Homozygous plasminogen-deficient (Plg<sup>-/-</sup>) mice had a significantly reduced thrombolytic capacity toward intravenously injected <sup>125</sup>I-fibrin labeled plasma clots prepared from Plg<sup>-/-</sup> murine plasma (9% ± 3% lysis after 8 hours; (mean ± SEM, n = 6), as compared with 82% ± 8% in wild-type mice; *P < .0001). Bolus injection of 1 mg purified murine plasminogen in 10- to 17-week-old Plg<sup>-/-</sup> mice increased the plasminogen antigen and activity levels at 8 hours to normal levels (130 ± 5 μg/mL). Plasminogen administration was associated with significant restoration of thrombolytic potential (64% ± 7% spontaneous clot lysis; *P < .0001 versus lysis without plasminogen injection). Bolus injection of 1 mg plasminogen in homozygous tissue-type plasminogen activator-deficient (t-PA<sup>-/-</sup>) mice doubled the plasminogen antigen and activity levels after 8 hours and increased <sup>125</sup>I-fibrin clot lysis at 8 hours from 13% ± 3% to 34% ± 5% (*P = .008). Fibrinogen, t-PA antigen and α<sub>2</sub>-antiplasmin activity levels after 8 hours were not significantly different in the groups with or without plasminogen injection. Injection of plasminogen induced a variable increase (on average 7- to 10-fold) of PAI-1, but no correlation with the extent of spontaneous clot lysis was observed. Histopathologic examination at the end of the experiments revealed that fibrin deposition in the liver of Plg<sup>-/-</sup> mice was slightly reduced 8 hours after bolus plasminogen injection (*P = .007) and markedly reduced after 24 hours (*P < .0001). Plasminogen antigen levels in liver extracts were comparable with those found in wild-type mice at 8 hours (130 ± 20 versus 110 ± 15 ng/mg protein) and decreased to 25 ± 3.2 mg/mg protein at 24 hours. Thus, restoration of normal plasminogen levels in Plg<sup>-/-</sup> mice normalized the thrombolytic potential toward experimentally induced pulmonary emboli, and resulted in removal of endogenous fibrin deposits within 24 hours.

© 1996 by The American Society of Hematology.

MATERIALS AND METHODS

Proteins, Reagents, and Assays

Homozygous t-PA<sup>-/-</sup> and Plg<sup>-/-</sup> mice were obtained and characterized as described previously.1,2 Mice were kept in microisolation cages on a 12-hour day-night cycle and fed a regular chow. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. Mice were anesthetized by intraperitoneal injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL) and blood was collected by vena cava puncture with a 24-gauge needle. Platelet poor plasma was prepared by centrifugation of blood collected on citrate at 4,000g for 5 minutes.

Murine Glu-plasminogen was purified from plasma and characterized as described elsewhere,4 and <sup>125</sup>I-labeled to a specific radioactivity of 1.7 × 10<sup>8</sup> cpm/μg, using lactoperoxidase.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without reduction was performed on 10% to 15% gradient
gels using the PhastSystem (Pharmacia, Uppsala, Sweden). Immunoblotting, after transfer to nitrocellulose sheets, was performed according to Towbin et al., using a polyclonal rabbit antiserum against murine plasminogen that was purified by immunoaffinity chromatography on insolubilized human plasminogen. Quantitative determination of plasminogen antigen in murine plasma and in liver extracts was performed by enzyme-linked immunosorbent assay (ELISA) using purified rabbit polyclonal antibodies and with murine plasminogen for calibration. Liver extracts were prepared by homogenization and extraction with 0.1 mol/L Tris, 0.25% Triton X-100, pH 8.0, and protein concentration was determined according to Bradford.

Plasminogen activity in murine plasma was determined by activation with human u-PA after acidification and neutralization to inactive protease inhibitors, and quantitation of generated plasmin with the chromogenic substrate S-2403 (Chromogenix, Antwerp, Belgium). α₂-Antiplasmin activity levels in murine plasma were determined by addition of human plasmin (final concentration 5 nmol/L) and measurement of residual plasmin with S-2403 (final concentration 0.3 mmol/L) after incubation at 37°C for 30 seconds. Plasminogen and α₂-antiplasmin activity levels are expressed as a percentage of the value obtained in pooled plasma from wild-type mice.

