). Dade's kits for the determination of plasma fibrinogen and activated partial thromboplastin time (APTT) were obtained from American Scientific Products (McGraw Park, IL).

Expression of recombinant LACl in mouse C127 cells. A bovine papillomavirus virus-based vector, pMON1123, was used to express LACl. pMON1123 consists of the entire bovine papilloma virus genome cloned in the pBR322 derivative pML2. This vector uses the mouse metallothionein I promoter and the SV40 late poly A addition site to direct the expression of proteins encoded by DNA...
fragments inserted into a unique BamHI site. For the expression of the LACI cDNA, pMON1123 was digested with BamHI and the 5'-overhanging ends were filled in with Klenow fragment (Boehringer Mannheim, Indianapolis, IN) and deoxyribonucleotides (dNTPs). Similarly, the LACI cDNA was isolated as an EcoRI fragment and the ends were rendered blunt by Klenow fill-in. The LACI fragment was ligated into pMON1123 to yield the plasmid pMON1456. Mouse C127 cells were grown and cotransfected with pMON1456 and pSV2neo as previously described. Following selection with G418, resistant colonies were picked and seeded into 24-well plates. Conditioned media from each well was then assayed for recombinant LACI (rLACI) expression by an enzyme-linked immunosorbent assay. One clone, 1455-15, expressing approximately 1 to 2 μg LACI/10^6 cells/24 h, was expanded for isolation of rLACI.

Isolation of recombinant C127 cell LACI. The rLACI-producing cell line 1455-15 was cultured in Dulbecco's Modified Eagle's Medium (GIBCO Laboratories, Grand Island, NY) containing 10% fetal bovine serum. The cells were grown in 150-cm² flasks to confluency. Each flask was then trypsinized and used to seed one 850-cm² roller bottle. After confluency, the cells from each roller bottle were used to seed one 10-chamber cell factory (6,000 cm²). On reaching confluency, the cells were washed with phosphate-buffered saline and incubated in a serum-free medium consisting of Dulbecco's Modified Eagle's Medium, supplemented with 0.2 μg/mL menadione, 2.5 mM/L sodium butyrate, and 50 U/mL aprotinin. The serum-free conditioned medium was collected every 2 days and replaced with fresh medium. The pooled medium was adjusted to 50 mM/L (NH₄)₂SO₄, filtered through a 3 μm filter, and concentrated approximately 30 fold, using an Amicon YM30 radial cartridge. The concentrate was subjected to ammonium sulfate precipitation. The proteins precipitated between 23% to 90% saturation of ammonium sulfate were collected and dialyzed against a solution of 0.15 mol/L NaCl and 20 mmol/L Na₂SO₄. Using a fibrometer, one tenth of 1 mL of plasma was preincubated with 0.1 mL of 25 mmol/L sodium sulfate, and the absorbance measurement was recorded. Absorbance measurement was at 280 nm, assuming an absorbance of 1.0 for a solution of 1.0 mg/mL in a 1-cm cell. Detailed characterization of the purified rLACI will be described elsewhere.

