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Platelets inhibit the lysis of pulmonary microemboli

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pulmonary embolism; plasminogen activators; platelets; fibrinolysis; animal model; glycoprotein IIb/IIIa

THE LUNG SERVES as a natural filter for thromboemboli of diverse sizes (12). Thrombotic occlusion of the main or lobar pulmonary arteries by large clots (massive pulmonary embolism, PE) is a common cause of morbidity and mortality (15, 20). In contrast, pulmonary deposition of microemboli (ME) often does not cause acute symptoms (silent ME). Nevertheless, pulmonary ME is a relatively common accompaniment to acute lung injury (adult respiratory distress syndrome), hyperoxia, bleomycin toxicity, sepsis, trauma, surgery on the lower extremities, and angioplasty (2, 21, 30, 33, 36, 39, 40, 44, 52, 53). Microembolism may lead to inflammation and edema acutely and contribute to the development of pulmonary fibrosis and hypertension over time (4, 9, 22, 27, 31).

The treatment of PE, as with other venous thromboembolic conditions, is typically restricted to agents such as heparin that retard fibrin clot formation, with a relatively limited use of agents that promote fibrinolysis. Despite the effectiveness of such therapy, PE remains a common cause of mortality and morbidity. Thus there is a continuing need for more effective means to prevent and treat both PE and ME, based on a more thorough understanding of the mechanisms that regulate clot dissolution in the lung.

For example, the role of platelets in PE and ME has received relatively little study. Recently, it has been shown that platelet activation by antibodies in mice that are transgenic for expression of the signal transducing receptor FcRγIIA leads to occlusive platelet-fibrin thrombi in the pulmonary vasculature (50). Inhibition of platelet function by aspirin, nitric oxide donors, and inhibitors of other signal transduction pathways may improve the outcome of thrombotic pulmonary vascular occlusion secondary to intravascular thrombosis in several animal models (16, 23, 34, 51). Venous thrombi and PE are also the most common sequelae of intravascular platelet activation among patients suffering from heparin-induced thrombocytopenia (55). Yet human studies to date have provided ambiguous results concerning the efficacy of antiplatelet agents in the treatment of PE and ME (35, 48). However, the recent introduction into clinical medicine of potent intravenous inhibitors of the platelet glycoprotein IIb/IIIa complex (GP IIb/IIIa), the receptor required for platelet aggregation, suggests the need and provides the opportunity to reinvestigate this question.

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Platelets have been proposed to suppress clot lysis in large arteries via diverse mechanisms, including mechanical cross-linking, promotion of clot stability (24), and release of fibrinolytic inhibitors (13, 25, 26). However, the extent to which platelets affect dissolution of thromboemboli in the pulmonary arterial circulation, the vasculature most commonly affected in humans, is unknown as is the efficacy of newly available potent inhibitors of platelet function. We hypothesized that incorporation of platelets into nascent pulmonary thromboemboli would make them less susceptible to fibrinolysis and that inhibition of platelet GP IIb/IIIa function may attenuate this effect.

We developed rodent models that allow quantitative assessment of pulmonary microemboli dissolution and examined the role of platelets in pulmonary fibrinolysis. The results of our study show that *1*) platelets inhibit lysis of pulmonary ME and *2*) antiplatelet agents facilitate both spontaneous and pharmacological fibrinolysis in the lungs.

MATERIALS AND METHODS

Materials. The following materials were used: Iodogen from Pierce (Rockford, IL), $Na^{125}I$ from NEN Life Sciences (Boston, MA), fatty acid-free bovine serum albumin (BSA) from Boehringer-Mannheim (Indianapolis, IN), human thrombin and epinephrine from Sigma (St. Louis, MO), horse collagen (type I) from Chrono-Log (Havertown, PA), human recombinant tissue-type plasminogen activator (tPA, Activase) from Genentech (San Francisco, CA), fibrinogen (Fg; FIB 1, 1310) from Enzyme Research Laboratories (South Bend, IN), Retavase (a mutant form of human tPA that lacks kringle and growth factor domains), and murine monoclonal antibody that cross-reacts with human and rat platelet glycoprotein IIb/IIIa complex [MAb 7E3 $F(ab')_2$] from Centocor (Malvern, PA). Adult Sprague-Dawley male rats (200–350 g) and BALB/c mice were used following a protocol approved in writing by the University of Pennsylvania Institutional Animal Care and Use Committee. Human blood was obtained by phlebotomy from healthy volunteers, following a protocol approved in writing by the University of Pennsylvania Institutional Review Board for Studies Involving Human Subjects. Fg and Retavase were radiolabeled with ^{125}I using Iodogen per the manufacturer's instructions, and the free iodine was removed using a Bio-Spin 30 column (Bio-Rad Laboratory, Hercules, CA).

Preparation and characterization of ^{125}I-labeled thrombi. Plasma was isolated by centrifugation of fresh human blood anticoagulated in citrate (0.32% final concentration). ^{125}I-Fg ($\sim 30 \times 10^6$ cpm/ml) was added to fresh plasma or Fg solution 30 mg/ml in Krebs-Ringer bicarbonate buffer (KRB): 119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, and 1.2 mM KH_2PO_4-KRB. Before adding ^{125}I-Fg, we supplemented plasma with unlabeled Fg to a final concentration of 10 mg/ml to avoid potential differences in Fg levels among donors, to increase clot density, to prevent damage during homogenization, and to increase the yield of ^{125}I-ME.

$CaCl_2$ (20 mM final concentration) and human thrombin (Sigma; 0.2 U/ml final concentration) were added to plasma or fibrin containing ^{125}I-Fg to induce clotting. After 1-h incubation at room temperature, the clots were incubated overnight at 4°C to promote retraction. Clot stability and susceptibility to lysis by streptokinase and tPA did not change over the next 48 h at 4°C (data not shown); clots stored for longer times began to undergo spontaneous lysis. Therefore, all clots were used within 24 h of formation.

All subsequent steps were performed at 4°C. The procedure described above produces ^{125}I-labeled clots consisting of extensively cross-linked fibrin (1), which were dissected into either large emboli (^{125}I-clots, 5 mm in diameter) or homogenized to form a suspension of microemboli (^{125}I-ME). Before homogenization, clots were decanted on a plastic lid and cut into small pieces. The pieces were resuspended in 2 ml KRB and homogenized at 26,000 rpm for 1 min in a PT-3100 Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After the first homogenization step, the samples were centrifuged for 15 min at 2,000 *g*. The supernatant was removed, and the residual particles were pooled, resuspended in KRB, and rehomogenized. This procedure was repeated twice, and the resulting ^{125}I-ME were suspended in 8 ml KRB-BSA (3 mg/ml). The suspension was allowed to sediment at 4°C for 5 min to eliminate large particles. Four milliliters from the supernatant were used for the final preparation of the ^{125}I-ME suspension; it was divided into 200-μl aliquots that were stored at 4°C and used within 48 h. Random aliquots were selected to characterize the size distribution of the microparticles using a ZM Coulter counter (Coulter Electronics, Hialeah, FL). Approximately 80% of ME ranged from 1.5 to 3.5 μm in diameter, and <5% exceeded 10 micron in diameter (1). Large, single clots and ^{125}I-ME formed from platelet-rich plasma were always used on the day they were prepared. The accumulation and dissolution of ^{125}I-ME stored at 4°C remained stable for at least 3 days (not shown). Thus, in all experiments, fibrin or plasma ^{125}I-ME were injected within 3 days of preparation.

