# Plasminogen Activator Inhibitor-1 Gene-deficient Mice

## II. Effects on Hemostasis, Thrombosis, and Thrombolysis

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## Abstract

**The effects of plasminogen activator inhibitor-1 (PAI-1) gene inactivation on hemostasis, thrombosis and thrombolysis were studied in homozygous PAI-1-deficient (PAI-1$^{-/-}$) mice, generated by homologous recombination in $D_3$ embryonic stem cells. Diluted (10-fold) whole blood clots from PAI-1$^{-/-}$ and from PAI-1 wild type (PAI-1$^{+/+}$) mice underwent limited but significantly different ($P < 0.001$) spontaneous lysis within 3 h (6±1 vs 3±1%, respectively). A 25-μl $^{125}$I-fibrin-labeled normal murine plasma clot, injected into a jugular vein, was lysed for 47±5, 66±3, and 87±7% within 8 h in PAI-1$^{+/+}$, heterozygous PAI-1-deficient (PAI-1$^{+/-}$), and PAI-1$^{-/-}$ mice, respectively ($P = 0.002$ for PAI-1$^{+/+}$ vs PAI-1$^{-/-}$ mice). Corresponding values after pretreatment with 0.5 mg/kg endotoxin in PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice, were 35±5 and 91±3% within 4 h, respectively ($P < 0.001$). 11 out of 26 PAI-1$^{+/+}$ but only 1 out of 25 PAI-1$^{-/-}$ mice developed venous thrombosis ($P = 0.004$) within 6 d after injection of 10 or 50 μg endotoxin in the footpad. Spontaneous bleeding or delayed rebleeding could not be documented in PAI-1$^{-/-}$ mice after partial amputation of the tail or of the caecum.**

**Thus, disruption of the PAI-1 gene in mice appears to induce a mild hyperfibrinolytic state and a greater resistance to venous thrombosis but not to impair hemostasis. (*J. Clin. Invest.* 1993. 92:2756-2760.) Key words: embryonic stem cells • homologous recombination • fibrinolysis • gene targeting**

## Introduction

Clot lysis in vivo results primarily from the enzymatic action of the fibrinolytic system (1-2). This system comprises an inactive proenzyme plasminogen, which can be converted to the active enzyme plasmin, that will degrade fibrin into soluble fibrin degradation products. The enzymatic action of tissue-type plasminogen activator (t-PA)[1] and of urokinase-type plasminogen activator, the two physiological plasminogen activators, is controlled by several plasminogen activator inhibitors (PAIs) (3). Of those, plasminogen activator inhibitor-1 (PAI-1) is believed to be of primary importance.

A role of PAI-1 in hemostasis is deduced from studies, correlating PAI-1 deficiency with an hemorrhagic tendency (4-8). The five presently known patients with reduced (4-7) or absent (8) plasma PAI-1 levels indeed appear to suffer from delayed rebleeding after trauma or surgery.

PAI-1 may also participate in the development of thrombotic events. Elevated plasma levels correlate with deep venous thrombosis (9), as well as disseminated intravascular coagulation associated with sepsis and endotoxemia (10). In addition, increased PAI-1 levels have been correlated with a higher risk of thrombosis in patients during acute phase reactions after surgery (11) and trauma (12). PAI-1 might also participate in acute coronary thrombosis as suggested by the increased PAI-1 levels in patients with coronary artery disease (13), angina pectoris (14), and recurrent myocardial infarction (15). A possible causal relation between high PAI-1 levels and the occurrence of thrombosis may be inferred from the observation that transgenic mice overexpressing PAI-1 suffer from spontaneous thrombosis of tail and hind leg veins (16) and that PAI-1 specific antibodies enhance endogenous thrombolysis and reduce thrombus extension in rabbits (17).

