Regulation of Arterial Thrombolysis by Plasminogen Activator Inhibitor-1 in Mice

Peter M. Farrehi, MD; C. Keith Ozaki, MD; Peter Carmeliet, MD, PhD; William P. Fay, MD

Background.—Platelet-rich arterial thrombi are resistant to lysis by plasminogen activators. However, the mechanisms underlying thrombolysis resistance are poorly defined. Plasminogen activator inhibitor-1 (PAI-1), which is present in plasma, platelets, and vascular endothelium, may be an important determinant of the resistance of arterial thrombi to lysis. However, in vitro studies examining the regulation of platelet-rich clot lysis by PAI-1 have yielded inconsistent results.

Methods and Results.—We developed a murine arterial injury model and applied it to wild-type (PAI-1+/+) and PAI-1−/− deficient (PAI-1−/−) animals. FeCl₃ was used to induce carotid artery thrombosis. Thrombi consisted predominantly of dense platelet aggregates, consistent with the histology of thrombi in large-animal arterial injury models and human acute coronary syndromes. To examine the role of PAI-1 in regulating endogenous clearance of platelet-rich arterial thrombi, thrombi were induced in 22 PAI-1+/+ mice 14 PAI-1−/− mice. Twenty-four hours later, the amount of residual thrombus was determined by histological analysis of multiple transverse sections of each artery. Residual thrombus was detected in 55 of 85 sections (64.7%) obtained from PAI-1+/+ mice compared with 19 of 56 sections (33.9%) from PAI-1−/− mice (P=.009). Computer-assisted planimetry analysis revealed that mean thrombus cross-sectional area was 0.033±0.027 mm² in PAI-1+/+ mice versus 0.016±0.015 mm² in PAI-1−/− mice (P=.048).

Conclusions.—PAI-1 is an important determinant of thrombolysis at sites of arterial injury. Application of this model to other genetically altered mice should prove useful for studying the molecular determinants of arterial thrombosis and thrombolysis. (Circulation. 1998;97:1002-1008.)

Key Words: thrombosis | thrombolysis | carotid arteries | plasminogen activators
Methods

Animals

C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, Me. PAI-1–deficient mice were generated by homologous recombination in embryonic stem cells, as described previously. To eliminate potential effects of strain differences on experimental results, consecutive generations of mice carrying the null PAI-1 allele were backcrossed repeatedly to C57BL/6J mice. Only mice that were the product of five or more backcrosses to C57BL/6J were used in experiments comparing PAI-1–deficient (PAI-1−/−) mice with wild-type C57BL/6J (PAI-1+/+) mice. Genotyping of mice was performed by polymerase chain reaction analysis of tail DNA as described previously. All animal care and experimental procedures complied with The Guide for Care and Use of Laboratory Animals, Department of Health, Education, and Welfare Publication No. NIH 78-23, and were approved by the University of Michigan Committee on Use and Care of Animals.

Thrombosis Protocol

Mice (6 to 8 weeks old; weight, ~25 g) were anesthetized by inhalation of 1% isoflurane and secured in the supine position under a dissecting microscope (Zeiss). A midline cervical incision was made, and the left common carotid artery was isolated by blunt dissection. FeCl3 (Mallinkrodt Chemical) was used to induce vascular injury. A 1×2-mm strip of filter paper saturated with 10% FeCl3 solution was applied to the adventitial surface of the surgically exposed carotid artery for 3.0 minutes, then removed. The surgical incision was sutured with 4–0 monofilament, and the animal was returned to its cage. Operators were blinded to PAI-1 genotype while performing the thrombosis protocol. Twenty-four-hour postoperative, mice were anesthetized, and the injured carotid segments were isolated and perfusion-fixed immediately after inducing the procedure on 3 to 5 mice, operators could routinely isolate the carotid artery in <10 minutes. The Wilcoxon rank-sum test was used to determine whether a significant difference existed in mean thrombus cross-sectional areas between experimental groups. Student’s unpaired t test was used to compare in vitro aggregation of wild-type and PAI-1–deficient platelets.