Our preliminary studies revealed no difference in lysis of clots formed from human plasma or from pure human Fg by tPA or when fresh rat or murine plasma was used as a source of plasminogen and its activators, under the experimental circumstances employed in our studies (not shown). In vivo studies showed no significant difference in the extent of pulmonary deposition or rate of spontaneous dissolution of ^{125}I-ME prepared from human plasma or pure Fg in rats and mice (not shown). No acute changes in the respiratory or circulatory status in mice or rats injected with human ME was observed suggestive of an allergic reaction to heterologous protein. All experiments were terminated within 1–5 h postinjection.

In vivo experiments. The external jugular vein was accessed in 300- to 350-g male rats via a neck incision under anesthesia and catheterized with a 14-gauge angiocatheter. This allowed injection of single large ^{125}I-clots (~5 mm diameter), which adapted the diameter of the catheter and vein due to their high elasticity. ^{125}I-ME (200-μl aliquots containing 30–100,000 cpm) were resuspended several times just before extraction into a 27.5-gauge syringe and injected via either jugular (through the pectoral muscle) or the tail vein. Only tail vein injection was used in mice. In a separate series, ^{125}I-Fg was coinjected with the indicated dose of collagen and epinephrine (^{125}I-Fg/coll/epi mixture) via the jugular or tail vein in both species.

At the indicated times after injection of ^{125}I-clots, ^{125}I-ME or ^{125}I-Fg/coll/epi mixture, animals were killed, and the major organs were harvested immediately, rinsed in saline, and dried on a filter paper; the radioactivity in each tissue was measured (Perkin-Elmer, Gaithersburg, MD). The injected dose (ID, cpm/animal) of injected radioactivity was calculated after subtracting the residual radioactivity remaining in the tube, syringe, and injection site.

Perfusion of isolated rat lung. Lungs were isolated from anesthetized rats (200–250 g) and perfused using recirculating perfusate (KRB, pH 7.4, containing 10 mM glucose and 3% fatty acid-free BSA, KRB-BSA) as described (37). Lungs were ventilated with a humidified gas mixture containing 5% CO_2 and 95% air with a SAR-830 rodent ventilator (CWE, Ardmore, PA) at 60 cycles/min, 2 ml tidal volume, and 2 cm H_2O end-expiratory pressure. The lungs were perfused at a flow rate of 10 ml/min at 37°C, followed by nonrecirculating perfusion for 5 min with KRB-BSA.

In vitro assay of degradation of ^{125}I-labeled clots. Diluted homogenates of organs (1 g/10 ml KRB) were obtained from intact anesthetized rats. Homogenate (200 μl) was then added to ^{125}I-fibrin-labeled human plasma clots (200 μl) enriched with 10 mg/ml of human Fg. Clots were incubated at 37°C, and radioactivity in the supernatants, representing degradation of the fibrin clot, was measured at the indicated time points.

Statistics. Data were analyzed using the unpaired, nonparametric Mann-Whitney test. Differences between groups were considered significant when $P < 0.05$.

RESULTS

The purpose of our studies was to examine the effect of platelets on the lysis of pulmonary emboli and to examine the impact of platelet inhibitory therapy on the rate of clot dissolution in the pulmonary vasculature. To address this issue, our first task was to develop rodent models in which the effect of platelets on fibrinolysis could be examined quantitatively in vivo.

Pulmonary deposition of large emboli and ME in vivo. We first compared the biodistribution of radiolabeled Fg, ^{125}I-Fg, as well as ^{125}I-clots and ^{125}I-ME prepared from ^{125}I-Fg, 5 min after intravenous injection in intact rats (Fig. 1). Most ^{125}I-Fg remained in the plasma or was taken up by the liver. Little radioactivity accumulated in the lungs (Fig. 1*A*), consistent with the expected hepatic clearance of large (molecular weight >60 kDa) radiolabeled proteins.

^{125}I-clots injected via the jugular vein followed either of two distinct patterns of distribution. Approximately 40% of the animals died within 5 min of injection. Postmortem examination of these animals revealed that ~60% of injected ^{125}I-labeled protein was found in the heart and in the main pulmonary arteries, but outside of the lung parenchyma proper (Fig. 1*B*). In contrast, among the survivors, 90% of the injected radioactivity was found in the lungs (Fig. 1*C*).

Injection of ^{125}I-ME led to quite a different pattern of distribution and clinical outcome. Fifty to sixty percent of the injected dose (%ID) was found in the lungs in all animals (Fig. 1*D*). The percentage of radioactivity in blood and other organs did not exceed 5%ID after injection of ^{125}I-ME, similar to that seen for ^{125}I-plasma clots in survivors (Fig. 1, *C* and *D*). Injection of ^{125}I-ME gave similar results in rats and in mice, with 50–60% of injected radioactivity found in the lungs 10 min postinjection (Table 1). Tail vein and jugular vein injection of ^{125}I-ME prepared from fibrin or human plasma was distributed similarly in rats (not shown).