Deficient fibrinolysis caused by overexpression of PAI-1, may also participate in the development and/or progression of atherosclerosis (18). Known risk factors for atherosclerosis including obesity, noninsulin-dependent diabetes, hyperinsulinemia, and hypertriglyceridemia have been correlated with increased plasma levels of PAI-1 (reviewed in reference 18). Furthermore, elevated PAI-1 levels constitute a risk factor for coronary atherosclerosis in survivors of myocardial infarction with glucose intolerance (19). Recently, expression of PAI-1 mRNA in severely atherosclerotic human arteries has been related to the progression of the atherosclerotic disease (20, 21).

Collectively, these data suggest that PAI-1 plays an important role in hemostasis, thrombosis, and possibly in the progression of atherosclerosis. Since PAI-1 is an acute phase reactant (22), it is, however, not clear whether increased PAI-1 expression in these pathological processes is a cause or a consequence of the disorder.

To obtain new information on the role of PAI-1 in vivo, we have investigated the effects of PAI-1 gene inactivation in mice (23) on hemostasis, thrombosis, and thrombolysis.

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1. *Abbreviations used in this paper:* PAI, plasminogen activator inhibitor; PAI-1, murine PAI-1; PAI-1$^{+/+}$, wild type PAI-1 mice; PAI-1$^{+/-}$, heterozygous PAI-1-deficient mice; PAI-1$^{-/-}$, homozygous PAI-1-deficient mice; t-PA, tissue-type plasminogen activator.

## Methods

### *Animals and blood collection*

The mice were kept in microisolation cages on a 12-h day-night cycle and fed a regular chow. Unless otherwise stated, the 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.

### *Whole blood clot lysis*

Diluted whole blood clot lysis was determined essentially as described by Gallimore et al. (24). Briefly, 100 μl of fresh blood, collected by vena cava puncture, was diluted 10-fold with saline containing ~ 10,000 cpm human $^{125}$I-fibrinogen (0.01 μCi $^{125}$I, Amersham, Buckinghamshire, United Kingdom) and was coagulated at 37°C with 10 μl bovine thrombin (60 U/ml) (Sigma Immunochemicals, St. Louis, MO), 0.75 M $CaCl_2$ solution. At various time points, the $^{125}$I content of 50-μl aliquots of supernatant was measured using a gamma counter (model BF300; Berthold, Vilvoorde, Belgium).

### *Hemostasis analyses*

For differential cell counts, blood was collected in 0.01 M trisodium citrate, and cells were counted using an automated analyzer (Cell-Dyn 610U-Hematology Analyzer; Sequoia-Turner Co, Mountain View, CA). Cell counts are expressed per milliliters of whole blood. The activated partial thromboplastin time and thrombin time were determined using standard clinical laboratory procedures. Fibrinogen was determined by a coagulation rate assay as previously described (25).

### *$^{125}$I-fibrin labeled plasma clot lysis in vivo*

Lysis of $^{125}$I-fibrin-labeled murine plasma clots injected via a jugular vein and embolized into the pulmonary arteries was monitored essentially as previously described (26). Briefly, a 25-μl $^{125}$I-fibrin-labeled plasma clot, containing ~ 70,000 cpm human $^{125}$I-fibrinogen (corresponding to 0.07 μCi $^{125}$I), was prepared from a plasma pool of wild type PAI-1 (PAI-1$^{+/+}$) or homozygous PAI-1-deficient mice (PAI-1$^{-/-}$) mice, respectively, and injected into the jugular vein. Clot lysis was evaluated in groups of five animals each by measuring the residual radioactivity in the heart and lungs ex vivo at various time points, and was defined as the amount of radioactivity which had disappeared, expressed in percent of the total amount of radioactivity injected.

