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ENHANCEMENT OF THE THROMBOLYTIC EFFICACY OF PROUROKINASE BY LYS-PLASMINOGEN IN A DOG MODEL OF ARTERIAL THROMBOSIS

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ABSTRACT

Current findings suggest that the efficacy of thrombolytic therapy may be limited by the availability of active forms of plasminogen at the thrombus site (1, 2). The purpose of this study was to determine if the systemic administration of 0.5 mg kg^{-1} glu-plasminogen (glu-plg) or 0.5 mg kg^{-1} lys-plasminogen (lys-plg) could safely increase the efficacy of a single intravenous bolus injection of 50,000 U kg^{-1} prourokinase (proUK) in a dog model of arterial thrombosis. Thrombolysis was measured by monitoring the continuous decrement of ^{125}I-gamma emissions from a radiolabeled thrombus. Reflow was evaluated by direct visual examination. Forty dogs (mean wt 10.3 $\pm$ 2 kg) were randomly sorted into 4 groups of 10 each. The dogs in each group were given either saline plus saline, saline plus proUK, glu-plg plus proUK, or lys-plg plus proUK 60 minutes after formation of an occlusive arterial thrombus. Ninety minutes after drug administration the dogs receiving saline plus proUK, glu-plg plus proUK, and the lys-plg plus proUK showed greater thrombolysis (41%, 43%, and 66%, respectively) than the control (saline plus saline) group (15%, $P < 0.01$). The lys-plg plus proUK treatment caused greater lysis than the saline plus proUK or the glu-plg plus

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proUK treatment ($P < 0.05$). All of the dogs (10/10) receiving lys-plg plus proUK had patent vessels at the end of the 90 minute monitoring period, whereas only 4/10 and 5/10 vessels were patent in the saline plus proUK and glu-plg plus proUK groups, respectively. None of the dogs in the saline plus saline group had patent vessels. No significant changes were observed in the various coagulation parameters tested for any of the 4 treatment groups. The results show that lys-plg can safely increase the thrombolytic efficacy of proUK.

INTRODUCTION

Plasmin is a serine protease derived *in-vivo* from the proteolytic cleavage of the zymogen plasminogen. In turn, plasmin is responsible for the proteolytic degradation of fibrin and fibrinogen. Plasminogen is present in the plasma at a concentration of approximately 200 ug ml^{-1} and exists mainly in two forms (3). The native form of plasminogen which is present in the plasma at a concentration of approximately 200 ug ml^{-1} has glutamic acid as its NH_2-terminal residue and is referred to as "glu-plasminogen" (glu-plg). However, limited plasmic digestion of this native form leads to small amounts of truncated plasma plasminogen with NH_2-terminal lysine, valine, or methionine residues, which have been collectively termed "lys-plasminogen" (lys-plg). Lys-plg does not appear to occur in plasma under normal conditions and is generated in very small amounts during thrombolytic therapy (4, 5). The reactivity of lys-plg with various plasminogen activators such as streptokinase, urokinase, and proUK is greater than that of glu-plg (1, 6, 7) and the adsorption of lys-plg to fibrin is superior to that of glu-plg (8, 9, 10). It has been demonstrated that activation of glu-plg to lys-plasmin by urokinase and tissue-type plasminogen activator occurs by two pathways (11):

1. glu-plg --> glu-plasmin --> lys-plasmin

2. glu-plg --> lys-plg --> lys-plasmin.

Pathway 1 is thought to predominate in plasma, while pathway 2 is initiated when fibrin is present. Therefore, lys-plg represents a possible intermediate in the plasminogen to plasmin activation reaction. However, at early stages of thrombolysis the accumulation of lys-plg is inhibited by the action of α_2-antiplasmin. Therefore the kinetics of plasminogen to plasmin conversion tend to be regulated by the properties of glu-plg rather than lys-plg.