Histological and Morphometric Analyses

To quantify the amount of thrombus present in carotid arteries 24 hours after injury, four evenly spaced transverse sections (distance between sections, ~1 mm) were prepared from each paraffin-embedded carotid artery and subjected to hematoxylin-eosin staining. Each transverse section was scored for the presence or absence of intraluminal thrombus. In addition, a computer-assisted planimetry program (Image-Pro Plus, Media Cybernetics) was used to measure the cross-sectional area of thrombus for each transverse section, and the mean of these values was calculated for each artery. Examiners were blinded to the genotype (ie, PAI-1+/+ or −/−) of specimens during all histological analyses. To determine the composition of thrombi, some carotid artery specimens were analyzed by transmission electron microscopy as described previously.

Platelet Aggregation Studies

Blood was collected into citrate anticoagulant from anesthetized mice by inferior vena cava puncture with a 25-gauge needle. PRP was prepared by centrifuging blood (120g for 6 minutes) at room temperature in a swing-out rotor. The platelet count of PRP was adjusted with citrated platelet-poor plasma to 2×108 platelets/mL. Samples (200 µL) of PRP were placed in 7.5×55-mm siliconized flat-bottom tubes, and standard aggregometry was performed with a model PAP-4 aggregometer (Bio/Data Corp.).

Statistical Analyses

Data are presented as mean±SD. The generalized estimating equations method was used to determine whether a significant difference existed between experimental groups in the prevalence of intraluminal thrombus. The Wilcoxon rank-sum test was used to determine whether a significant difference existed in mean thrombus cross-sectional areas between experimental groups. Student’s unpaired t test was used to compare in vitro aggregation of wild-type and PAI-1–deficient platelets.

Results

Murine Model of Acute Arterial Injury

We performed a series of studies to determine the feasibility of using mice to study arterial thrombosis and thrombolysis. The common carotid artery was selected for analysis because it was easily approached via a midline cervical incision. After performing the procedure on 3 to 5 mice, operators could routinely isolate the carotid artery in <10 minutes. We used topical application of FeCl3 to the exposed carotid artery to induce vascular injury and thrombosis. Completely occlusive carotid artery thrombosis was well tolerated by mice, presumably because of collateral cerebral blood flow provided by the contralateral carotid artery. After recovery from anesthesia, mice appeared healthy, demonstrating normal ambulation, feeding, and grooming. The operative mortality rate of carotid artery surgery was <5%. Fig 1 demonstrates the use of Whatman 1 filter paper to apply FeCl3 to the arterial surface. Control experiments performed in >50 wild-type mice revealed that 10% FeCl3 induced completely occlusive thrombosis within 30 minutes (determined by visual inspection of the artery under the dissecting microscope) in >90% of mice, whereas application of filter paper alone (ie, no FeCl3) to carotid arteries did not induce thrombosis. To determine the composition of thrombi, some carotid artery specimens were analyzed by transmission electron microscopy as described previously.
from 10 consecutive mice revealed that thrombus composition was uniform from animal to animal (Fig 2A). Transmission electron microscopy confirmed that thrombi consisted predominantly of dense platelet aggregates (Fig 2B), consistent with the composition of arterial thrombi observed in humans and large-animal thrombosis models.