### *Venous thrombosis after injection of endotoxin in the foodpad*

8-wk-old mice were anesthetized with ether and their hind footpads injected with 10 or 50 μg of endotoxin (Lipopolysaccharide W, *Escherichia coli* 0111:B4, LD 28.3 mg/kg; Difco Laboratories, Detroit, MI)[2] in a total volume of 20 or 50 μl sterile saline using a 27-gauge needle (27). After 6 d, the mice were killed. Footpad tissue was fixed for 2 h at room temperature in B5-fixative (10% dilution of 40% formaldehyde in saturated mercuric chloride), dehydrated through graded methanol and xylol baths, and embedded in paraffin. 5-μm sections were cut at various intervals, mounted, and stained with hematoxylin and eosin for routine light microscopy. Sections were analyzed by two investigators unaware of the genotype of the mice.

### *Bleeding and rebleeding*

*Amputation of the tail tip.* The tails of mice anesthetized with 60 mg/kg Nembutal were prewarmed for 5 min at 37°C in a water bath and a 5-mm segment was amputated with a razor blade. The tail was immersed immediately in 10 ml 37°C prewarmed saline, and the time required to stop spontaneous bleeding was determined. To evaluate rebleeding, after bleeding had stopped, the tail was submerged for another 60 min in 10 ml prewarmed (37°C) saline containing 0.01 mM trisodium citrate. The number of red blood cells and the amount of hemoglobin released from the tail wound during this 60-min period were determined.

*Partial removal of the caecum.* A 5-mm incision through the abdominal wall was made in mice, anesthetized with 60 mg/kg Nembutal, and the caecum was exposed. A first suture with resolvable thread (Maxon 5.0; Davis and Geck, Danbury, CT) was applied between the upper and middle third of the caecum. The distal half of the caecum was then amputated, the mucosa was folded inside the lumen of the remaining half, and a second suture was placed to prevent exposure of intestinal mucosa to the peritoneum. After rinsing of the peritoneal cavity with saline, the peritoneum and the abdominal wall were closed with resolvable Maxon and nonresolvable It-Cron 5.0 (Davis and Geck) thread, respectively. Care was taken to obtain optimal hemostasis. Mice were allowed to recover and were observed for 6 d. After 6 d, the mice were anesthetized and the abdominal skin was removed. An 18-gauge catheter (Vialon Insyte-W; Becton Dickinson, Erembodegem, Belgium) connected to a syringe containing 5 ml sterile saline was introduced into the peritoneum, carefully avoiding any blood vessels, and the peritoneal cavity was rinsed. The peritoneal lavage fluid was assayed for red blood cells and hemoglobin. Then, the peritoneum was opened and blood collected by puncture of the vena cava into 0.1 vol of 4% trisodium citrate to determine hemoglobin, hematocrit, red blood cells, and platelet count.

### *Statistical analysis*

The statistical significance of differences between groups was determined using Student's *t* test for paired or unpaired values and two-sided *P* values or by ANOVA, as indicated.

## Results

*Fibrinolytic activity in plasma.* As illustrated in Fig. 1, diluted whole blood clots of PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice underwent progressive although limited lysis reaching a plateau of 3±1 and 6+1%, respectively, after ~ 3 h ($P < 0.001$ by ANOVA).

*$^{125}$I-fibrin labeled plasma clot lysis in vivo.* Spontaneous in vivo clot lysis in PAI-1$^{-/-}$, heterozygous PAI-1-deficient (PAI-1$^{+/-}$), and PAI-1$^{+/+}$ mice was studied after injection of a