Thromboplastin-induced coagulopathy model in the rabbit. A rabbit thromboplastin-induced coagulopathy model was developed by modifying a procedure originally designed for studying thrombolysis in the rat, as described by Scheu et al. The experimental setup is schematically illustrated in Fig 1. Male and female New Zealand White rabbits weighing 2.5 to 3.5 kg were anesthetized by intramuscular injection of ketamine hydrochloride (25 mg/kg) and Innovar-Vet (0.02 mg fentanyl/kg and 1 mg droperidol/kg), and intravenous injection of a 50% solution of pentobarbital was given to effect. Indwelling catheters were introduced into two superficial jugular veins and one femoral vein. A lead shield was placed over the rabbit with an opening over the lung field (3.5 × 4 cm). A 2-inch iodide probe was mounted directly over the opening to the lungs 1 cm above the rabbit. Radioactivity was determined using a Ludlum scale-rate meter #2200. Radioactivity determinations are based on 1-minute counts from a digital readout. Sodium iodide (0.5 mL of a 2% solution) was initially administered through the femoral vein to block uptake of free 125I to the thyroid. At −30 minutes, 125I fibrobrin (2 μCi in 1 mL of saline) was given via the femoral vein, followed by 2 mL of saline. At −15 minutes, 6-aminohexanoylic acid (200 mg/kg) was administered subcutaneously to inhibit intrinsic fibrinolysis. Administration of rLACI was started at −1 minute, followed by coinfusion with thromboplastin at zero time by bolus injection followed by thromboplastin infusion through a jugular vein. In the coinfusion study, 1 mL of rLACI solution (dissolved in saline solution containing 20 mmol/L sodium sulfate) was infused through one jugular vein over a 1-minute period, followed by coinfusion of rLACI and thromboplastin solutions into opposite jugular veins. The infusions were run side by side from the same syringe pump at a rate of 0.6 mL/min. The amount of thromboplastin used was determined by the weight of the rabbit, and was given at a dose of 1.5 mL/kg body weight. The total time for infusion was approximately 8 to 9 minutes. When rLACI was administered in the bolus study, it was given at −1 minute into the femoral vein in 3 mL of saline containing 20 mmol/L sodium sulfate, followed by 2 mL of saline, and allowed to circulate for 1 minute before the start of the thromboplastin infusion at time zero. The administration of thromboplastin was performed in the same way as in the infusion model. In both studies, 2.7 mL blood samples were drawn into 0.3 mL of 3.8% citrate solution before any injection, and at 10, 20, 30, 60, and 90 minutes. Plasma was separated by centrifugation and stored at −80°C until assayed. Radioactivity over the lung field was determined at 2-minute intervals. At time zero, the radioactive counts were normalized to a value of 20,000 cpm and all other counts were adjusted to this time point. The normalized counts per minute were then plotted versus time, and the standard error of the mean was calculated for all time points.

Fibrinogen determination. The fibrinogen levels of the plasma samples were determined by the thrombin time method, using a Fibrometer (Becton Dickinson, Mountain View, CA) and a Fibrinogen determination kit from Dade (Aguada, PR).

Activated partial thromboplastin time. Dade's activated cephaloplastin reagents were used to determine the APTT of the plasma using a fibrometer. One tenth of 1 mL of plasma was preincubated with 0.1 mL of activated cephaloplastin reagent for exactly 2 minutes at 37°C. A calcium solution (0.1 mL of 25 mmol/L CaCl₂) was added to the mixture and the time to clotting was recorded.

Prothrombin time. One tenth of 1 mL of thromboplastin reagent (Ortho Diagnostic) was preincubated with 0.1 mL of 25 mmol/L CaCl₂ for 2 minutes at 37°C. The plasma (0.1 mL) was added to the mixture and the time to clotting was recorded.

RESULTS

Comparison of the effect of rLACI on the thromboplastin-induced coagulation in rabbit and human plasmas in vitro. The cDNA used for the expression of recombinant protein encodes human LACI. Testing the efficacy of the recombinant molecule requires that the human LACI inhibit the thromboplastin-induced coagulation in the plasma of the animal to be tested. In preliminary in vitro studies comparing the effects of human rLACI on the thromboplastin-induced clotting in plasmas from various species, we found that rLACI prolonged the prothrombin time of various plasmas in the following order: human plasma > dog plasma > rabbit plasma.
plasma = rat plasma = mouse plasma (unpublished results). Human rLACI is a very poor inhibitor of TF in rat or mouse plasmas, which precludes the use of these animals. rLACI inhibits the TF in the rabbit moderately well, but the effect is not as pronounced as in human or dog plasmas. Due to consideration of material requirements and access to animal studies, we first investigated whether rabbits can be used for testing the effect of human rLACI. Figure 2 shows the comparison of the effect of rLACI on the thromboplastin-induced clotting in rabbit and human plasmas in vitro. rLACI prolonged the prothrombin time in a dose-dependent manner in both the rabbit and the human plasmas. Assuming that the slope of the dose versus clotting time curve reflects the relative potencies, the human rLACI is approximately 4.1-fold more potent in human plasma than in rabbit plasma. It should be noted that the recalcification time in the absence of thromboplastin is 400 seconds for the human plasma and 103 seconds for the rabbit plasma. Furthermore, the prothrombin time of the rabbit and the human plasma in the presence of identical concentrations of thromboplastin are 20 and 34 seconds, respectively. These results indicate that the coagulation system of the rabbit plasma is more active than the human counterpart. Therefore, the higher coagulatory activity of the rabbit plasma may be partly responsible for the requirement of higher concentrations of human rLACI in prolonging the prothrombin time in rabbit plasma compared
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Fig 3. Thromboplastin-induced radioactivity change over the lung field and the effect of 6-aminohexanoic acid. The experiments were performed as described in the experimental section and Fig 1, except that the rabbits were injected either with or without 6-aminohexanoic acid. Group 1 rabbits were injected with saline (controls). Group 2 rabbits were infused with thromboplastin (TF) and 6-aminohexanoic acid. Group 3 rabbits were injected with thromboplastin. The number of rabbits in each group is indicated.

with human plasma. In addition, human rLACI may be less active in a rabbit system due to cross-species specificity. These results suggest that human rLACI is capable of inhibiting thromboplastin-induced coagulation in the rabbit, although higher doses of rLACI may be required to be as effective in the rabbit as in the human system.