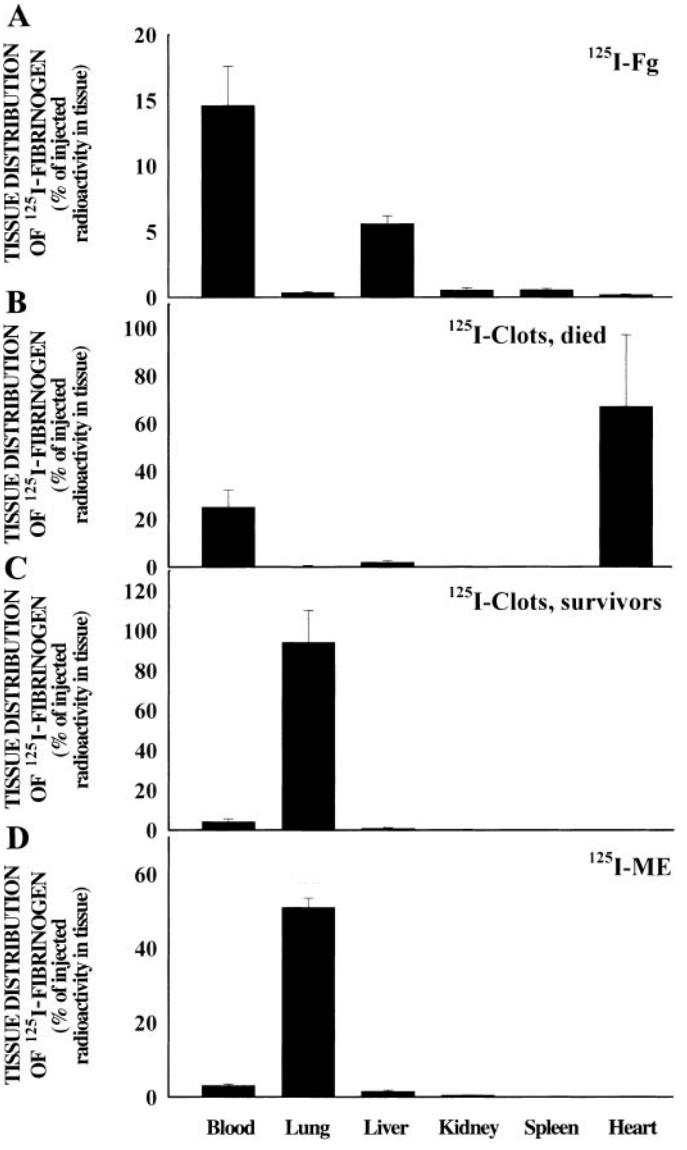

Fig. 1. Distribution of radiolabeled fibrinogen (Fg) and fibrin clots in rats after injection via the jugular vein. Animals were killed 5 min postinjection. *A*: ^{125}I-labeled monomer fibrinogen, $n = 4$. *B*: ^{125}I-clots, 5–6 mm in diameter, in animals that did not survive 5 min postinjection, $n = 4$. *C*: the same injection as in *B*, showing the survivors, $n = 7$. *D*: ^{125}I-Microemboli (^{125}I-ME) injection, $n = 6$. The data are calculated as percentages of injected doses of radioactivity recovered in the organs (%ID/organ). Here and in all following figures, the data are shown as means ± SE, unless indicated otherwise.

Distribution and spontaneous dissolution of ^{125}I-clots and ^{125}I-ME in rat lungs. ^{125}I-clots were heterogeneously distributed in the lungs and tended to lodge in a single lobe. For example, 70–90% of injected radioactivity was found in the left lobe (the largest in this species) in six out of ten animals in one experiment. In these animals, <0.5% of ID was found in the other lobes. However, in two other animals, 50–80% of injected ^{125}I-clots were found in the right lower lobe (the second largest), with no more than 0.5% in the other lobes. In the remaining two rats, ^{125}I-clots were found in two lobes, likely due to disintegration and emboli-

Table 1. *Distribution of ^{125}I-ME in rats and mice after intravenous injection*

%ID/organ	*n*	Blood	Lung	Liver	Kidney	Spleen	Heart
Mice	24	4.4 ± 0.4	50.8 ± 1.9	4.7 ± 0.2	1.23 ± 0.06	0.8 ± 0.1	0.33 ± 0.02
Rats	14	0.23 ± 0.05	54.6 ± 3.5	0.13 ± 0.04	0.47 ± 0.11	0.12 ± 0.03	0.04 ± 0.01

The data, shown as percentage of injected radioactivity per organ recovered 10 min postinjection via the tail vein in intact anesthetized animals, are means ± SE. Anesthetized animals were killed 10 min postinjection of ^{125}I-microemboli (ME) via the tail vein. Note that in both animal species, the major fraction of radioactivity was found in the lungs, clearly indicating that ME accumulate in the pulmonary vasculature after intravenous injection.

zation from the heart or pulmonary artery bifurcation. In the latter cases, a single clot was identified in the left and the lower right lobes.

In contrast, ^{125}I-ME were distributed homogeneously throughout the lobes. Table 2 shows the lobar distribution of ^{125}I-ME in rat lungs from several separate experiments. The data are expressed as percentages of ID per gram, to compensate for differences in lobe size. The results clearly show that ME are evenly distributed among the lobes in rats. These data also show that the pulmonary deposition of ^{125}I-ME in intact rats is highly reproducible. These data are consistent with the results of a previous study, in which we documented the homogenous distribution of ^{125}I-ME in lobes of the lung after tail vein injection in mice followed by gamma-radioautography (1).

Injection of 10^5 microparticles per animal in 100 μl saline did not lead to an increase in respiratory rate or other overt behavioral change in rats or mice throughout the experimental period (up to 5 h), nor was any evidence of lung tissue damage or inflammation seen at postmortem examination. The death rate after injection of ^{125}I-ME was low (<3%, $n > 100$). In contrast, when a larger dose of ^{125}I-ME (10^6 microparticles per animal) was injected in mice, acute pulmonary edema was evident 5 h postinjection (wet-to-dry ratio 6.4 ± 0.5 vs. 4.4 ± 0.3 in control mice), and disseminated lung hemorrhage was visible at postmortem examination (data not shown). Rats tolerated an injection of 10^6 microparticles without evidence of gross injury.

Pulmonary ^{125}I-ME underwent marked spontaneous dissolution within the first hour after injection in rats in contrast to the behavior of large ^{125}I-clots (Fig. 2). Figure 3 compares the kinetics of pulmonary ^{125}I-ME dissolution in rats and mice. In both species, pulmonary radioactivity did not change significantly in the first 30 min, arguing against nonspecific wash-out, but dropped precipitously over the next few hours. Dissolution of ^{125}I-ME approached 70 and 50% in mice and rats 1 h postembolization, respectively. By 5 h, dissolution of ^{125}I-ME was practically complete in both species. The first phase of pulmonary ^{125}I-ME dissolution was accompanied by an elevation in the radioactivity found in the plasma, likely reflecting the release of ^{125}I-fibrin degradation products from the lungs (Fig. 3, *insets*). However, plasma radioactivity was not elevated in proportion to the extent of pulmonary fibrinolysis and declined after 1 h, suggesting rapid clearance of radiolabeled fibrin degradation products from the bloodstream.

Dissolution of ME ex vivo in perfused rat lungs. Pulmonary emboli represent a substrate for plasmin generated by plasminogen activators both circulating in plasma and expressed in lung tissue. To dissect the relative contributions of pulmonary and plasma-derived fibrinolytic factors in spontaneous dissolution of ME, we characterized lysis of ^{125}I-ME lodged in isolated perfused lungs (IPL) and in vitro lysis of ^{125}I-clots by lung tissue homogenates.

First, we compared the rate of degradation of ^{125}I-ME lodged in rat lungs in vivo with their dissolution in isolated rat lungs perfused with KRB (Fig. 4). Initial pulmonary deposition of ^{125}I-ME 10 min postinjection was comparable in each model (61 ± 5% in intact rats, 75 ± 2% in the isolated lungs). Spontaneous fibrinolysis was very effective in vivo: almost no residual ^{125}I-ME was detected in the lungs 3 h postinjection in intact rats (2.3 ± 1.1%). In contrast, fibrino-

Table 2. *Distribution of ^{125}I-ME in the lung lobes after intravenous injection in rats*

	n	Right Lower	Right Middle	Right Upper	Lingular	Left	Lung Total
Experiment							
1	4	46.4 ± 1.9	54.4 ± 5.5	48.1 ± 4.6	50.8 ± 5.1	42.8 ± 3.5	48.5 ± 2.5
2	3	39.1 ± 11.2	28.9 ± 7.1	34.1 ± 8.3	33.5 ± 4.1	38.7 ± 7.2	35.7 ± 7.5
4	3	45.5 ± 10.1	34.8 ± 5.8	37.2 ± 4.5	34.2 ± 5.8	47.5 ± 3.5	39.9 ± 1.8
11	3	50.8 ± 6.1	54.1 ± 3.9	42.1 ± 6.3	38.6 ± 6.2	50.5 ± 11.2	47.2 ± 5.9
Total	25	48.0 ± 3.7	52.3 ± 3.2	45.6 ± 7.2	49.8 ± 5.4	50.0 ± 3.2	49.2 ± 4.0
Weight, g		0.3	0.15	0.1	0.1	0.45	1

The results are calculated as means ± SE. Anesthetized rats were killed 10 min postinjection of ^{125}I-ME via the tail vein. Note a high reproducibility of the results of several separate experiments indicating that radiolabeled ME are homogenously distributed throughout the lung lobes. The data are shown as percentage of injected dose of radioactivity recovered per gram of lung tissue (%ID/g) to compensate for size difference between the lobes. The last column showing this parameter for the total lung was calculated as a sum of radioactivity in all lobes divided by total lung weight. The total lung uptake also can be calculated as a sum of %ID/g in individual lobes after multiplying the data on the lobe weight (see the last row that shows an average lobe weight in grams).

