Overexpression of the Cell Cycle Inhibitor $p16^{INK4a}$ Promotes a Prothrombotic Phenotype Following Vascular Injury in Mice

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Objective—Age-associated cellular senescence is thought to promote vascular dysfunction. $p16^{INK4a}$ is a cell cycle inhibitor that promotes senescence and is upregulated during normal aging. In this study, we examine the contribution of $p16^{INK4a}$ overexpression to venous thrombosis.

Methods and Results—Mice overexpressing $p16^{INK4a}$ were studied with 4 different vascular injury models: (1) ferric chloride ($FeCl_3$) and (2) Rose Bengal to induce saphenous vein thrombus formation; (3) $FeCl_3$ and vascular ligation to examine thrombus resolution; and (4) lipopolysaccharide administration to initiate inflammation-induced vascular dysfunction. $p16^{INK4a}$ transgenic mice had accelerated occlusion times (13.1 ± 0.4 minutes) compared with normal controls (19.7 ± 1.1 minutes) in the $FeCl_3$ model and 12.7 ± 2.0 and 18.6 ± 1.9 minutes, respectively in the Rose Bengal model. Moreover, overexpression of $p16^{INK4a}$ delayed thrombus resolution compared with normal controls. In response to lipopolysaccharide treatment, the $p16^{INK4a}$ transgenic mice showed enhanced thrombin generation in plasma-based calibrated automated thrombography assays. Finally, bone marrow transplantation studies suggested increased $p16^{INK4a}$ expression in hematopoietic cells contributes to thrombosis, demonstrating a role for $p16^{INK4a}$ expression in venous thrombosis.

Conclusion—Venous thrombosis is augmented by overexpression of the cellular senescence protein $p16^{INK4a}$. (***Arterioscler Thromb Vasc Biol*. 2011;31:827-833.**)

Key Words: aging ■ coagulation ■ pathology ■ thrombosis

Aging is an important risk factor for developing cardiovascular disease, and also the least understood.[1,2] Venous thromboembolism (VTE) is characterized by the development of thrombi in the deep veins of the legs; these thrombi are prone to dislodging and embolizing to the lungs. This condition accounts for 140 000 to 200 000 deaths each year in the United States.[3,4] The risk of developing VTE substantially increases with age, and individuals over the age of 55 years have an annual incidence 5 to 7 times higher than young adults.[5] Although VTE in the younger population is often explained by mutations in hemostatic genes, mechanisms behind the increased risk of VTE in the elderly are less well understood.

Senescence is one cellular phenomenon known to be associated with aging. Cellular senescence is a stress-induced process that is controlled by cell cycle inhibitors and that promotes an irreversible growth arrest.[6–9] $p16^{INK4a}$, a cell cycle inhibitor that promotes senescence, binds to cyclin-dependent kinases 4 and 6 to disrupt phosphorylation of the retinoblastoma protein, causing a G_1 cell cycle arrest.[10] Expression of $p16^{INK4a}$ increases with age in many tissues and is a biomarker of aging.[11–14] Furthermore, $p16^{INK4a}$ expression correlates with biomarkers of senescence, such as senescence-associated β-galactosidase expression, and expression is associated with gerontogenic activities, such as smoking, physical inactivity, and ad libitum feeding in humans or mice.[11,14] In some tissues such as pancreatic β-cells, neural stem cells, and hematopoietic stem or progenitor cells, the age-induced increase in $p16^{INK4a}$ expression is associated with reduced cellular proliferation coupled with an impaired tissue response to injury.[15–17] Additionally, senescent cells are thought to contribute to aging pathology through the production of cytokines (interleukin-6) that further promote inflammation and cellular dysfunction.[18]

The contribution of senescence to disease in the venous circulation and how it may be involved in age-related VTE or a possible prothrombotic phenotype remain largely uncharacterized. The aim of this study was to ascertain whether

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overexpression of $p16^{INK4a}$ modified venous thrombus formation in several well-defined animal models. Our results demonstrate that $p16^{INK4a}$ overexpression augments vascular occlusion and delayed thrombus resolution relative to wild-type controls. Furthermore, $p16^{INK4a}$ transgenic mice display enhanced thrombin generation, increased thrombin-antithrombin, and increased plasminogen activator inhibitor-1 (PAI-1) levels when exposed to low-dose lipopolysaccharide (LPS). Additionally, bone marrow transplantation between wild-type and $p16^{INK4a}$ transgenic mice demonstrated a substantial contribution of hematopoietic cells to this phenotype. Overall, these results show that expression of $p16^{INK4a}$ is involved in promoting a prothrombotic environment in the venous vasculature.