The thromboplastin-induced coagulopathy model in the rabbit. An in vivo model system of intravascular coagulation in rabbits was developed by adapting the procedure previously described by Scheu et al for rats. Details of the experimental setup are described in Materials and Methods and in Fig 1. In our rabbit model, thromboplastin (Ortho Diagnostic system, lot OBT203; the vial contents were dissolved in 12 mL of water as recommended) was infused into rabbits to induce intravascular coagulation. The infusion of 1.5 mL of thromboplastin/kg body weight in 8 to 9 minutes reproducibly caused a change in radioactivity over the lung field. The use of lower amounts of thromboplastin caused a smaller or undetectable change in radioactivity over the lungs, whereas infusion of larger amounts of thromboplastin carried a greater risk of killing the animal. Figure 3 shows the time course of the thromboplastin-induced radioactivity change over the lung field in rabbits and the effect of a fibrinolysis inhibitor, 6-aminohexanoic acid. In rabbits infused with control buffers alone, the radioactivity over the lung steadily declined. In rabbits infused with thromboplastin alone, the radioactive counts over the lungs increased for approximately 10 minutes, then declined at a rate similar to the control rabbits. The radioactivity increase and the subsequent decrease in the thromboplastin-infused rabbits possibly reflects the formation of microclots that were initially trapped in the lung and were subsequently removed. Pathologic examination of lung sections of these rabbits killed 10 minutes after the start of thromboplastin infusion revealed the presence of trapped fibrin (data not shown). In rabbits pretreated with 6-aminohexanoic acid and infused with thromboplastin, the radioactivity profile over the lung field was similar to the rabbits treated with thromboplastin alone. This result is different from the data obtained in rats in which 150 mg of 6-aminohexanoic acid/kg body weight prevented or delayed the decline of radioactivity over the lungs.

Effect of rLACI on the thromboplastin-induced coagulopathy. Having established a model that allowed monitoring of fibrin micro clot formation in the lung, we attempted to examine the effect of coinfusion or prior bolus injection of rLACI on thromboplastin-induced coagulopathy. Figure 4 shows the effect of the coinfusion of rLACI (800 µg rLACI/kg body weight) with thromboplastin on the radioactivity profile over the lungs. The results show that coinfusion of 800 µg rLACI/kg with thromboplastin completely suppressed the increase in radioactivity associated with infusion of thrombo-
plastin alone. Figure 5 shows the effect of prior bolus injection of LACI on the thromboplastin-induced changes in the lung radioactivity profile. This result indicates that prior rLACI bolus treatment (800 μg rLACI/kg) similarly prevented a thromboplastin-induced radioactivity increase over the lungs. It is noteworthy that in either the coinfusion study (Fig 4) or the rLACI bolus study (Fig 5), there is an initial decrease in radioactivity over the lungs just after the start of thromboplastin infusion that does not occur in control rabbits or rabbits treated with thromboplastin alone. The cause of this initial decrease in radioactivity is not understood but may be related to the fact that the inhibition of TF by LACI requires the formation of a small amount of activated factor X[3,10]. The dual processes of initiation and feedback inhibition of coagulation may manifest as an initial decrease in radioactive counts over the lung field. In contrast, injection of thromboplastin alone will cause an uninhibited coagulation cascade that may obscure the initial decrease of radioactivity. Lower doses of rLACI (270 and 135 μg LACI/kg) did not completely prevent the thromboplastin-induced radioactivity increase in the lungs. A typical result is shown in Fig 6.