Application of Carotid Injury Model to PAI-1–Deficient Mice

To examine the role of PAI-1 in regulating endogenously mediated arterial thrombolysis, we applied the FeCl₃ model to wild-type (PAI-1 +/-) and PAI-1–deficient (PAI-1 –/-) mice. Twenty-two PAI-1 +/- and 14 age- and sex-matched PAI-1 –/- mice were studied. Each group was subjected to identical carotid artery injury with 10% FeCl₃ as described in “Methods.” There was no difference in operative bleeding between the two experimental groups. Twenty-four hours later, the injured carotid segments were perfusion-fixed and excised. Examiners were blinded to PAI-1 genotype during all evaluations of tissue samples. To determine whether the amount of residual intraluminal thrombus differed between experimental groups, each segment of injured carotid artery was cross-sectioned at four evenly spaced intervals as described in “Methods,” and transverse sections were prepared for histological analysis (Fig 3). Residual thrombus (either completely or partially occlusive) was detected in 55 of 85 sections (64.7%) obtained from PAI-1 +/- mice, compared with 19 of 56 sections (33.9%) from PAI-1 –/- mice (P=0.009, Fig 4A). The cross-sectional area of thrombus was measured for each transverse section by computer-assisted planimetry, and the mean thrombus area for each artery was calculated (Fig 4B). Mean residual thrombus area was 0.033±0.027 mm² (95% CI, 0.022 to 0.044 mm²) in PAI-1 +/- mice versus 0.016±0.015 mm² (95% CI, 0.008 to 0.024 mm²) in PAI-1 –/- mice (P=.009). Subgroup analysis of patent and occluded vessels revealed an equal proportion of patent arteries in both groups (9 of 22 PAI-1 +/- mice versus 6 of 14 PAI-1 –/- mice). However, significantly less residual mural thrombus was observed in patent vessels from PAI-1 –/- mice than in those from PAI-1 +/- mice (0.001±0.002 versus 0.011±0.015 mm², respectively, P<.05). Similarly, increased thrombus was present in occluded vessels from PAI-1 +/- mice compared with PAI-1 –/- mice (0.052±0.021 versus 0.027±0.010 mm², respectively, P<.05).

The significant difference in residual thrombus observed between wild-type and PAI-1–deficient mice 24 hours after injury could be explained by reduced thrombus formation and/or by enhanced thrombolysis in PAI-1 –/- mice. To address this issue, we studied thrombus generation after vascular injury in PAI-1 +/- (n=8) and PAI-1 –/- (n=4) mice. Mice were subjected to arterial injury with 10% FeCl₃, for 3 minutes. Thereafter, a miniature Doppler flow probe (model 0.5VB, Transonic Systems) that interfaced with a Transonic model T106 flowmeter was applied to the carotid artery, and...
blood flow was recorded with a computerized data acquisition program (WinDaq, DATAQ Instruments). All 4 vessels of PAI-1−/− mice occluded within 30 minutes of initiation of injury, whereas 7 of 8 vessels from PAI-1+/+ mice occluded within 30 minutes. These results suggested that PAI-1−/− mice did not exhibit a major defect in thrombus formation after injury compared with PAI-1+/+ mice. Because several minutes were necessary to properly position the flow probe on the artery, precise determination of the time of arterial occlusion was not possible in 7 of 12 mice studied, because the vessel had already thrombosed by the time the flow probe was properly positioned. Recorded times to occlusion after initiation of injury were <7.5, <9.5, 15.5, and 21 minutes in PAI-1−/− mice versus <6.0, <6.5, <8.5, <15, <15, 17, and 16 minutes in PAI-1+/+ mice (blood flow was monitored for a total of 45 minutes after injury in the 1 PAI-1+/+ animal that did not develop occlusive carotid artery thrombosis). To determine whether PAI-1 deficiency was associated with a primary defect in platelet aggregation, pooled platelet-rich plasma was prepared from PAI-1+/+ mice (n=2) and PAI-1−/− mice (n=2), and in vitro platelet aggregation studies were performed (Fig 5). There was no significant difference in ADP-induced aggregation between PAI-1+/+ and PAI-1−/− platelets (64.5±4.9% in PAI-1+/+ mice versus 58±4.2% in PAI-1−/− mice, P=.29). We also compared thrombus composition in PAI-1+/+ and PAI-1−/− mice, because this parameter is an important determinant of the rate of clot lysis.24 No gross differences were observed—ie, all thrombi appeared to be platelet-rich. Because leukocytes migrate into thrombi within 24 hours after initial formation and leukocyte proteases, such as elastase, degrade fibrin,25 we compared thrombus leukocyte density between experimental groups. No significant differences were observed (1.4±0.4 leukocytes per high-power field in PAI-1+/+ mice versus 1.4±0.4 leukocytes per high-power field in PAI-1−/− mice). Similarly, no differences in the histological appearance of injured arterial walls was observed between PAI-1+/+ and PAI-1−/− mice. Topical application of FeCl3 produced full-thickness, noncircumferential vascular injury (Fig 3). Modifying Injury Model for Study of Thrombus Formation