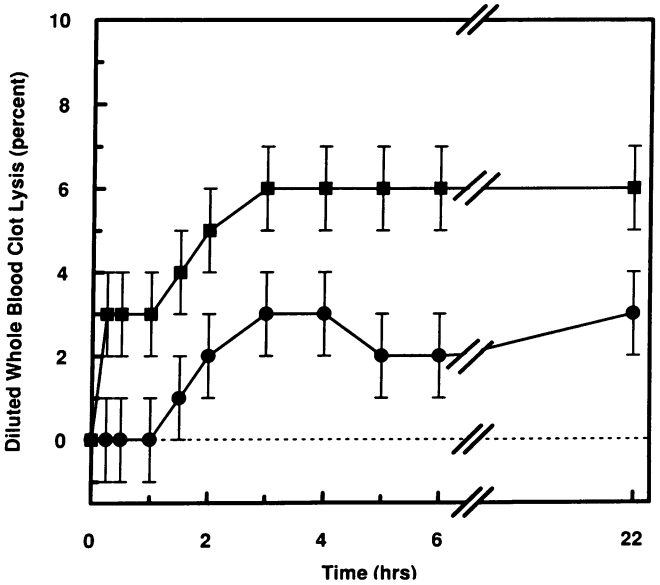


*Figure 1.* Diluted whole blood clot lysis in PAI-1$^{-/-}$ (*squares*) and PAI-1$^{+/+}$ (*circles*) mice. Data represent mean±SEM of lysis of five replicate blood clots from four different animals.

2. The dose of endotoxin and the time required to develop venous thrombosis was found to be dependent on the type of endotoxin tested. Significant lot-to-lot variations have also been observed by others (27).

$^{125}$I-fibrin–labeled murine plasma clot (prepared from PAI-1$^{+/+}$ mice) into the jugular vein. PAI-1$^{-/-}$ mice lysed the $^{125}$I-fibrin–labeled murine plasma clot at a significantly higher rate than their PAI-1$^{+/-}$ and PAI-1$^{+/+}$ littermates: 87±7 vs 66±3 vs 47±5% lysis during 8 h, respectively ($P$ = 0.03: PAI-1$^{-/-}$ vs PAI-1$^{+/-}$; $P$ = 0.002: PAI-1$^{-/-}$ vs PAI-1$^{+/+}$; $P$ = 0.02 PAI-1$^{+/-}$ vs PAI-1$^{+/+}$) (Fig. 2).

The difference in the rate of experimental clot lysis between the PAI-1$^{-/-}$ and PAI-1$^{+/+}$ mice was even more pronounced when the mice had been pretreated with 0.5 mg/kg endotoxin 2 h before the experiment (91±3 vs 35±5% lysis during 4 h, respectively, $P < 0.001$) (Fig. 3).

The lysability of plasma clots prepared from PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice was found to be similar. PAI-1$^{+/+}$ mice lysed a plasma clot prepared from PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice for 44±4 and 42±5%, respectively within 4 h ($P$ = NS). Corresponding values in PAI-1$^{-/-}$ mice were 66±13 and 49±9%, respectively within 4 h ($P$ = NS).

*Thrombus formation after endotoxin injection in the footpad.* 6 d after injection of endotoxin in the footpad, venous thrombosis was observed by light microscopic examination in 11 out of 26 PAI-1$^{+/+}$, but only in 1 out of 25 PAI-1$^{-/-}$ mice ($P$ = 0.004 by chi-square analysis). Thrombi of various calibers were observed in deep, medium-sized veins (Fig. 4). The thrombi were adherent to the vessel wall, covered to a variable extent by endothelium, and in various stages of organization. Arterial thrombi were not observed. Variably sized inflammatory infiltrates, consisting primarily of lymphocytes and monocytes, were observed both in PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice. This inflammatory infiltrate frequently extended into the subcutaneous fatty tissue and striated muscle, but not into the overlying epidermis.

*Haemostasis analyses and bleeding assays.* No differences were observed between PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice in aPTT, TT, and blood cell count.