Effect of rLACI on the thromboplastin-induced changes in the blood fibrinogen level and other coagulation parameters. The results obtained by monitoring the radioactivity over the lungs strongly suggest that fibrin was formed as a result of the activation of coagulation by thromboplastin. To further confirm this, we also measured plasma fibrinogen levels in response to thromboplastin and rLACI infusion, as shown in Fig 7. In rabbits infused with control buffers, fibrinogen levels did not change significantly within the 90-minute observation period. In rabbits treated with a bolus injection of 800 pg rLACI/kg before infusion of thromboplastin, fibrinogen levels essentially remain unchanged throughout the observation period. Apparently, this dose of rLACI effectively suppressed the thromboplastin-induced intravascular coagulation. The amount of rLACI used was serially reduced to investigate the effect of rLACI dosage in the control of thromboplastin-induced coagulation. It was determined that 135 to 270 μg rLACI/kg still provides essentially complete prevention of fibrinogen decrease, while 23 μg rLACI/kg protects the fibrinogen decrease by approximately 50% (Fig 7). Interestingly, we found that 135 to 270 μg rLACI/kg prevented systemic fibrinogen decrease but did not prevent the thromboplastin-induced radioactivity increase over the lungs (Fig 6). This suggests that there is local formation of fibrin in the lung that is not accompanied by significant changes in systemic fibrinogen levels. We have also compared two other coagulation parameters, APTT and the prothrombin time (PT), on plasmas collected from these rabbits (Table 1). In the saline control rabbits, the APTT

Fig 5. Effect of prior bolus injection of rLACI on the lung radioactivity changes induced by thromboplastin. The experiments were performed as described in Fig 1. 6-Aminohexanoic acid was used in all rabbits. rLACI was injected as a bolus through the femoral vein 1 minute before thromboplastin infusion. Group 1 rabbits were infused with saline (controls). Group 2 rabbits were infused with thromboplastin alone. Group 3 rabbits were given a bolus of rLACI (800 μg/kg) 1 minute before the thromboplastin infusion. The number of rabbits in each group is indicated.

Fig 6. Effect of low and high doses of rLACI on the lung radioactivity changes induced by thromboplastin. The experiments were performed as described in Fig 4. 6-Aminohexanoic acid was used in all rabbits. The rabbits were injected with a bolus of 800, 270, or 135 μg/kg rLACI 1 minute before the thromboplastin infusion. Note that the rabbits injected with 800 μg rLACI/kg did not show a radioactivity increase over the thromboplastin control, while lower doses of rLACI (270 or 135 μg/kg) did not prevent the radioactivity increase over the control group.
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Fig 7. Effect of thromboplastin and rLACl on systemic fibrinogen levels in rabbits. The experiments were performed as described in Fig 4. 6-Amino-hexanoic acid was used in all rabbits. Various amounts of rLACl were injected into the rabbits 1 minute before thromboplastin infusion, as indicated. A group of rabbits were treated with saline (controls). A second group of rabbits was infused with thromboplastin without injection of rLACI, as indicated. Blood samples were taken at various time points, and the fibrinogen levels of the separated plasmas were measured using a fibrinogen determination kit (Dade).

and PT essentially remain constant throughout the observation period. In rabbits infused with thromboplastin alone, the APTT and PT were prolonged to some extent. The exact reasons for this prolongation are not clear, but may be partly caused by the systemic decrease of fibrinogen. In rabbits infused or bolus injected with 800 µg rLACI/kg body weight, the APTT and PT showed the highest values after the end of the rLACI treatment (after 10 minutes). This was followed by a slow decline with time. It should be noted that the APTT and PT values at the end of the experiment (after 90 minutes) were still significantly higher than values before any injection. Because the fibrinogen levels in these rabbits remain constant, the prolongation may be due to the presence of rLACI in circulation.

DISCUSSION

Several techniques have been used to induce a pathologic state that mimics disseminated intravascular coagulation in humans. These include the injection of thromboplastin, thrombin, snake venoms, red blood cell stroma, and endotoxin. We have chosen to use thromboplastin to induce

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<th>Time (min)</th>
<th>Control*</th>
<th>+ TF‡</th>
<th>+ TF, + LACI§ (10-min infusion)</th>
<th>+ TF, + LACI§ (10-s, Bolus)</th>
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<tr>
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<td>90</td>
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<td>13.3 ± 1.0</td>
<td>32.6 ± 5.0</td>
<td>15.3 ± 0.8</td>
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The experiments were performed as described in the experimental section and Figure 1. The standard error in this table is represented by standard error of the mean.