Data are reported as mean ± SEM and the statistical significance of differences between groups was determined using Student's t-test. Correlations between data obtained in the experimental groups were determined by linear regression analysis. Histopathologic data on fibrin deposition in liver sections of animals with or without plasminogen injection were compared by chi square analysis, using a two by four contingency table.

**Pharmacokinetic Analysis**

Pharmacokinetic parameters describing the disposition of plasminogen from plasma following bolus injection of ¹²⁵I-labeled plasminogen (700,000 cpm, corresponding to 410 ng protein) in wild-type mice were determined by quantitation of residual plasma radioactivity at different time points (0 to 48 hours) after injection. Before injection of ¹²⁵I-labeled plasminogen, the animals received a bolus injection of 50 μL of a 1% NaCl solution. Groups of three to four mice were killed at the indicated time points and the plasma concentration of ¹²⁵I-labeled plasminogen was measured before and after precipitation with CCl₄-CO₂H in order to correct for the reappearance of radiolabeled breakdown products in plasma. The results were plotted on semilogarithmic paper, analyzed by graphical curve peeling and the pharmacokinetic parameters were calculated using standard formulas derived by Gibaldi and Perrier. The distribution of radioactivity was determined in the blood, the liver, the lungs, and the kidney-urine. The radioactivity in the rest of the body was estimated by adding the radioactivity in spleen, head, legs, tail, back, and intestines.

**¹²⁵I-Fibrin Labeled Plasma Clot Lysis In Vivo**

Lysis of ¹²⁵I-fibrin labeled murine plasma clots injected via a jugular vein and embolized into the pulmonary arteries was determined essentially as previously described. Briefly, a 25-μL ¹²⁵I-fibrin- labeled plasma clot, containing ~70,000 cpm human ¹²⁵I-fibrinogen (corresponding to 0.07 μCi ¹²⁵I), was prepared from plasminogen-deficient (Plg⁻⁻⁻) murine plasma, and injected into the jugular vein of Plg⁻⁻⁻, t-PA⁻⁻⁻, or wild-type mice. Spontaneous clot lysis was evaluated by measuring the residual radioactivity in the heart and lungs ex vivo 8 hours after injection, and was defined as the amount of radioactivity that had disappeared, expressed as the percent of the total amount of radioactivity injected. In separate experiments, spontaneous clot lysis was measured in Plg⁻⁻⁻, t-PA⁻⁻⁻, and wild-type mice following bolus injection of 0.2 mL of a 5 mg/mL solution of murine plasminogen 5 minutes before injection of the labeled plasma clot. The endpoint in the plasminogen solution, as determined with the Limulus Amoebocyte lysate assay (Chromogenix, Möndlal, Sweden) corresponded to a dose of 2 endotoxin units (0.16 ng) administered per mouse.

**Histopathologic and Immunohistochemical Examination**

Plg⁻⁻⁻ mice (n = 6) and Plg⁻⁻⁻ mice injected with plasminogen (n = 6) of either sex, age 10 to 17 weeks, were anesthetized (8 or 24 hours after the injection) and perfused via cardiac puncture with 0.9% NaCl, followed by 4% formalin in 0.07 mol/L Na-phosphate buffer, pH 7.0. Liver and lungs were removed, postfixied in the same fixative for 20 hours, and embedded in paraffin. Four 7 μm cross sections, with 250 μm between sections, were examined after hematoxylin-eosin staining or immunostaining.

Immunostaining for fibrin(ogen) was performed by incubating the sections with goat antiserum against murine fibrinogen/fibrin (Nordic, The Netherlands, working dilution 1/500), in 0.01 mol/L Tris, pH 7.6 containing 0.9% NaCl and 0.1% Triton X-100 for 3 hours at room temperature. After rinsing, the sections were incubated consecutively for 60 minutes, with biotinylated rabbit antigoat IgG (Dako, Prosan, Gent, Belgium, dilution 1/400) and with peroxidase labeled avidin-biotin complex (Dako). Immunostaining for plasminogen was performed on liver sections using the polyclonal rabbit antiserum described above, with peroxidase labeled swine antirabbit IgG (Dako, dilution 1/100) as secondary antiserum.