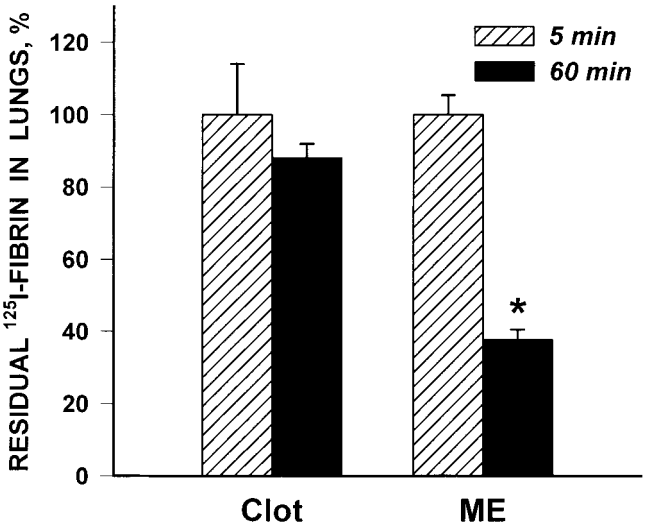

Fig. 2. Spontaneous dissolution of ^{125}I-ME, but not ^{125}I-clots, lodged in the rat lungs. *Statistically different vs. 5-min value ($P < 0.01$).

lysis in the isolated lungs was not as effective (45 ± 10% residual ^{125}I-ME by 3 h).

This result might suggest that rat lung tissue possesses relatively low fibrinolytic or considerable antifibrinolytic activity. However, this does not appear to be the case. To compare fibrinolytic activity in different organs, we added aliquots of various tissue homogenates to ^{125}I-labeled fibrin clots in vitro. The Fg preparation used to form these fibrin clots contained trace amounts of plasminogen that can be converted to plasmin by exogenous plasminogen activators. Fibrinolysis was monitored by measuring the release of radioactivity into the supernatant fluid at various times. After a 60-min incubation with ^{125}I-labeled clots, only homogenates prepared from buffer-perfused rat lungs induced significant lysis that markedly exceeded the background level (Fig. 5*A*, difference with all other tissues is statistically significant, $P < 0.05$). After a more prolonged incubation, homogenates of other organs (kidney, heart, and brain) also induced significant lysis, yet lung homogenate still caused the most extensive fibrinolysis among the organs tested (Fig. 5*B*).

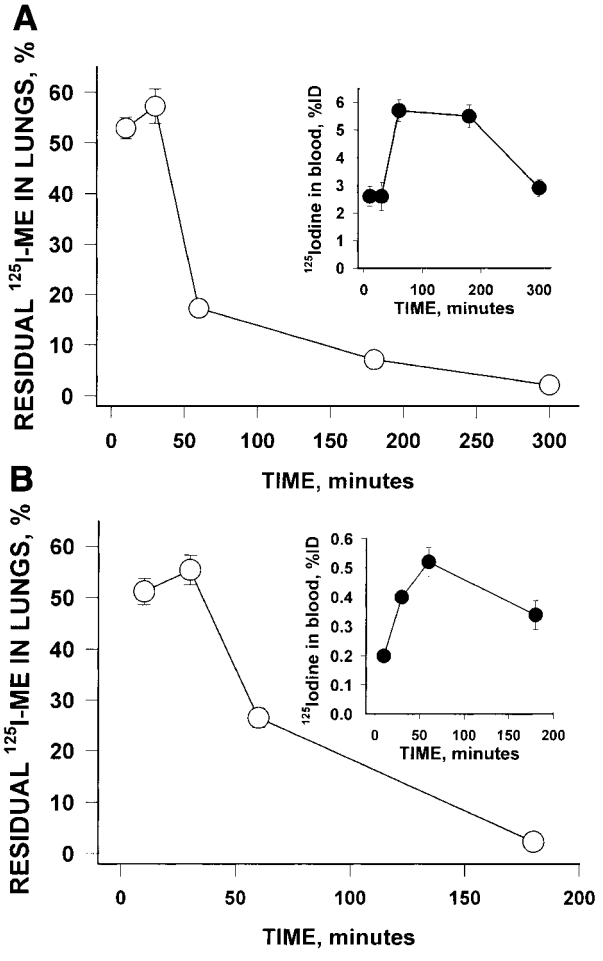

Fig. 3. Kinetics of spontaneous dissolution of ^{125}I-ME lodged in the lungs of mice (*A*) and rats (*B*). *Insets*: blood level of ^{125}I.

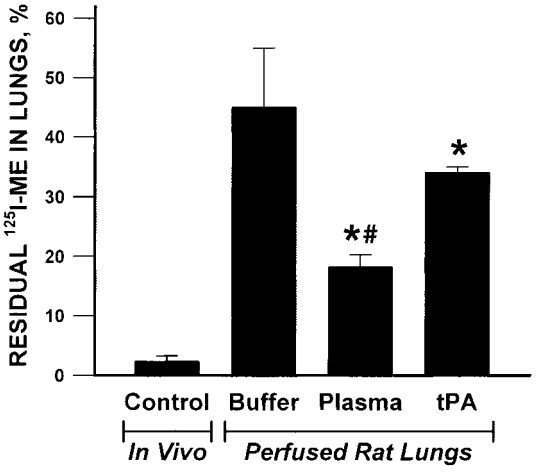

Fig. 4. Dissolution of ^{125}I-ME lodged in the isolated rat lungs perfused with Krebs-Ringer buffer (KRB). The data are shown as residual radioactivity (% of initial load) in the perfused lungs 3 h post-^{125}I-ME administration. Bars: control, ^{125}I-ME dissolution after lodging in intact rats in vivo; buffer, isolated lungs perfused with KRB; plasma, isolated lungs perfused with KRB containing 20% diluted fresh rat plasma; tPA, isolated lungs perfused with KRB containing tissue-type plasminogen activator. *Statistically different vs. values obtained in isolated perfused lungs (IPL) perfused with buffer ($P < 0.01$). #Statistically different vs. values obtained in IPL perfused with buffer enriched in tPA ($P < 0.01$).