The basic principal of thrombolytic therapy is to enhance the body's own natural fibrinolytic system by the administration of exogenous plasminogen activators. Ideally, thrombolytic therapy would provide: 1) complete lysis, 2) rapid reperfusion, 3) easy route of administration, 4) limited systemic fibrinogenolysis, and 5) low incidence of rethrombosis. To date, several different plasminogen activators, such as urokinase (UK), streptokinase (SK), tissue plasminogen activator (t-PA), and prourokinase (proUK) have been investigated; however, none have proven clearly superior in meeting all of the above criteria when given alone. At high levels of any activator the rate and extent of clot lysis may become limited by the rate of binding of plasminogen or the rate at which

plasminogen can achieve an open conformation in which it is accessible to activation. Based upon the known properties of lys-plg, it may be desirable to increase the plasma concentration of lys-plg at the onset of thrombolytic therapy. Several studies have been performed to investigate the effect of infusing plasminogen prior to or after thrombolytic therapy with the non-fibrin selective plasminogen activators UK and SK in order to substantially enhance the total available substrate (plg) (1, 2, 12-14). The majority of these trials suggest that such combination therapy enhances thrombolytic efficacy without increasing the incidence of bleeding complications. The purpose of this study was to investigate a distinct modification of the combination therapy approach. More specifically, this study examines the effect of human glu-plg and lys-plg when administered as an intravenous bolus dose, equivalent to only a small fraction of the total available plasminogen, immediately prior to an intravenous bolus dose of the fibrin-selective plasminogen activator proUK in a dog model of arterial thrombosis.

MATERIALS AND METHODS

Description of Animal Model

The dog model of arterial thrombosis used for this study has been described in detail elsewhere (15); however, a brief outline of the procedure is provided here for convenience. Each dog was anesthetized with pentobarbital sodium (30 mg kg^{-1}, intravenous injection) and then maintained at a surgical plane of anesthesia with inhaled methoxyfluorane and oxygen. A 2.0 cm long segment of the right femoral artery was then isolated and the luminal surface denuded of endothelium by a 5 minute exposure to boiling saline. A small branch vessel was cannulated, without disturbance of proximal or distal blood flow, and ^{125}I-fibrinogen (0.15 uC in 0.05 ml physiologic saline solution) and thrombin (100 U in 0.05 ml physiologic saline solution) were added to the isolated segment of the blood filled artery to create a totally occlusive thrombus. The thrombus was allowed to mature and develop adhesions to the damaged luminal surface for 30 minutes before the ligatures which demarcated the arterial segment were released. The thrombus was then exposed to the systemic circulation for an additional 30 minutes to permit washout of nonadherent labeled fibrinogen and fibrin and to determine the rate of autolysis (i.e., endogenously mediated, spontaneous thrombolysis) before the administration of any thrombolytic agents. Thrombolysis was monitored continuously before, during, and for 90 min after drug administration by measuring the ^{125}I-gamma emissions from the thrombus with an ^{125}I-specific gamma probe (Eberline Co., Santa Fe, NM). Both jugular veins were catheterized; one for the collection of blood samples for clinicopathologic testing (described below), and the other for the administration of the thrombolytic agents.

Preparation of Plasminogens and ProUK

Human glu-plg was obtained from Enzyme Research Laboratories, S. Bend, Indiana. This native form of plasminogen contained less than 0.2% plasmin and could be activated by urokinase to give 24.5 $\pm$ 0.5 I.U. of plasmin activity per mg protein. Human lys-plg also was obtained from Enzyme Research Laboratories and was freed of plasmin by chromatorgraphy at neutral pH on a column of aprotinin-agarose (Sigma). The product was immediately acidified to pH 4.5 on sephadex G-75. Chromogenic assay

using S2251 showed that the product contained less than 0.2% plasmin. After activation with urokinase the same chromogenic assay gave 24 U/mg protein. The lys- and glu-plasminogen preparations yielded single bands representing molecular weights of 88,000 and 93,000 grams per mole respectively when analyzed by SDS (sodium dodecyl sulfate) polyacrylamide gel (4% stacking gel and 12% separating gel) electrophoresis (17). N-terminal analysis by Edman degradation gave an average yield of 92% of the amount applied over the first 4 cycles. The only obsserved sequences over 15 cycles began with either lys-77 or val-78 of the plasminogen sequence. From the average recoveries over the first 4 cycles, the species beginning with val-78 was estimated to represent 30% of the total protein.