Our experiments with the vascular flow probe suggested that the carotid artery model would prove useful for quantitatively studying blood flow and thrombus formation in mice. Such a system would be useful in the analysis of mice with genetic modifications in factors that regulate carotid artery blood flow (eg, adrenergic receptors) or platelet deposition after arterial injury (eg, platelet or endothelial cell integrins). Carotid artery blood flow in anesthetized mice (n=14) was 1.1±0.7 mL/min. Mice could be maintained under general anesthesia for at least 1 hour with stable heart and respiratory rates and stable carotid artery blood flows. As shown in Fig 6A, high-quality flow tracings could be obtained from the mouse carotid artery. To better study thrombosis after injury, we modified our protocol to allow induction of vascular injury while the flow probe was positioned on the artery. This was done with a smaller strip of filter paper (0.5×1.0 mm) saturated with higher concentrations of FeCl3 (25% to 50%) applied proximal to the flow probe. Mean time to occlusion was 13.6±5.1 minutes after 25% FeCl3 injury (n=4) and 5.6±4.9 minutes after 50% FeCl3 injury (n=5). Cyclic flow reductions, which result from repetitive formation and embolization of thrombi at sites of vascular injury,26 were observed (Fig 6B). These observations indicate that the carotid artery model demonstrates several features observed in large-animal models and therefore should prove useful in murine studies of blood flow regulation and thrombosis after vascular injury.

**Discussion**

Platelet-rich thrombi are more resistant to lysis by t-PA than platelet-poor thrombi.24 However, the mechanisms underlying this phenomenon are not well defined. In this report, we studied the role of PAI-1 in regulating the endogenous clearance of platelet-rich arterial thrombi in mice. PAI-1 is abundant in platelets, and its secretion from vascular endothelial cells is stimulated by factors released from activated platelets.27 Several in vitro studies suggest that PAI-1 is the dominant factor underlying platelet-mediated clot lysis resis-
PAI-1 and Arterial Thrombolysis

Figure 6. Monitoring of carotid artery blood flow. Miniature Doppler flow probe (7.2 MHz) was used to monitor carotid artery blood flow in mice. A, Phasic carotid artery flow before and after FeCl3 injury. B, Mean carotid artery blood flow in different mouse subjected to FeCl3 injury (50%) for 1.5 minutes. Artifactual reduction in flow during induction of injury is due to removal of saline from surgical site to allow application of FeCl3. Note that mean blood flow initially declines gradually and that repetitive, transient increases in flow are observed. Thereafter, flow declines rapidly, with complete loss of flow occurring 14 minutes after injury. After loss of flow in left carotid artery was documented, flow probe was placed on right carotid artery. Flow rate of 1.6 mL/min was observed (data not shown).