The in vitro fibrinolysis assay shows that rat lungs possess relatively high fibrinolytic activity. However, both alveolar and vascular plasminogen activators contribute to in vitro lysis, whereas only those accessible from the endothelial lumen can contribute to the lysis of ME in the perfused lungs. Figure 4 shows that infusion of tPA (300 μg/45 ml perfusate) had some impact on fibrinolysis (34 ± 1% residual ^{125}I-ME vs. 45 ± 10% in plasma-free buffer-perfused lungs). However, infusion of fresh 20% rat plasma stimulated fibrinolysis more effectively (18 ± 2% residual ^{125}I-ME; differences with plasma-free control and tPA-supplemented perfusion are statistically significant; $P < 0.01$).

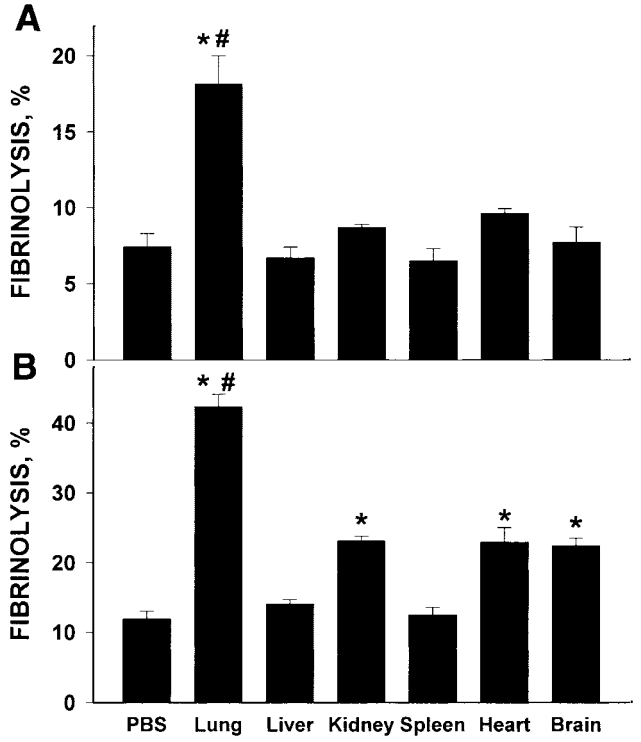

Fig. 5. Effect of homogenate prepared from the rat organs on in vitro lysis of ^{125}I-labeled fibrin clots. *A*: 60-min incubation at 37°C. *B*: 390-min incubation at 37°C. *Extent of fibrinolysis is statistically different from background level obtained by adding PBS ($P < 0.01$); #extent of fibrinolysis is statistically different from that attained by homogenates of other organs tested ($P < 0.01$).

Effect of exogenous tPA on dissolution of pulmonary emboli in intact rats. In the next series of experiments, we characterized the dissolution of PE in rats by exogenously added plasminogen activators. In pilot experiments, we found that intravenous injection of a relatively high dose of tPA (Activase, 500 μg/kg) 10 min after injection of large ^{125}I-clots caused ~50% fibrinolysis. (Residual radioactivity in the lungs 1 h after ^{125}I-clots injection was 38.4 ± 5.8 vs. 81.5 ± 4.5%ID in control rats). However, the high mortality and low reproducibility that we observed, although reflecting the clinical experience with massive PE, limited the utility of this approach as a model.

Therefore, we explored the more reproducible ME model to examine the effect of plasminogen activators in greater detail with Retavase (a variant of human recombinant tPA that lacks kringle and growth factor domains and demonstrates slower clearance from the circulation in humans). Figure 6*A* shows that even a relatively low dose of Retavase (30 μg/kg) injected in rats 10 min post-^{125}I-ME administration reduced the residual pulmonary radioactivity by 50% (17% of injected ^{125}I at 1 h). Higher doses (100 μg/kg and above) caused 90–95% dissolution of pulmonary ^{125}I-ME in both rats and mice by 1 h.

We then asked whether Retavase could be administered in a prophylactic mode. However, Retavase injected into mice even 10 min before ^{125}I-ME had no effect on the rate of pulmonary clot lysis, even at very

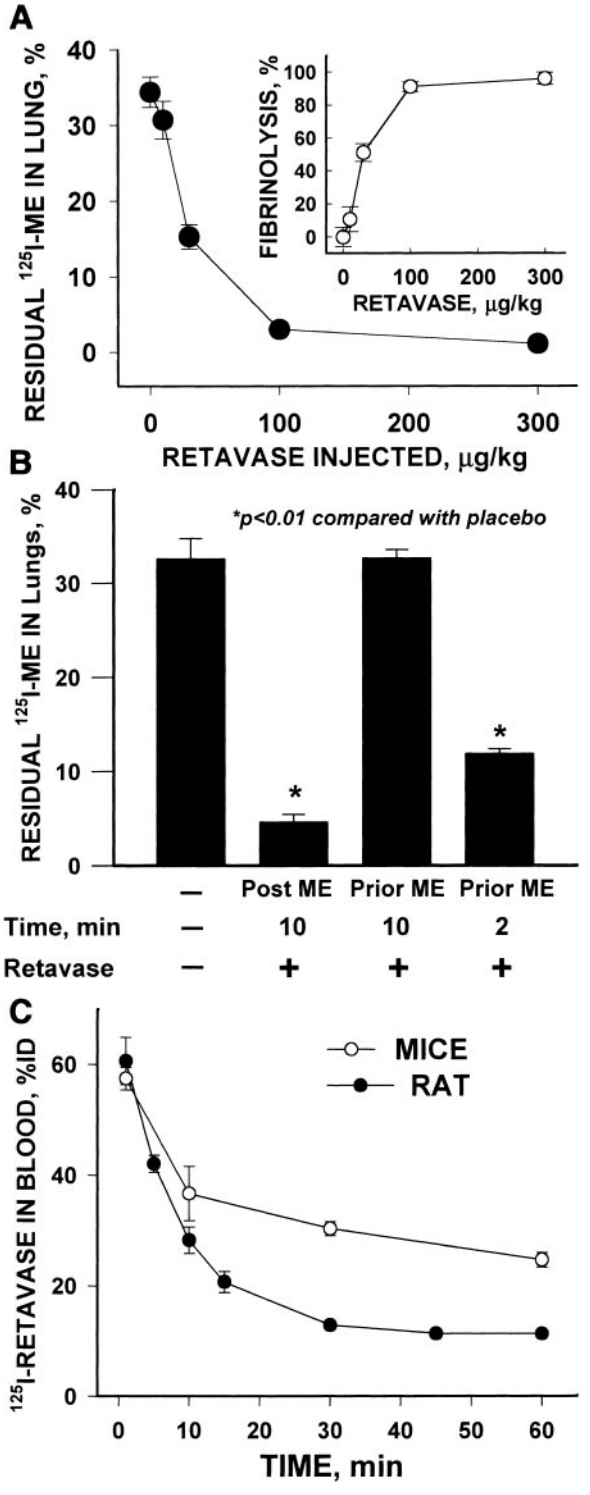

Fig. 6. Dissolution of pulmonary microemboli in rodents by Retavase (tPA). *A*: dependence of dissolution of pulmonary ^{125}I-ME on the dose of Retavase injected in rats 10 min post-^{125}I-ME. *Inset*: data expressed as percentage of fibrinolysis in the lungs, where 0% corresponds to the level of residual ^{125}I-ME in animals injected with placebo (saline). *B*: effect of time of Retavase injection on lysis of pulmonary ^{125}I-ME in mice. First bar, placebo (saline); second bar, 500 μg/kg Retavase 10 min post-^{125}I-ME injection; third bar, 500 μg/kg Retavase 10 min before ^{125}I-ME injection; fourth bar, 500 μg/kg Retavase 2 min before ^{125}I-ME injection. In experiments shown in *A* and *B*, residual ^{125}I-ME radioactivity was determined in the lungs 60 min post-^{125}I-ME injection. *C*: the kinetics of blood clearance of ^{125}I-labeled Retavase after intravenous injection in mice (○) and rats (●).