ProUK was obtained from Abbott Laboratories, North Chicago, Illinois. It was prepared from conditioned cell culture media of human kidney (HEK) cells grown under urokinase (Abbokinase) type production conditions but with aprotinin present to preserve the single chain product. Purification was accomplished essentially by the method of Stump and co-workers (16). The final product (0.5 to 1.0 mg ml^{-1}) was stored in Tris-HCl, 0.1 M NaCl, pH 7.5, with 10 I.U. ml^{-1} aprotinin. It was shown by SDS-PAGE gel electrophoresis (17) to be more than 95% pure and to have a molecular weight of approximately 54,000 grams per mole. The proUK contained less than 0.5% two-chain UK as shown by assay with chromogenic substrate (S-2444) (18). By preincubation with plasminogen, 1.0 mg of the proUK could be made maximally equivalent in the S-2444 assay to 109,000 I.U. of international standard (WHO) urokinase.

Experimental Design

Forty beagle dogs (average wt $\pm$ 2SD = 10.3 $\pm$ 2.0 kg) were randomly sorted into four equal groups. A thrombus was produced in the right femoral artery of each dog as described above. After monitoring autolysis for 30 minutes, dogs in Group 1 and Group 2 received a bolus intravenous injection of isotonic saline (5 ml), dogs in Group 3 received a bolus intravenous injection of 0.50 mg kg^{-1} glu-plg, and dogs in Group 4 received a bolus intravenous injection of 0.50 mg kg^{-1} lys-plg. Five minutes later, dogs in Group 1 received another equivalent volume of isotonic saline (5 ml), while dogs in Groups 2, 3, and 4 received a bolus injection of 50,000 U kg^{-1} proUK. The decrement in ^{125}I-gamma emissions from each thrombus, indicating thrombolysis, was summated and recorded for every 60 second interval for 90 minutes following drug administration. Results are expresssed in terms of percent thrombolysis which was determined from the 90 minute reading. Patency of the arterial segment (at time = 90 min) was determined by palpation, doppler signalling techniques, vessel color, and by severing the vessel distal to the original thrombus site and checking for blood flow as previously described (19). The arterial segment was then excised for macroscopic pathologic examination. Figure 1 represents a timeline diagram of the experimental protocol.

Blood samples were collected from the jugular vein contralateral to the drug administration site at the following times: prior to administration of anesthesia (baseline value); and 5 minutes, 25 minutes, and 60 minutes after administration of saline or the test drugs. Blood samples (9 ml) were collected into tubes containing 3.8% sodium citrate (1 ml) and centrifuged. The plasma was then separated into aliquots and used for the immediate determination of the PT, APTT, and the TT using commercially available reagents (Dade Actin for the APT, Aguada, Puerto Rico; Coagachek Thromboplastin for

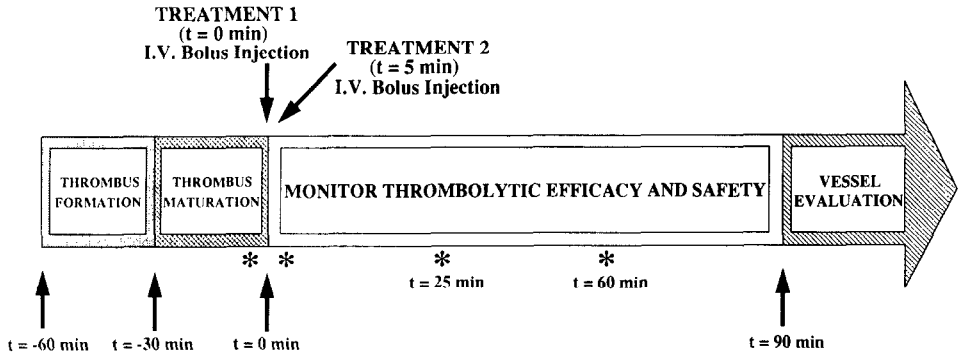

Figure 1. This time line diagram shows the chronologic sequence of thrombus formation and maturation followed by experimental therapy and monitoring of thrombolysis and reflow. The details of treatment 1 and treatment 2 for the individual 4 groups are described in the text.