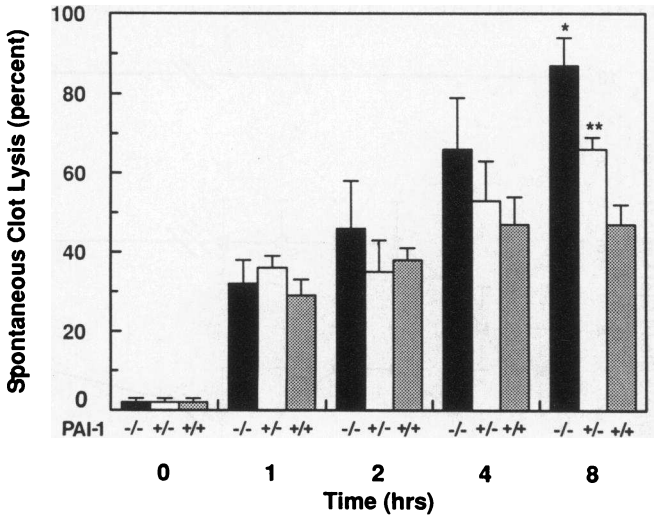


*Figure 2.* Spontaneous lysis of $^{125}$I-fibrin labeled pulmonary emboli in PAI-1$^{+/+}$, PAI-1$^{+/-}$, and PAI-1$^{-/-}$ mice. A 25-μl $^{125}$I-fibrin–labeled murine plasma clot was injected into the jugular vein, and the residual radioactivity in the heart and lungs was measured ex vivo at the indicated time points. Values are expressed as percent of total radioactivity injected. −/−, PAI-1$^{-/-}$ mice; +/−, PAI-1$^{+/-}$ mice; +/+, (PAI-1$^{+/+}$) mice. *$P$ = 0.03 vs PAI-1$^{+/-}$ and $P$ = 0.002 vs PAI-1$^{+/+}$ mice; **$P$ = 0.02 vs PAI-1$^{+/+}$ mice.

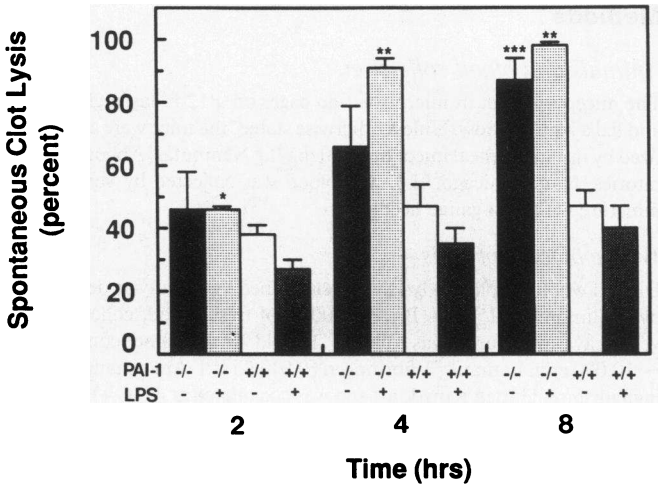


*Figure 3.* Effect of intraperitoneal injection of endotoxin on spontaneous lysis of $^{125}$I-fibrin–labeled pulmonary emboli in PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice. A 25-μl $^{125}$I-fibrin–labeled murine plasma clot was injected into the jugular vein, and the residual radioactivity in the heart and lungs was measured ex vivo at the indicated time points. Values are expressed as percent of total radioactivity injected. −/−, PAI-1$^{-/-}$ mice; +/+, PAI-1$^{+/+}$ mice; LPS (+), with endotoxin injection; LPS (−), without endotoxin injection. *$P$ = 0.008 vs PAI-1$^{+/+}$ mice after endotoxin injection. **$P < 0.001$ vs PAI-1$^{+/+}$ mice after endotoxin injection. ***$P$ = 0.002 vs PAI-1$^{+/+}$ mice without endotoxin injection.

Two models were used to document a potential hemorrhagic tendency. When a 5-mm segment of the tail was amputated, no significant difference was observed between PAI-1$^{+/+}$ and PAI-1$^{-/-}$ animals both with respect to bleeding time and rebleeding as defined in Methods (Table I). Furthermore, all of the 145 PAI-1$^{-/-}$ of the 623 $F_3$ PAI-1 littermates that underwent a partial tail amputation for genotyping survived the procedure without apparent excessive blood loss.