high doses (500 μg/kg; Fig. 6*B*). This result is consistent with the kinetics of elimination of Retavase from the bloodstream in mice and rats (Fig. 6*C*), a characteristic feature of all known plasminogen activators despite genetic deletion of epitopes that have been implicated in clearance. In support of this notion, Retavase (500 μg/kg) injected in mice 2 min before ^{125}I-ME significantly facilitated clot dissolution in the lungs (Fig. 6*B*).

Involvement of platelets and effect of antiplatelet GP IIb/IIIa. Having established reproducible, nonlethal, models of pulmonary embolization in rodent, we then turned to examine the role of platelets in controlling the rate of clot dissolution. To do so, we first compared the dissolution of ^{125}I-ME prepared from platelet-poor plasma (the standard preparation used throughout this study) with that of ^{125}I-ME prepared from platelet-rich plasma. Figure 7*A* shows that incorporation of platelets into ^{125}I-ME significantly retarded the lysis (compare residual ^{125}I 1 h post-ME injection). Thus even in this model of ME, which are intrinsically highly susceptible to endogenous fibrinolysis, the impact of platelets is evident within the often critical first hour.

One common clinical setting in which diffuse ME develop is in disorders characterized by disseminated intravascular platelet activation. To simulate the pulmonary deposition/dissolution of ^{125}I-thrombi (both large and ME) induced by intravascular platelet aggregation in vivo, rodents were injected intravenously with ^{125}I-Fg/coll/epi mixture. Collagen and epinephrine activate platelets and have been shown previously to cause the pulmonary deposition of endogenously formed intravascular platelet-rich clots in mice (58). In our pilot studies, ^{125}I-Fg/coll/epi mixture injection also caused pulmonary deposition of ^{125}I-Fg in rats to 3.46 ± 0.76%ID compared with 0.71 ± 0.09%ID in control rats injected with ^{125}I-Fg in saline. However, collagen/epinephrine also caused high lethality in rats, characterized by patchy pulmonary hemorrhages and pulmonary edema detectable by marked elevation of lung wet-to-dry ratio and pulmonary deposition of ^{125}I-labeled albumin (not shown).

In an attempt to avoid these poorly controlled and severe adverse effects of collagen/epinephrine in rats, we carried out these studies in mice. We found that injection of ^{125}I-Fg mixed with collagen (90 μg/kg) and epinephrine (600 μg/kg) led to rapid pulmonary deposition of ^{125}I-Fg in mice without significant lethality and pulmonary edema. In mice, pulmonary radioactivity was markedly elevated ($P < 0.05$) 10 min after injection of ^{125}I-Fg/coll/epi mixture, whereas the radioactivity in other organs (e.g., liver) and blood was reduced (Fig. 7*B*). The lung-blood radioactivity ratio increased threefold after ^{125}I-Fg/coll/epi mixture injection, from 0.48 ± 0.08 to 1.45 ± 0.22.

Figure 7*C* shows that there was practically no dissolution of ^{125}I-Fg labeled clots formed in the murine lungs after injection of ^{125}I-Fg/coll/epi mixture (open bars), and fibrinolysis was markedly retarded compared with dissolution of preinjected ^{125}I-ME in control BALB/c mice (solid bars). Figure 7*D* shows that 18.9 ±

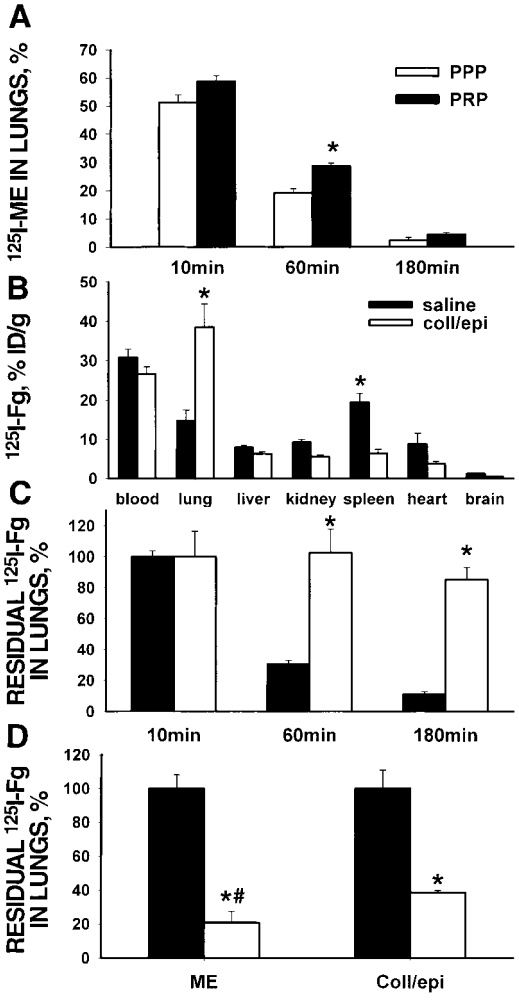

Fig. 7. Platelets inhibit dissolution of ME and fibrin lodged in rodent lungs in vivo. *A*: comparison of dissolution of ME prepared from platelet-poor (PPP, open bars) and platelet-rich (PRP, solid bars) human plasma, $n = 10$ (PPP) and 5 (PRP). *Difference between PRP and PPP ME is statistically significant ($P < 0.01$). *B*: deposition of ^{125}I-fibrinogen (^{125}I-Fg) in murine lungs induced by injection of collagen/epinephrine mixture (coll/epi). Data are shown as ^{125}I tissue level in mice 10 min postinjection of ^{125}I-Fg in saline (control, solid bars) or ^{125}I-Fg/coll/epi mixture (open bars), $n = 5–6$. Note that pulmonary radioactivity significantly increases, whereas that in other organs and blood is reduced (*difference between coll/epi and saline-injected mice is statistically significant, $P < 0.01$). *C*: spontaneous dissolution of ^{125}I-Fg deposits in the murine lungs after injection in form of ^{125}I-ME (solid bars) or ^{125}I-Fg/coll/epi mixture (open bars); $n = 5–27$, with at least 2 different experiments on each time point. Note that compared with ME, dissolution is markedly and significantly (*$P < 0.01$) retarded in the case of intravascular platelet activation by coll/epi. *D*: therapeutic dissolution of ^{125}I-Fg deposits in the murine lungs after injection in form of ^{125}I-ME or ^{125}I-Fg/coll/epi mixture. Retavase (500 μg/kg in ME group or 1 mg/kg in ^{125}I-Fg/coll/epi mixture group, open bars) or saline (solid bars) was injected in mice via tail vein 10 min after injection of ^{125}I-ME or ^{125}I-Fg/coll/epi mixture, and residual pulmonary radioactivity was determined 50 min later. The results are calculated as %control level obtained in saline-injected mice (solid bars). $n = 4–12$. Note that Retavase effectively dissolves pulmonary deposits in both models, although the amplitude of the fibrinolysis is higher in case of ^{125}I-ME (the difference with saline control is statistically significant in both Retavase-treated groups, *$P < 0.01$, whereas the difference between the Retavase-treated groups is statistically significant, $^{\#}P < 0.05$).