the PT, Technicon Instruments, Elmhurst, IL; and Biodata Corp. Thrombinex for the TT, Hatboro, PA). A 2-fold increase over baseline values for the PT, APTT, and TT was considered to be significant. The mean reference values (± SEM) for PT, APTT, and TT in our laboratory are 14.4 ± 0.8 s, 10.7 ± 1.3 s, and 31.8 ± 1.3 s, respectively. Additional blood samples were collected at the same times for the determination of the hematocrit, platelet count, and fibrin degradation products (FDPs). The Thrombo-Wellcotest method (Wellcome Laboratories, Triangle Park, NC) was used for the semi-quantitative determination of FDPs. This method utilizes latex particles coated with antibodies against fibrinogen fragments D and E in serum. The aliquots that were used for α_2-antiplasmin (α_2-AP) determination contained the protease inhibitor P-PACK to prevent *in-vitro* generation of plasmin and loss of α_2-AP (20, 21). α_2-AP concentration was assayed by the Coatest Antiplasmin method (Helena Laboratories, Beaumont, TX) which utilizes the chromogenic substrate S-2251 (22). Decreased α_2-AP concentration was used as an indicator of systemic plasminogen activation.

Statistical Analysis

An analysis of variance (ANOVA) method was used to test the null hypothesis that there was no difference in the percent lysis recorded 90 minutes following drug administration for the 4 groups. If the null hypothesis proved to be false, then the Neuman-Keuls method was used to compare the mean values of the groups and identify which groups had a significantly different thrombolysis value. The number of dogs showing a 2-fold increase over baseline for the PT, APTT, or TT value was recorded for each group at each of the 3 post-drug administration blood collection times. The Fisher Exact test was used to test the null hypothesis that there was no difference between each of the drug-treated groups and the control group in the number of dogs with a 2-fold increase in these values at any of the sample collection times.

RESULTS

Percent Thrombolysis

There was no significant difference between the control group (No. 1) and any of the other 3 groups in the amount of autolysis which was recorded during the initial 30 minutes after the thrombus was formed and exposed to the circulation (i.e., prior to saline or drug administration). Ninety minutes following drug administration, the total decrement in ^{125}I counts for Group 1 showed 15 $\pm$ 2% thrombolysis, Group 2 showed 41 $\pm$ 4% thrombolysis, Group 3 showed 43 $\pm$ 7% thrombolysis, and Group 4 showed 66 $\pm$ 8% thrombolysis (Figure 2). The percent lysis values for Groups 2, 3, and 4 were all significantly greater ($P < 0.01$) than Group 1 (control group). Group 4 (lys-plg plus proUK) but not Group 3 (glu-plg plus proUK) showed significantly greater ($P < 0.05$) thrombolysis than Group 2 (saline plus proUK).

Patency

Ninety minutes following treatment none of the dogs receiving saline plus saline (Group 1) had a patent arterial segment. Four of the 10 dogs (40%) receiving saline plus proUK (Group 2) had a patent vessel at 90 minutes; however, all but one of these vessels showed the presence of a large residual mural thrombus and this caused a non-quantified partial reduction in flow. Although not quantified, blood flow was observed to be brisk and pulsatile in all vessels identified as patent. Patency of the arterial segment was also observed in 5 of 10 dogs (50%) receiving glu-plg plus proUK (Group 3). Of the 5 patent vessels, 4 contained large amounts of residual red thrombus. Ten of the 10 dogs (100%) receiving lys-plg plus proUK (Group 4) had patent vessels, all of which showed only small fragments, or no fragments, of residual thrombus after 90 minutes.

Clinicopathologic Test Values

None of the dogs in any of the 4 groups showed a 2-fold increase in either the PT, APTT, or the TT at any of the blood collection times. In addition, no changes were seen in the hematocrit or platelet counts, and no dogs showed increased FDP concentrations. The mean α_2AP concentrations for Groups 2, 3, and 4 showed their lowest values 60 min following drug administration (Figure 3). The lowest measured value was 80% at 60 min in Group 2 which is consistent with values previously reported with this dose