Antibody binding was visualized with diamobenzidine, resulting in a brown staining of the immunoreactive sites. All sections were briefly counterstained with Harris’ hematoxylin, dehydrated and mounted with DePeX. The specificity of the primary antibodies was tested by adsorption of the antiserum with murine fibrinogen or plasminogen.

The extent of fibrin deposition was given a severity score of 0 to 3. Score 0 indicated the absence of fibrin deposits; score 1, the appearance of a few small fibrin deposits; score 2, the presence of many small to large fibrin deposits; score 3, the presence of large fibrin deposits with calcification.

**RESULTS**

**Pharmacokinetics of Murine Plasminogen**

The clearance of murine plasminogen from blood following bolus injection of ¹²⁵I-labeled protein in wild-type mice could be described as the sum of three exponential terms by graphical curve peeling (Fig 1). Injection of 410 ng plasminogen resulted in a plasma concentration 2 minutes after injection of 48 ± 1 ng/mL (mean ± SEM; n = 4). From the coefficients and exponents in the equation C(t) = Ae⁻ᵃᵗ + Be⁻ᵇᵗ + Ce⁻ᶜᵗ with A = 33 ng/mL, B = 13 ng/mL, C = 2.4 ng/mL, and α = 0.035 min⁻¹, β = 0.004 min⁻¹, and γ = 0.001 min⁻¹, the following kinetic parameters were derived: t₀/₂ (α) = 20 minutes, t₀/₂ (β) = 3 hours, t₀/₂ (γ) = 15 hours, area under the curve = 6.6 μg/min/mL and plasma clearance = 0.063 mL/min. This pharmacokinetic analysis applies to a total plasminogen preparation, and may be somewhat different for molecular forms of plasminogen with different glycosylation and/or NH₂-terminal amino acid sequences. The organ distribution of radioactivity in mice killed at different time points after injection of ¹²⁵I-labeled plasminogen.
from various organs followed its disappearance rate from the blood.

Clot Lysis and Hemostasis Parameters

Plasma clot lysis. As shown in Table 2, spontaneous lysis within 8 hours of a 125I-fibrin labeled pulmonary plasma clot prepared from plasminogen-deficient murine plasma was almost abolished in Plg<sup>-/-</sup> mice (9% ± 3%; mean ± SEM; n = 6) and in t-PA<sup>-/-</sup> mice (13% ± 3%; n = 6), as compared with wild-type mice (82% ± 8%; n = 5) (P < 0.0001 for both Plg<sup>-/-</sup> and t-PA<sup>-/-</sup> versus wild-type mice).

Following supplementation of Plg<sup>-/-</sup> mice with murine plasminogen, spontaneous clot lysis after 8 hours was 64% ± 7% (P < 0.0001 versus Plg<sup>-/-</sup> mice without supplementation), and was not significantly different from the lysis observed with wild-type mice without plasminogen supplementation (P = .13). Spontaneous clot lysis in wild-type mice after bolus injection of murine plasminogen was not different from that without its administration (P = .58). In contrast, injection of plasminogen in homozygous t-PA<sup>-/-</sup> mice resulted in a significantly enhanced spontaneous clot lysis (34% ± 5%; P = .008 versus the experiments without injection of plasminogen); the observed lysis remained, however, significantly lower than that obtained in Plg<sup>-/-</sup> mice with injection of plasminogen (64% ± 7%; P = .006) and also lower than in wild-type mice with supplementation of plasminogen (73% ± 14%; P = .02).