6.6% of the radiolabeled fibrin(ogen) remained in the lungs of mice injected with ME followed 10 min later by Retavase (0.5 mg/kg), whereas 39.5 ± 1.5% residual clot remained in the lungs of mice treated with twice as much Retavase after injection with ^{125}I-Fg/coll/epi mixture ($P < 0.05$). These results indicate that incorporation of activated platelets into endogenously formed pulmonary thrombi/emboli lessens their susceptibility to endogenous and pharmacological fibrinolysis.

As a third approach, we asked whether inhibition of platelet function would facilitate resolution of pulmonary ME. To do so, we examined the effect a murine MAb 7E3 F(ab′)$_2$ fragment that blocks the Fg receptor, GP IIb/IIIa, expressed on rat and human, but not on mouse, platelets (7). Injection of 6–10 mg/kg MAb 7E3 in rats inhibits the aggregation of rat platelets for several hours in vitro and in vivo (17, 45). We could not examine the effect of 7E3 on collagen/epinephrine-induced in situ clot formation because the mouse platelets were nonreactive and the model is lethal in rats. However, we have previously shown that ^{125}I-ME formed from platelet-poor plasma accumulate in the pulmonary vasculature as aggregates larger in size than the injected particles themselves and are invested with blood elements (1). This finding raised the possibility that endogenous platelets are incorporated into the clot during its growth and extension in vivo. In support of this notion, we found that intravenous injection of 10 mg/kg MAb 7E3 F(ab′)$_2$ enhanced the dissolution of ^{125}I-ME by 20–25% (Fig. 8*A*). Finally, we injected rats with ME followed by a low dose of Retavase (10 μg/kg), 7E3 F(ab′)$_2$ (6 mg/kg), or both. Figure 8*B* shows that addition of 7E3 F(ab′)$_2$ enhanced Retavase-induced fibrinolysis by 50%.

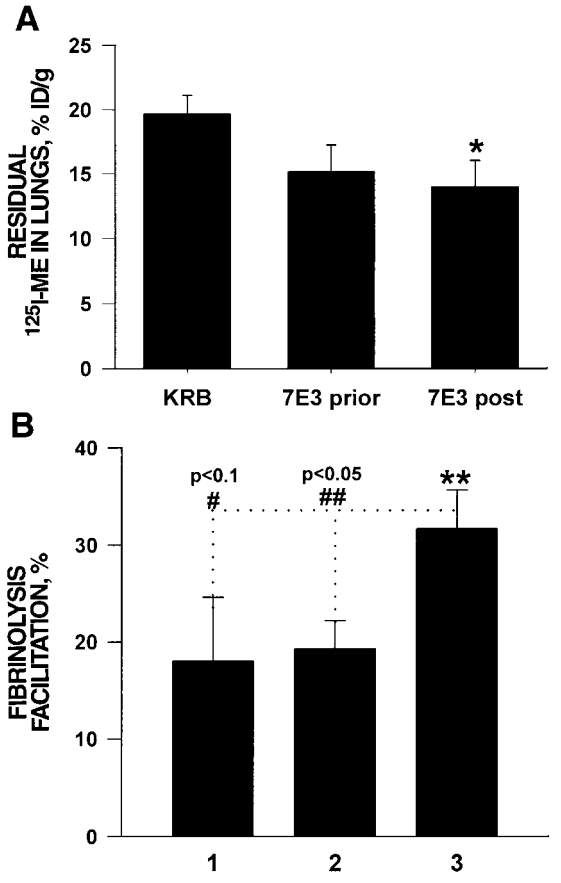

Fig. 8. Effect of glycoprotein (GP) IIb/IIIa monoclonal antibodies (MAb) on spontaneous and fibrinolytic dissolution of microemboli lodged in rat lungs. *A*: anti-GP MAb 10 mg/kg 7E3 F(ab)$_2$ was injected intravenously 10 min before (7E3 prior) or 10 min after (7E3 post) injection of ^{125}I-ME in rats. Control mice were injected with equal volume of KRB 10 min post-^{125}I-ME (KRB). Residual radioactivity in the lungs was determined 60 min post-^{125}I-ME injection. Data shown as means ± SE, $n = 4–7$ (*difference with control group is statistically significant, $P < 0.05$). *B*: effect of combined administration of Retavase and the GP IIb/IIIa inhibiting antibody MAb 7E3 on residual ME in the rat lungs. Ten min post-ME injection, rats were injected via the tail vein with Retavase (10 μg/kg, *bar 1*); MAb 7E3 (6 mg/kg, *bar 2*); or mixture of these two proteins at the same doses (*bar 3*). One hour post-ME injection, the residual ME was measured in the lungs. The data are expressed as %stimulation of fibrinolysis over the placebo (saline). Statistical significance: with placebo group (**$P < 0.01$), with single-drug groups (#$P < 0.1$; ##$P < 0.05$).

DISCUSSION

The risk of developing clinically significant pulmonary embolization in humans depends on underlying disease, the balance between coagulation and fibrinolysis, and preexisting pulmonary function, among other factors. Some of these factors cannot be adequately addressed in currently available animal models. Even relatively straightforward parameters, such as size and composition of the clot(s), are challenging to investigate in vivo. To carry out such investigations, one needs an in vivo model that permits accurate and reproducible measurements of fibrin deposition and dissolution caused by emboli of known size and content lodged in a vascular bed of interest. Ideally, these models should be applicable to a variety of animal species ranging from small rodents such as mice (which permits experiments to be conducted in diverse and defined genetic backgrounds) to primates (to evaluate species-specific pathways, as well as potential side effects relevant to human patients).

Models that are based on vascular trauma or injection of endotoxin to initiate intravascular fibrin formation are useful (10, 11, 18, 47, 56, 57) but difficult to control in terms of clot size, prevalence, and destination. Injection of preformed clots overcomes some of these limitations (3, 6, 29). We have used this latter approach to study PE in laboratory rodents and have established a model that enables us to study clot dissolution in a reproducible and quantitative manner. Theoretically, measurement of plasma radioactivity may permit a continuous nonlethal monitoring of fibrinolysis within the lung (44). However, this approach is limited by potential alterations in clearance mechanisms, the results of unintended changes in hepatic perfusion, renal elimination, and peripheral receptor saturation. In contrast, direct measurement of the residual radioactivity in the lungs on postmortem examination provides unambiguous data on fibrinolysis in the organ of interest.