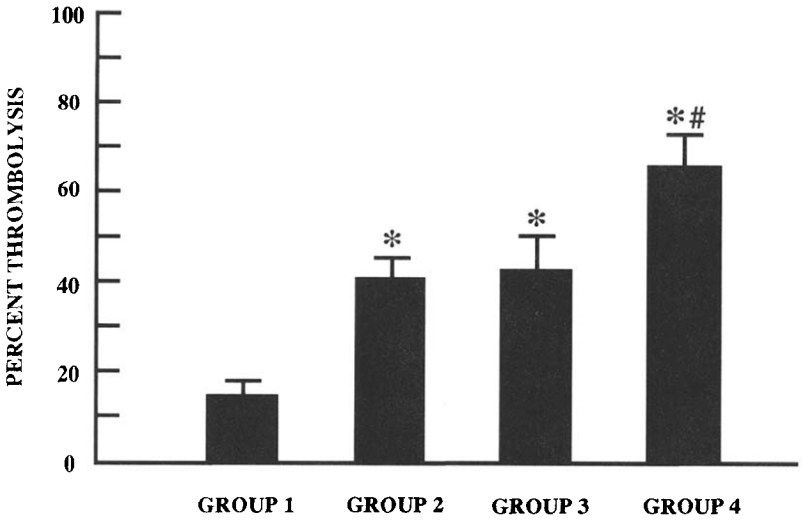

Figure 2. This graph shows the quantitative thrombolysis results for the 4 groups at 90 minutes after drug administration. Group 1 (control) received saline plus saline; Group 2 received saline plus 50,000 U/kg proUK; Group 3 received 0.5 mg glu-plg plus 50,000 U/kg proUK; and Group 4 received 0.5 mg lys-plg plus 50,000 U/kg proUK. The asterisk represents a p-value less than 0.01 vs. Group 1, and the cross represents a p-value less than 0.05 vs. Groups 1, 2, and 3.

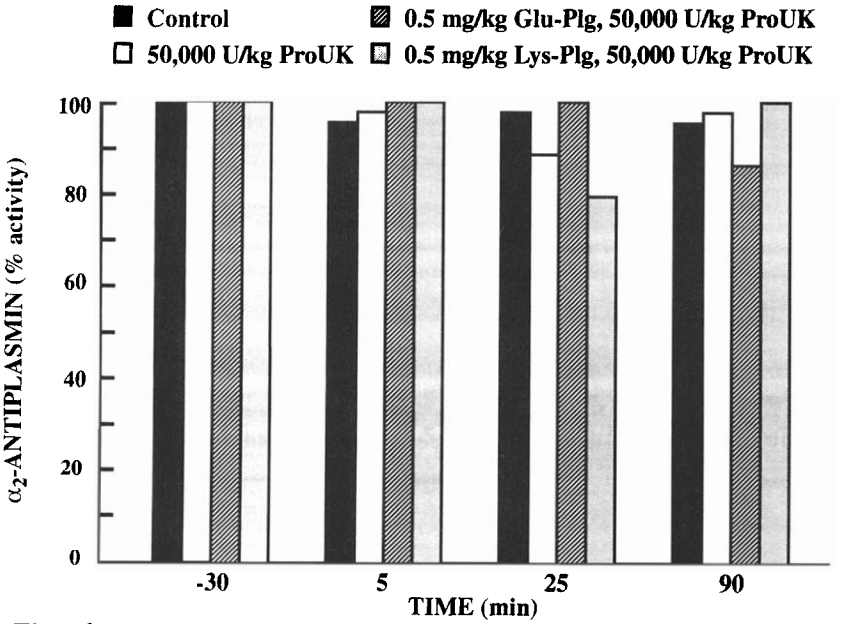

Figure 3. The plasma concentration of α_2-antiplasmin did not change significantly at any time after drug administration in any of the 4 groups. This finding indicates that minimal systemic activation of plasminogen occurred in the treated animals.

of proUK in the dog model (23). Finally, none of the dogs in any of the 4 treatment groups showed signs of bleeding from the surgical incisions sites or catheter access sites.

DISCUSSION

This study shows that systemic administration of lys-plg, but not glu-plg, can safely increase the thrombolytic efficacy of proUK in a dog model of arterial thrombosis. The dose of glu-plg and lys-plg (0.50 mg kg^{-1}) administered to the dogs in this study represents approximately 5% of the normal plasma concentration of plasminogen. The dose of proUK (50,000 U kg^{-1}) administered was chosen based on previous dose-response studies performed in our laboratory and provided a degree of thrombolysis (41%) which allowed any synergistic or antagonistic effects of the plasminogen forms to be readily noticeable (23).