When a partial amputation of the large caecum was performed and the extent of blood loss into the peritoneal cavity measured, PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice showed similar blood cell counts and numbers of red blood cells in their peritoneal fluid 6 d after surgery (Table I). The hematocrits in PAI-1$^{+/+}$ and PAI-1$^{-/-}$ mice at 6 d were also similar.

## Discussion

The effects of PAI-1 gene disruption on hemostasis, thrombosis, and thrombolysis were studied in PAI-1–deficient mice, generated by homologous recombination in embryonic stem cells (23).

Disruption of the PAI-1 gene enhanced endogenous thrombolysis in a gene-dose–dependent way. Lysis of a pulmonary plasma clot was significantly faster in PAI-1$^{+/-}$ mice, but even faster in PAI-1$^{-/-}$ mice compared to PAI-1$^{+/+}$ mice, consistent with an inhibitory role of PAI-1 on plasminogen activation in vivo (3). Intraperitoneal injection of endotoxin augmented the difference in reactivity between PAI-1$^{-/-}$ and PAI-1$^{+/+}$ mice, possibly because of stimulation, not only of PAI-1 secretion (28–30), but to a lesser extent, also of t-PA secretion (30).

PAI-1$^{-/-}$ mice were found to develop significantly less frequently venous thrombi than PAI-1$^{+/+}$ mice after local injection of endotoxin in the footpad, although a similar extent of

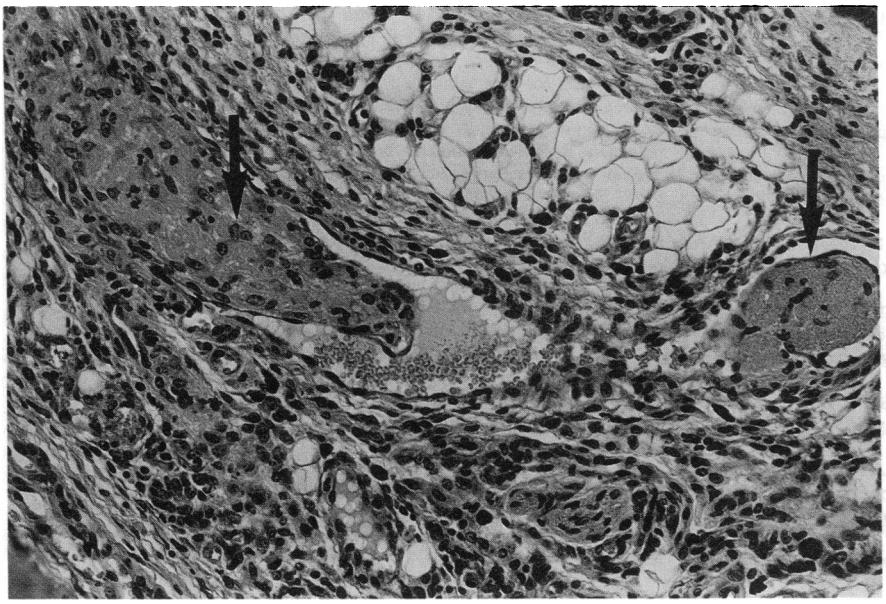

*Figure 4.* Light micrograph of a footpad section of a PAI-$1^{+/+}$ mouse killed 6 d after injection of 50 $\mu$g endotoxin. A large organized thrombus containing fibrinoid material, polymorphs, and monocytes, and that is lined with an intact endothelium is filling most of the lumen of the sectioned vein. A section through two veins, each filled with a mural thrombus (*arrow*) is shown. Red blood cells surround the thrombus. A cellular infiltrate, consisting predominantly of polymorphs and monocytes, is present in the surrounding tissue. Hematoxylin and eosin, ×87.

inflammation was observed. The greater resistance of PAI-$1^{-/-}$ mice to venous thrombosis is also consistent with their increased capacity to lyse experimental plasma clots in the pulmonary embolus model. Taken together, these findings indeed are suggestive of a significant causal role for PAI-1 in the development of venous thrombosis. Previous studies did not always allow to determine whether the acute phase protein PAI-1 was a cause or consequence of the thrombotic phenomena.