Our data show that it is possible to model both massive PE and ME in rats and mice by varying the size of the infused fibrin clots. Injection of large (5–6 mm diameter) clots, as in humans, is associated with considerable lethality. Either embolization of the right ventricle or passage of clots through a patent foramen ovale leading to embolization of the coronary or cerebral vasculature could contribute to lethality. However, the location of large clots cannot be controlled, and their destination appears to track the size of the primary lobar vessels. In contrast, ME are nonlethal and produce a highly reproducible, homogenous deposition of fibrin in lungs in mice and rats alike. Using this model, we demonstrated that clot dissolution is impaired in mice genetically deficient in either tPA or urokinase-type plasminogen activator (1).

The purpose of this study was to begin to examine the physical, biochemical, and cellular factors that contribute to the rate of clot lysis in the pulmonary circulation. We found that large ^{125}I-labeled pulmonary clots do not undergo significant spontaneous dissolution in intact rats (Fig. 2), whereas pulmonary ^{125}I-ME undergo complete dissolution in intact rats and mice within the first 5 h (Fig. 3). The 30-min lag phase before appreciable lysis of ME is seen argues against mechanical elimination as a major contributor to the observed reduction in lung radioactivity. More likely, the lag time reflects the requirement for endogenous plasminogen activators to be activated, plasmin to be generated, and especially permeation of plasmin into the clot and fibrinolysis proper to occur (43).

Dissolution of PE in intact animals represents the outcome of a complex interplay between systemic (blood) and local (pulmonary) factors. Our data indicate that total fibrinolytic activity in rat lung in vitro is greater than in other organs (Fig. 5). This result corroborates studies by others using lungs from diverse other species (12, 57). A substantial fraction of total plasminogen activator activity in the lungs is generated within the alveolar space (38, 46). The pulmonary vasculature also expresses plasminogen activators (28, 49). Thus the lungs seem to be well equipped to function as blood filters that trap and dissolve emboli (12).

However, despite the presence of considerable plasminogen activator activity in lung tissue, the dissolution of ^{125}I-ME by isolated rat lungs was relatively inefficient in the absence of plasma. This result implies that the rate-limiting step in pulmonary fibrinolysis may be the diffusion of plasminogen from the plasma to the inner layers of the fibrin clot. This speculation would also help to explain the relatively poor spontaneous and pharmacological dissolution of large clots lodged in rat lungs, notwithstanding the intravenous infusion of high concentrations of tPA. The surface-to-volume ratio of the larger clots is much lower than it is for ME, which would be expected to impair plasminogen supply to internal portions of the clot (43). However, other factors (e.g., local and general hypoxia due to vascular occlusion, redistribution of blood flow, etc.) may also impair dissolution of large clots.

Conversely, the higher surface-to-volume ratio of ME compared with massive PE permits more effective delivery of plasminogen activators, as well as permeation of the ME by plasminogen. We previously reported that intravenous infusion of recombinant human urokinase markedly accelerates dissolution of pulmonary ^{125}I-ME in mice (1). In the present study, even a single injection of a relatively low dose of Retavase ($<$100 μg/kg) was highly effective (Fig. 6). However, no effect was observed when even a high (500 μg/kg) dose of Retavase was administered just 10 min before ^{125}I-ME injection, likely the result of the extremely fast elimination of the drug from the bloodstream. Thus even tPA variants that have been modified to prolong their lifetime in the blood, such as Retavase (31), are unlikely to provide prophylaxis even when given in high doses or modifications that cannot be used for thromboprophylaxis.

Our studies provide additional support for the concept that platelets represent an abundant and important component of PE (54). Fg and platelets colocalize in venules as well as in arterioles after ischemia/reperfusion injury (32). Clots are dynamic, and the outcome of our prior studies also suggest that fibrin ME incorporate both hematopoietic cells and additional fibrin after they adhere to the vessel wall in vivo (1). Platelets not only accelerate thrombin production but also are a source of plasminogen activator inhibitor 1 (PAI-1) and induce endothelial cells in the vicinity of PE to express this inhibitor (25, 26) through the elaboration of transforming growth factor-β and other constituents (13). Vitronectin, also secreted by activated platelets, may help to stabilize thrombi after vascular injury (24). Platelets also cause clot retraction generating a physical barrier to the perfusion of plasminogen and plasminogen activators (5, 8).

We found that both inclusion of platelets during the formation ^{125}I-ME in vitro and intravascular activation of platelets by collagen/epinephrine leading to thrombus production in vivo render PE more resistant to lysis (Fig. 7). Consistent with these observations, MAb 7E3 F(ab′)$_2$, a monoclonal antibody that inhibits Fg binding to platelet GP IIb/IIIa, facilitates the lysis of ^{125}I-ME in rat lungs and augments the efficacy of a suboptimal dose of Retavase in vivo (Fig. 8). These data imply that platelets promote the resistance of clots to fibrinolysis. Recent in vitro findings by others revealed that an anti-GP IIb/IIIa MAb inhibits tissue factor-induced thrombin production, platelet factor-4 release, formation of procoagulant microparticles (42), and accelerates fibrinolysis (8, 19). Taken together with these in vitro data, our results obtained in intact animals imply that it may be possible to accelerate pulmonary fibrinolysis or even lessen the propensity to form occlusive thrombi by impairing platelet function. However, the kinetics of GP IIb/IIIa occupancy (41), as well as thrombocytopenic and potential bleeding effects of MAb 7E3 (17), must be carefully tested to evaluate the safety of this maneuver.

The results of this study, therefore, extend understanding of potential involvement of platelets in fibrinolysis to the pulmonary circulation, the most com-

monly affected organ in man. There is considerable vascular heterogeneity in the expression of plasminogen activators and receptors (14), and vascular beds also differ in terms of local mechanisms involved in formation and dissolution of clots. Therefore, the present results warrant further study addressing mechanism of platelet-mediated retardation of pulmonary fibrinolysis, as well as potential risk/benefit balance of MAb 7E3 interventions in more detail. For example, our pilot experiments showed that the rate of spontaneous dissolution of ^{125}I-Fg deposits after ^{125}I-Fg/coll/epi mixture injection does not differ in PAI-1-deficient mice from that in control littermates, despite the notion that PAI-1 is one of the thrombi-stabilizing factors in platelets that suppresses lysis in large vessels. We also observed that injection of MAb 7E3 in rats treated with ^{125}I-Fg/coll/epi mixture did not change pulmonary level of ^{125}I (that may reflect extravascular deposition of radiolabeled materials due to pulmonary edema) but changed the character of lung hemorrhages from relatively large patches to smaller, less profound and more homogenous pattern.

In summary, this paper presents what, to our knowledge, is the first quantitative analysis addressing the role of platelets on the dissolution of PE. The data suggest that there may be circumstances in which administration of antiplatelet therapy combined with a lower dose of a fibrinolytic agent may represent a useful and safer approach to treat some PE. This paradigm clearly requires more rigorous pharmacological, toxicological, and effectiveness studies to define its benefits and limitations in this vascular bed. Additional studies, including those in the animal models described in this work, may help to identify the settings in which combined therapy would be effective and safe.

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