Hemostasis parameters. Bolus injection of 1 mg purified murine plasminogen in Plg<sup>-/-</sup> mice resulted in plasma antigen and activity levels after 30 minutes of (mean ± SEM; n = 3) 460 ± 39 µg/mL and 370% ± 33%, as compared with undetectable levels of both antigen (<1 µg/mL) and activity (<2%) in Plg<sup>-/-</sup> mice without injection of plasminogen. Antigen and activity levels after 30 minutes in t-PA<sup>-/-</sup> mice were 550 ± 20 µg/mL and 550% ± 36% with injection of plasminogen, as compared with 130 ± 10 µg/mL and 93% ± 12% without injection of plasminogen. Corresponding antigen and activity levels in wild-type mice were 550 ± 39 µg/mL and 440% ± 80% with injection of plasminogen and 120 ± 17 µg/mL and 88% ± 15% without injection. At the end of the experiments with injection of plasminogen (8 hours), plasminogen antigen and activity levels were (mean ± SEM; n = 6) 130 ± 5 µg/mL and 97% ± 6% in Plg<sup>-/-</sup> mice, 280 ± 24 µg/mL and 240% ± 13% in t-PA<sup>-/-</sup> mice and 280 ± 18 µg/mL and 230% ± 7% in wild-type mice (Table 2). Twenty-four hours after injection of plasminogen in Plg<sup>-/-</sup> mice, plasminogen antigen and activity levels were (mean ± SEM; n = 6) 35 ± 2.5 µg/mL and 14% ± 2%. SDS-PAGE and immunoblotting of plasma samples taken at the end of the experiments (Fig 2) confirmed the presence of intact plasminogen in plasma from Plg<sup>-/-</sup> mice injected with murine plasminogen and did not reveal the generation of detectable levels of plasmin-α<sub>2</sub>-antiplasmin complex. In none of the experimental groups was clot lysis associated with systemic fibrinolytic activation as indicated by virtually unchanged fibrinogen levels at the end of the experiments (Table 2).

α<sub>2</sub>-Antiplasmin activity levels after 30 minutes were (mean ± SEM; n = 3) 104% ± 2%, 83% ± 6%, and 98% ± 15% in Plg<sup>-/-</sup>, t-PA<sup>-/-</sup>, and wild-type mice with injection of plasminogen, as compared with 106% ± 7%, 100% ± 9%, and 107% ± 6% without injection. Also at the end of the experiments, residual α<sub>2</sub>-antiplasmin activity levels in the three groups with injection of plasminogen were ≥75% (Table 2). Plasma t-PA antigen levels after 30 minutes were (mean ± SEM; n = 3) 1.7 ± 0.56 ng/mL, <0.3 ng/mL, and 1.9 ± 1.2 ng/mL in Plg<sup>-/-</sup>, t-PA<sup>-/-</sup>, and wild-type mice with injection of plasminogen, as compared with 2.6 ± 0.84 ng/mL, <0.3 ng/mL, and 1.6 ± 0.39 ng/mL without injection.

![Fig 1. Disposition of 125I-labeled murine plasminogen from plasma following bolus injection in wild-type mice. 125I-labeled protein was quantitated in plasma samples from mice that were killed at different time intervals after injection. The data represent mean ± SEM of three or four experiments.](image-url)

Table 1. Organ Distribution of Radioactivity After Bolus Injection of 125I-labeled Murine Plasminogen in Mice

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0.5</th>
<th>1.5</th>
<th>4.0</th>
<th>8.0</th>
<th>16</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td>48 ± 1</td>
<td>28 ± 2</td>
<td>14 ± 1</td>
<td>8 ± 1</td>
<td>4 ± 1</td>
<td>1 ± 1</td>
<td>1 ± 0</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>18 ± 1</td>
<td>10 ± 2</td>
<td>4 ± 0</td>
<td>2 ± 0</td>
<td>1 ± 0</td>
<td>1 ± 0</td>
<td>—</td>
</tr>
<tr>
<td><strong>Lungs</strong></td>
<td>3 ± 0</td>
<td>2 ± 1</td>
<td>1 ± 0</td>
<td>1 ± 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Kidney-urine</strong></td>
<td>6 ± 1</td>
<td>6 ± 4</td>
<td>4 ± 2</td>
<td>8 ± 2</td>
<td>1 ± 0</td>
<td>1 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td><strong>Rest</strong></td>
<td>34 ± 2</td>
<td>43 ± 2</td>
<td>49 ± 1</td>
<td>40 ± 4</td>
<td>13 ± 3</td>
<td>8 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>109 ± 1</td>
<td>90 ± 2</td>
<td>72 ± 1</td>
<td>58 ± 6</td>
<td>19 ± 2</td>
<td>11 ± 1</td>
<td>8 ± 1</td>
</tr>
</tbody>
</table>

The data represent mean ± SEM of three or four determinations and are expressed in percent of the injected radioactivity.