*Table I. Hematological and Hemostatic Analysis of $F_3$ PAI-1 Littermates*

| | PAI-$1^{+/+}$ | PAI-$1^{-/-}$ |
|---|---|---|
| Blood analysis | | |
| aPTT (s) | 58±3 (18) | 63±4 (8) |
| TT (s) | 25±1 (18) | 26±5.5 (3) |
| Fibrinogen (g/liter) | 0.91±0.04 (18) | 0.81±0.3 (3) |
| RBC ($\times 10^{12}$/liter) | 8.8±0.2 (18) | 10±1.1 (3) |
| Hemoglobin (g/percent) | 13±0.3 (18) | 17±1.4 (3) |
| Hematocrit (percent) | 43±1 (18) | 52±6.3 (3) |
| Platelets ($\times 10^9$/liter) | 640±26 (18) | 660±140 (3) |
| Partial tail amputation | | |
| Bleeding time (min) | 0.95±0.15 (5) | 1.2±0.33 (5) |
| Blood loss* (mg hemoglobin) | 48±24 (5) | 32±17 (5) |
| Appendectomy (at 6 d) | | |
| RBC ($\times 10^{12}$/liter) | 8.2±0.6 (7) | 8.9±1.0 (6) |
| Hemoglobin (g/percent) | 12±0.9 (7) | 14±1.7 (6) |
| Hematocrit (percent) | 38±3.2 (7) | 42±5.7 (6) |
| Platelets ($\times 10^9$/liter) | 550±61 (7) | 600±68 (6) |
| RBC in peritoneal fluid‡ ($\times 10^6$) | 100±38 (7) | 100±54 (6) |

Data represent mean±SEM of the number of measurements indicated between brackets. aPTT, activated partial thromboplastin time; TT, thrombin time; RBC, red blood cell. * Blood loss represents the total amount of hemoglobin in milligrams recovered from the 10-ml saline recipient in which the injured tail was submersed for 1 h at 37°C (see Methods). ‡ RBC in the peritoneal fluid represent the total number of RBC recovered from rinsing the peritoneal cavity with 5 ml of saline (see Methods).

Although delayed rebleeding after trauma or surgery is a consistent clinical finding in patients with reduced (4–7) or absent (8) PAI-1 levels, spontaneous bleeding, or delayed rebleeding could not be documented after partial amputation of the tail or of the caecum. Thus, it is possible that PAI-1 deficiency may cause a milder hyperfibrinolytic state in mice than in man. Our observations that lysis of a pulmonary plasma clot in PAI-$1^{-/-}$ mice became only statistically faster after 8 h and that lysis of a diluted whole blood clot in vitro progressed to only ~ 6% are consistent with this interpretation.

Possible explanations for the difference in phenotype between the mouse (no rebleeding) and man (delayed rebleeding) may be the lower basal plasma levels of active PAI-1 in wild type mice (~ 2 ng/ml) than in man (~ 10 ng/ml). Species-dependent differences in the plasma levels of t-PA and possibly other components of the fibrinolytic system might also contribute to the phenotypic dissimilarity between mouse and man.

The effects of disruption of the PAI-1 gene on venous thrombosis became only apparent after injection of endotoxin. This suggests that the biological consequences of PAI-1 gene inactivation might be more significant in disease states with a disrupted coagulation/fibrinolytic balance such as atherosclerosis (18–21), glomerulonephritis (31), tumor invasion, and metastasis (32). The circumstantial evidence that PAI-1 may be involved in these pathological processes, as reviewed elsewhere (18–21, 31, 32), warrants further investigation.

In conclusion, disruption of the PAI-1 gene in mice does not appear to impair hemostasis, but is associated with increased resistance to thrombosis and with a mild hyperfibrinolytic state characterized by enhanced in vivo clot lysis.

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