This study indicated that when sequential bolus doses of lys-plg and proUK are administered, the resultant thrombolytic efficacy is substantially greater than that when only proUK is administered. It should also be noted that although lys-plg enhanced the thrombolytic efficacy of proUK, no significant changes in the various blood coagulation test values were detected. In addition, plasma α_2-AP concentrations, an indirect indicator of systemic plasminogen activation, did not decrease below 80% of the baseline value at any of the blood collection times. Therefore, the increased thrombolytic efficacy of lys-plg was not associated with any significant systemic plasminogen activation nor the associated detrimental alterations of the hemostatic mechanism. It appears that augmented plasminogen activation was confined to the thrombus site and thus protected from rapid inactivation by circulating α_2-AP. This localized thrombolytic activity can probably be attributed to the fibrin-specificity of proUK. According to previous dose-response studies performed in the same animal model, the thrombolytic efficacy and the incidence of vessel patency achieved with lys-plg plus proUK are equivalent to those achieved with 4 times the dose (or 200,000 U kg^{-1}) of proUK alone (23). However, this high dose of proUK caused a significant decrease in α_2-antiplasmin concentration (72% decreased from the baseline), whereas no significant decrease was noted for the combination therapy used in the present study. The combination lys-plg plus proUK therapy is not only safe but offers an easy route of administration which is especially important in emergent situations such as acute myocardial infarction.

The findings of this study are consistent with the results of several clinical studies that have been performed to investigate the thrombolytic efficacy and safety of lys-plg when used in conjunction with thrombolytic agents such as streptokinase (SK) and urokinase (UK) for treatment of deep venous thrombosis (12-14, 24), pulmonary embolism (25, 26), peripheral arterial thrombosis, and vascular graft occlusions (27-30). The majority of these clinical trials strongly suggested that infusing lys-plg prior to thrombolytic therapy increased thrombolytic efficacy without increasing the incidence of bleeding complications. However, the plasminogen activators involved in the investigation (SK and UK) are not "fibrin-specific"; that is, these thrombolytic agents activate systemic plasminogen as well as plasminogen localized at the thrombus site. Therefore, it is probable that systemic administration of lys-plg in combination with either UK or SK should result in enhanced fibrinolytic activity both at the clot site and systemically. The

present study supports the administration of subsequent intravenous bolus doses of lys-plg and proUK as a method for achieving safe and efficacious, clot-selective fibrinolytic activity. It should also be noted that the amount of lys-plg required to achieve these effects is very small, approximating less than 5% of the total circulating plasminogen.

These results are also in agreement with the *in-vitro* clot lysis studies performed by Watahiki and co-workers who found that the addition of lys-plg, but not glu-plg, at a concentration of 20% of normal plasma plasminogen increased thrombolytic efficacy of proUK (100 U/ml) 5 to 6 fold with only a small increase in fibrinogenolysis (31). Pannell and Gurewich, on the other hand, found that nonselective plasminogen activation by proUK and fibrinogenolysis occurred when plasma glu-plg was replaced by lys-plg (32). In this experiment nonselectivity referred to the ability of proUK to activate plasma lys-plg as well as plasminogen associated with the clot. However, the results of the present *in-vivo* study suggest an enhancement rather than a loss of clot specific thrombolytic activity by proUK. Such discrepancies may be attributed to the differences between *in-vitro* and *in-vivo* models. The *in-vitro* model may more closely mimic the thrombolytic activity at the clot site rather than that which occurs systemically. Our results indicate that fibrinogenolysis did not occur to a degree that was detectable by the methods used or that would be considered clinically significant.

The mechanism by which lys-plg, but not glu-plg, safely increases the thrombolytic efficacy of proUK has not been determined. Lys-plg may be able to occupy more lysine binding sites on the fibrin on the fibrin molecule than glu-plg. Furthermore, lys-plg provides a substrate for activation with higher fibrin affinity and greater activatability than glu-plg. Endogenous activator or trace two-chain UK may more readily form clot bound plasmin from lys-plg than glu-plg thus more rapidly initiating the local conversion of proUK into UK. It has been suggested that the "fibrin specific" action of proUK is a consequence of its selective activation of fibrin-bound plasminogen which has a conformation similar to that of lys-plg (32). Whatever the mechanism, it is important to note that although both lys-plg and proUK are administered systemically, fibrinolytic activity appeared to be confined to the thrombus site in this *in-vivo* model.

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