---

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
Also 8 hours after injection of plasminogen t-PA antigen levels were not different from the levels before injection (Table 2). Plasma PAI-1 antigen levels in the animals without plasminogen injection were (mean ± SEM) 14 ± 6.7 ng/mL (n = 5), 3.8 ± 0.72 ng/mL (n = 6), and 3.2 ± 0.70 ng/mL (n = 6) in Pig−/−, t-PA−/−, and wild-type mice, respectively. Injection of plasminogen was associated with a significant increase of PAI-1 antigen levels to 100 ± 36 ng/mL (n = 6), 52 ± 20 ng/mL (n = 3), and 26 ± 11 ng/mL (n = 6) 8 hours after injection in Pig−/−, t-PA−/−, and wild-type mice, respectively (all P < .01). In a separate set of experiments, PAI-1 antigen levels 24 hours after injection of plasminogen were 22 ± 9 ng/mL (mean ± SEM; n = 6), as compared with 14 ± 3.0 ng/mL before the injection. In the Pig−/− mice, linear regression analysis did not reveal a correlation between PAI-1 antigen levels in plasma and spontaneous clot lysis neither with or without plasminogen injection.

Histopathologic and Immunohistochemical Examination

Histopathologic examination and immunostaining revealed the presence of fibrin deposits in lung and liver sections of Pig−/− mice, as described previously. Eight hours after injection of plasminogen, no fibrin deposits (score 0) were detected in 7 of 24 liver sections (4 sections each of 6 animals), whereas without plasminogen supplementation all liver sections of Pig−/− mice showed some degree of fibrin deposition (score 1 to 3) (Table 3); chi square analysis using a two by four contingency table indicated a slight reduction of fibrin deposits in the experiments with plasminogen injection (P = .007). Twenty-four hours after injection of plasminogen no fibrin deposits were detected in 14 of 24 liver sections, and no sections with large calcified fibrin deposits (score 3) were observed (P < .0001 versus the experiments without plasminogen injection). Immunostaining of adjacent liver sections with the antiserum against murine plasminogen revealed specific binding of plasminogen to all the fibrin deposits in Pig−/− mice supplemented with plasminogen at 8 hours (Fig 3), whereas in liver sections taken at 24 hours, plasminogen was not detectable in any of the sections without fibrin deposits (not shown). This confirms that plasminogen is essentially fibrin-associated. Plasminogen antigen levels in extracts of liver sections taken at the end of the experiments with plasminogen injection in Pig−/− mice were (mean ± SEM; n = 6) 130 ± 20 ng/mg protein 8 hours after injection and 25 ± 3.2 ng/mg protein 24 hours after injection.
Fig 3. Light microscopic analysis of liver sections (200 × magnification) from Plg⁺⁻ mice without (I) or with (II) injection of murine plasminogen. Immunostaining of adjacent sections was performed with specific antisera against murine fibrinogen/fibrin (A) or against murine plasminogen (B).

as compared with 8.7 ± 0.6 ng/mg protein for experiments without plasminogen injection and 110 ± 15 ng/mg protein in wild-type mice, respectively. SDS-PAGE and immunoblotting of liver extracts with antibodies against murine plasminogen revealed that all the immunoreactive material migrated in the same position as native plasminogen. PAI-1 antigen levels in liver extracts of Plg⁻⁻ mice were (mean ± SEM) 0.13 ± 0.04 ng/mg protein (n = 5) in the group without plasminogen injection, and 3.5 ± 1.6 ng/mg protein (n = 5) or 0.40 ± 0.24 ng/mg protein (n = 6) 8 hours or 24 hours after injection of plasminogen, as compared with <0.05 ng/mg protein in wild-type mice. In Plg⁻⁻ mice, linear regression analysis did not reveal a significant correlation of the fibrin deposition score in the liver with PAI-1 antigen levels in liver extracts or with PAI-1 plasma antigen levels (data not shown).