Characterization of FeCl3-Induced Thrombosis and Comparison With Other Models

In addition to studying the biological function of PAI-1, a goal of our study was to develop a reliable model of arterial injury and thrombosis in mice. Kurz et al21 first described the use of FeCl3 to induce arterial thrombosis in rats weighing 375 to 450 g. Our studies demonstrate that this method can be applied effectively to animals weighing <25 g and that FeCl3 produces platelet-rich thrombi. The FeCl3 model exhibits several features that suggest that it will prove useful for studying the molecular determinants of arterial thrombosis and thrombolysis in transgenic mice. It allows for uniform injury from animal to animal, because the concentration of FeCl3, the size of the arterial segment being injured, and the duration of injury can be precisely controlled. Because FeCl3 does not produce circumferential vessel injury, it allows induction of thrombosis without complete destruction of vascular cells that mediate subsequent thrombolysis. Carotid artery blood flow can be monitored accurately in mice, and complete vascular occlusion is well tolerated because of collateral blood flow. In addition, the relevance of the murine model to large-animal thrombosis models is supported by the fact that cyclic flow variations are observed in the mouse carotid artery after FeCl3 injury.26

Detection of less residual thrombus after injury in PAI-1 −/− mice does not necessarily imply enhanced thrombolysis compared with PAI-1 +/+ mice, because it was not possible in our study to confirm equal amounts of thrombus deposition in each group. However, studies in which a flow probe was used to confirm thrombus formation did not suggest a diminished tendency to form thrombi in PAI-1 −/− mice. In addition, in vitro studies revealed no significant difference in platelet aggregation between experimental groups. Similarly, histological analysis of injured carotid segments did not reveal differences in thrombus composition or the extent of injury between groups. Carmeliet et al30 demonstrated that PAI-1 −/− mice exhibit normal tail bleeding times and normal blood loss after amputation of the caecum compared with PAI-1 +/+ mice. Together, these observations suggest that there is no significant difference in clot formation after injury between PAI-1 +/+ and PAI-1 −/− mice and that the reduction in residual thrombus that we observed 24 hours after injury in PAI-1 −/− mice resulted from enhanced thrombolysis. However, because thrombosis and thrombolysis can occur concurrently in vivo,2 it is possible that diminished thrombus formation also contributed to our results. Regardless of mechanism, our studies demonstrate the important role of PAI-1 in determining the net amount of platelet-rich thrombus present in arteries after injury.

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A variety of methods have been used to study thrombosis in rodents. These include application of electrical, mechanical, and other forms of energy to different vascular sites, intravenous injection of clots formed in vitro, and injection of blood/thrombin mixtures into isolated vascular segments.31,37–42 Only a few of these models have been applied to mice. Carmeliet et al30 and Palabrica et al43 modified the

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hamster pulmonary embolism model of Stassen et al.17 to study the regulation of thrombolysis in mice, whereas Pierangeli46 used “pinch” injury to induce femoral vein thrombosis. In contrast to these experimental systems, our model includes the key components of arterial injury and high-velocity laminar flow that are necessary to generate platelet-rich thrombi. The mechanism by which FeCl₃ induces thrombosis is not well defined. Iron induces formation of highly reactive oxidant species and potentiates the sensitivity of endothelial cells to oxidant damage.45 Recent studies indicate that FeCl₃ induces thrombosis by triggering expression of tissue factor, a major determinant of thrombosis at sites of atherosclerotic plaque rupture.46,47 Carmeliet et al.48 used perivascular electric injury to study vascular wound healing and neointima formation in mouse femoral arteries. Induction of thrombosis by electrical injury may depend on the generation of reactive iron species, because only iron-containing electrodes efficiently cause thrombus formation.29 Therefore, it is possible that topical FeCl₃ and electrical current delivered by iron-containing electrodes induce vascular injury by similar mechanisms.

In summary, we have developed a murine model of carotid artery injury that we have used to demonstrate the important role of PAI-1 in regulating the acute clearance of arterial, platelet-rich thrombi. The model is easy to perform and does not require the use of specialized surgical techniques or equipment to induce vascular injury. Because many other components of the murine blood coagulation and fibrinolytic system have been modified by gene targeting approaches, this model should prove useful for studying the function of other blood coagulation and fibrinolytic factors within the distinct environment of the acutely injured artery.

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