*Rabbits (N = 3) were infused with control buffers alone.
†Rabbits (N = 4) were infused with thromboplastin alone.
‡Rabbits (N = 3) were coinfused with thromboplastin and 800 µg LACI/kg body weight.
§Rabbits (N = 3) were injected with 800 µg LACI/kg body weight 1 minute before infusion of thromboplastin. One of the rabbits in this set had a significantly longer APTT and PT at each time point than the other two animals, which results in a larger standard error. The reason for this difference is not clear, but may be due to animal to animal variation.
intravascular coagulation for the following reasons. First, thromboplastin appears to be a direct mediator of intravascular coagulation associated with many underlying diseases; therefore, this treatment may closely simulate various disease states. Second, the mechanism of thromboplastin function in coagulation is well defined both in vitro and in vivo and, thus, interpretation of the results may be simpler. Finally, LACI is an inhibitor of thromboplastin function and is, therefore, expected to have a direct impact on thromboplastin-induced coagulation. In this study, we have found that thromboplastin infusion at a constant rate produced reproducible results both in terms of pulmonary fibrin deposition/dissolution and fibrinogen/fibrin conversion. We have also shown that rLACI suppresses these thromboplastin-induced changes in a dose-dependent manner. The use of rabbits in our study of the effect of human rLACI is a compromised choice. As shown in Fig 2, the amount of rLACI required for prolongation of the prothrombin time is several-fold higher in rabbit plasma than in human plasma. Nonetheless, our in vitro and in vivo data demonstrate that human rLACI effectively inhibits thromboplastin-induced coagulation in rabbits. In future studies, it will be interesting to determine whether the dosage requirement in higher species would be lower than that required in the rabbit, as suggested by the in vitro results.

The pulmonary thrombosis induced by a nonlethal dose of thromboplastin, as monitored by the radioactivity in the lungs, appears to last for only a short duration in rabbits, and the clearance of fibrin from the lungs is not inhibited by the fibrinolysis inhibitor, 6-aminohexanoic acid. These results are in contrast to the results observed in the rat, in which the pulmonary thrombosis is prolonged by 6-aminohexanoic acid. Different mechanisms may operate to remove the pulmonary thrombi in these two species. Despite the short duration of pulmonary thrombosis, thromboplastin caused a decrease of systemic fibrinogen within 10 minutes and fibrinogen levels remained depressed for a prolonged period. Under this rather drastic condition, rLACI at a dose of 135 µg/kg body weight has been shown to effectively prevent systemic fibrinogen decrease. For comparison, it is interesting to note that the dose of tissue plasminogen activator required to reperfuse a thrombus in animal models and humans is approximately 1 mg/kg body weight. Therefore, the dose requirement of rLACI as a therapeutic anticoagulant protein compares favorably with recombinant tissue plasminogen activator as a thrombolytic agent. It is unknown at this time, though, whether rLACI possesses any advantage over the widely used and much less expensive anticoagulant, heparin.

Anticoagulation of blood can be achieved by the use of a variety of compounds. These compounds can be grouped into several categories based on their mode of action: (1) compounds that directly inhibit thrombin activity, such as hirudin (a leech inhibitor), and synthetic thrombin inhibitors; (2) compounds that promote the inhibition of thrombin by the endogenous protease inhibitors, such as heparinoids and other polysaccharides; (3) compounds that affect the biosynthesis of vitamin K-dependent clotting factors, such as warfarin; (4) natural anticoagulant proteins such as antithrombin III, heparin cofactor II, protein C, protein S, thrombomodulin, placental (vascular) anticoagulant protein, and LACI; and (5) monoclonal antibodies against coagulation components. It is apparent that these compounds are directed against several different targets of the coagulation cascade, including the inhibition of the terminal products, factor X, and thrombin; the inhibition of the biosynthesis of functional coagulation components; the enhancement of the destruction of coagulation cofactors; the competitive binding of phospholipids; and the inhibition of the initiation of the coagulation cascade. Theoretically, inhibition of the initiating events in the coagulation cascade may be more advantageous than the control of coagulation at a later stage of the cascade, since consumption of coagulation components would occur to a lesser degree. Furthermore, compounds that directly bind to and inhibit TF may provide a targeted and localized control of coagulation at the site of blood vessel or cell damage. Whether rLACI, as an inhibitor of TF, is a better anticoagulant in terms of efficacy, bleeding tendency, and other side effects than other anticoagulants remains to be determined.

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Recombinant lipoprotein-associated coagulation inhibitor inhibits tissue thromboplastin-induced intravascular coagulation in the rabbit

KC Day, LC Hoffman, MO Palmier, KK Kretzmer, MD Huang, EY Pyla, E Spokas, GJ Jr Broze, TG Warren and TC Wun