DISCUSSION

Homzygous plasminogen-deficient (Plg⁻⁻) mice display normal viability but delayed growth and reduced survival, and show a greatly reduced spontaneous lysis of a ¹²⁵I-fibrin labeled pulmonary plasma clot and spontaneous fibrin deposition in the liver, lung, and stomach beyond the age of 6 weeks.⁶ ⁷ In this study, we have attempted to restore the thrombolytic potential in Plg⁻⁻ mice by bolus administration of purified murine plasminogen in order to demonstrate that plasminogen deficiency is causally related to the thrombotic phenotype.

Based on the clearance of ¹²⁵I-labeled murine plasminogen from plasma following bolus intravenous injection in wild-type mice, a bolus injection of 1 mg murine plasminogen was administered to Plg⁻⁻ mice in order to restore normal plasminogen levels after approximately 8 hours. This resulted in an increase in plasminogen antigen and activity levels that were very similar to the normal levels in wild-type mice at 8 hours after injection (Table 2). The same dose given to t-PA⁻⁻ or to wild-type mice resulted in a doubling of the normal levels of plasminogen antigen and activity at 8 hours.

Supplementation of Plg⁻⁻ mice with plasminogen resulted in significant spontaneous lysis after 8 hours of a ¹²⁵I-fibrin labeled pulmonary plasma clot prepared from Plg⁻⁻ murine plasma (64% ± 7%, as compared with 9% ± 3% without injection of plasminogen and with 82% ± 8% in wild-type mice). Interestingly, in previous studies using plasma clots prepared from wild-type mice, spontaneous clot lysis in Plg⁻⁻ mice was 0% at 24 hours. This would appear to indicate that fibrin clot structure may be different as a result of plasminogen deficiency and that these clots are more accessible to other nonplasmin fibrinolytic proteases.
It has previously been demonstrated that fibrin assembly and structure are influenced by the microenvironment.\textsuperscript{15-19} For instance, it has recently been shown that activation of the complement system has a profound effect on fibrin structure, resulting in a less permeable, more lysis resistant matrix.\textsuperscript{20} Thus, alterations in plasma constituents could have a profound effect on clot lysis by affecting the biophysical characteristics of the fibrin network.

Furthermore, our data show that in the absence of circulating plasminogen the plasminogen content of the clot (prepared from normal versus plasminogen-deficient plasma) is not sufficient to allow efficient clot lysis, thus emphasizing that recruitment of plasminogen from the circulating plasma onto the clot is a critical determinant of clot lysis. In essence these observations may represent the in vivo equivalent of the "plasminogen steal" phenomenon, which has previously been described in plasma in vitro.\textsuperscript{21-23}

t-PA antigen levels were not significantly different in the experiments with or without plasminogen, indicating that the enhanced thrombolytic potential in the Plg\textsuperscript{−/−} mice was not caused by an enhanced t-PA release. The enhanced thrombolytic potential also cannot be explained by lowered \(\alpha_2\)-antiplasmin levels as a result of systemic plasminogen activation. This is shown by our findings that (1) residual \(\alpha_2\)-antiplasmin activity levels were comparable in all experimental groups; (2) Western blot analysis of plasma samples from mice with plasminogen supplementation did not reveal the presence of plasmin-\(\alpha_2\)-antiplasmin complex; and (3) residual plasminogen antigen and activity levels at the end of the experiments corresponded well and were comparable in Plg\textsuperscript{−/−} mice with plasminogen administration relative to wild-type mice without plasminogen supplementation. In our previous study,\textsuperscript{7} heterozygous mice (Plg\textsuperscript{+/−}) with 35 ± 2 \(\mu\)g/mL plasminogen levels showed reduced lysis as compared with wild-type mice (62% ± 7% versus 85% ± 5% at 24 hours). This difference provides evidence of a plasminogen dose-response curve, which appears to saturate at normal plasminogen levels.

Plasma PAI-1 antigen levels in Plg\textsuperscript{−/−} mice were not lower than in wild-type mice, suggesting that circulating plasminogen does not upregulate plasma PAI-1. After injection of plasminogen, PAI-1 levels were significantly higher than before its injection, although the endotoxin contamination in the injected solution was very low. This increase, which was observed in Plg\textsuperscript{+/−}, t-PA\textsuperscript{−/−}, and wild-type mice, may be the result of an acute phase reaction, as PAI-1 is a known acute phase reactant protein, at least in humans.\textsuperscript{24} In agreement with our earlier observations in mice injected with a large dose of endotoxin,\textsuperscript{25} these increased PAI-1 levels did not prevent pulmonary clot lysis. Linear regression analysis of the data within each experimental group or of all data combined did not reveal a significant correlation between plasma PAI-1 antigen levels and spontaneous clot lysis. Recent studies, using adenovirus-mediated gene transfer of PAI-1 in mice, have shown that spontaneous clot lysis in the same model is virtually abolished at PAI-1 levels, which are more than 100-fold higher than those obtained in the present study (P Carmeliet, unpublished data, January 1995).

Control experiments with wild-type mice did not show an effect of doubling of the plasminogen concentration on pulmonary clot lysis. Similar experiments in t-PA\textsuperscript{−/−} mice, however, resulted in significantly higher clot lysis activity with plasminogen injection than without (34% ± 5% versus 13% ± 3%; \(P = .008\)). Thus, the availability of a higher substrate concentration in t-PA\textsuperscript{−/−} mice allows for an enhanced thrombolytic potential. Using the same model, it has been previously observed that spontaneous clot lysis progressively increases with time in t-PA\textsuperscript{−/−} mice, from <5% at 4 hours to approximately 50% at 72 hours.\textsuperscript{3} This may be explained by plasmin generation via alternative non-t-PA-dependent mechanisms, such as u-PA, which may partially compensate for the absence of t-PA.\textsuperscript{3}

Histopathologic examination of Plg\textsuperscript{−/−} mice at the end of the experiments indicated that the degree of fibrin deposition and calcification in the liver 8 hours after the start of the experiments was somewhat reduced as a consequence of the plasminogen injection. The plasminogen concentration in liver extracts from Plg\textsuperscript{−/−} mice supplemented with murine plasminogen was comparable with that observed in wild-type mice, which do not show fibrin deposits in the liver.

Immunoblotting of SDS-PAGE of liver extracts revealed a similar distribution of plasminogen-related antigen following plasminogen injection in Plg\textsuperscript{−/−} mice as observed in wild-type controls. Furthermore, immunostaining of adjacent liver sections showed binding of plasminogen to the fibrin deposits. These findings suggest that the relative resistance of fibrin deposits in the liver to lysis is not due to a poor accessibility with impaired binding of plasminogen. PAI-1 levels in liver extracts of Plg\textsuperscript{−/−} mice supplemented with plasminogen were significantly elevated at 8 hours, but did not correlate with the score given for fibrin deposition. Clot lysis resistance of fibrin deposits in the liver may be related to the age of the thrombus, which would be structurally more complex. It has been reported that aged and retracted or mechanically compressed fibrin clots are much more resistant to lysis than fresh clots.\textsuperscript{26,27} In agreement with this hypothesis, we have found that liver fibrin deposits were significantly reduced in Plg\textsuperscript{−/−} mice 24 hours after injection of plasminogen, indicating that ongoing fibrinolytic activity can induce delayed lysis of the fibrin deposits.

Thus, the present study directly shows that in vivo fibrin dissolution is critically dependent on the plasminogen/plasmin system. The study further establishes these mice as providing a useful model to examine resistance of aged clots to lysis as well as to analyze alternative thrombolytic agents.

ACKNOWLEDGMENT

Skillful technical assistance by J.-M. Stassen, B. Van Hoef, and M. Verstreken (Center for Molecular and Vascular Biology, Leuven) is gratefully acknowledged.

REFERENCES

3. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J,
15. Stassen JM, Vanlinthout I, Lijnen HR, Collen D: A hamster pulmonary embolism model for the evaluation of the thrombolytic and pharmacokinetic properties of thrombolytic agents. Fibrinolysis 4:15, 1990 (suppl 2)
Restoration of thrombolytic potential in plasminogen-deficient mice by bolus administration of plasminogen

HR Lijnen, P Carmeliet, A Bouche, L Moons, VA Ploplis, EF Plow and D Collen