Methods

A detailed description of the methods is presented in the Supplemental Materials, available online at http://atvb.ahajournals.org.

Mice

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of North Carolina–Chapel Hill. The bacterial artificial chromosome transgenic mice overexpressing $p16^{INK4a}$ used in this study have been described previously.[15]

Hemostasis Model

Hemostasis was assessed as previously described.[19] Briefly, wild-type and $p16^{INK4a}$ transgenic mice at 8 weeks of age were anesthetized with 2.5% tribromoethanol (T48402, Sigma-Aldrich, St. Louis, MO) at 0.1 mL/g body weight. The saphenous vein of anesthetized mice was exposed and transected with a 23-gauge needle. Once bleeding stopped, a longitudinal cut was made in the vessel, and the blood was gently wiped away with Kimwipes (Kimberly-Clark, Roswell, GA) until it clotted. The blood clot was disrupted using a 30-gauge needle, and the blood was gently wiped away. Clot disruption was repeated every time hemostasis occurred, and each hemostatic event was recorded using Chart software for 20 minutes.

$FeCl_3$ Vascular Injury

The saphenous vein thrombosis model was performed as previously described.[19] Briefly, the saphenous veins of anesthetized mice were exposed and dissected away from the saphenous artery. A 0.5×2-mm piece of filter paper was soaked in 2.5% (n=4), 5% (n=5), or 10% (n=4) $FeCl_3$ (F7134, Sigma-Aldrich) and laid over the saphenous vein for 3 minutes. The filter paper was then removed, and the tissue was washed 3 times with warm saline. Blood flow was monitored using a 20-MHz Doppler flow probe (Indus Instruments, Webster, TX). Occlusion was defined as the absence of blood flow for 1 minute. The time to flow restriction was defined as the time after injury at first cessation of blood flow.

Rose Bengal Photochemical Vascular Injury

Photochemical injury was performed as previously described.[20] Briefly, both right and left saphenous veins of anesthetized mice were exposed. The left saphenous vein was catheterized using catheters made in-house using pulled PE-10 tubing (Braintree Scientific, Braintree, MA). Rose Bengal (R-3877, Sigma-Aldrich), diluted to 30 mg/mL in normal saline, was infused through the catheter at a dose of 75 mg/kg through a gastight syringe (Hamilton Co, Reno, NV). Before infusion of Rose Bengal, a 1.75-mW green light (540 nm) (Prizmatix, Southfield, MI) was directed 0.5 cm over the injury site on the right saphenous vein. Light was applied to the vessel until a stable thrombus (defined as the absence of blood flow for 1 minute) was achieved.

Thrombus Resolution

We developed a new method to measure thrombus resolution using the saphenous vein. Wild-type and $p16^{INK4a}$ transgenic mice (n=3 per genotype at each time point) were subjected to 10% $FeCl_3$ injury to the saphenous vein. The tissue was then washed 3 times with warm saline, a single ligature was placed upstream of the thrombus using a 8-0 monofilament polypropylene suture to prevent embolization, and the leg was sutured closed. Mice were euthanized at various time points, and the saphenous neurovascular bundle was removed and fixed overnight in 4% paraformaldehyde and paraffin embedded. Five-micrometer sections were cut and hematoxylin and eosin (H&E) stained to visualize the presence of a thrombus under light microscopy. Vessels were sectioned through, and those sections showing the greatest area of occlusion were chosen for analysis. Such sections typically occurred near the center of the injured vessel. Images were analyzed using ImageJ software to calculate the percentage of the vessel lumen that remained occluded by a thrombus.

Low-Dose LPS Treatment

Wild-type and $p16^{INK4a}$ transgenic mice were treated with 2 mg/kg intraperitoneal injection of LPS (L3012, Sigma-Aldrich). At various times (1, 3, and 5 hours), the mice (n=5 per genotype each time point) were anesthetized, and 1 mL of blood was collected from the inferior vena cava into 3.8% sodium citrate at a ratio of 1:9 using a 25-gauge needle. Whole blood was spun at 4000*g* for 15 minutes, and the platelet-poor plasma was collected and stored at −80°C until it was analyzed.

Bone Marrow Transplantation

This procedure was performed as described previously.[21] Briefly, mice were irradiated using a cesium-137 irradiator (JL Shepherd, San Fernando, CA) with a total of 11 Gy (2 doses of 550 rad, with a 4-hour rest) to abolish endogenous hematopoietic cells. Bone marrow cells were isolated from donor mice,[21] and 1×10^7 cells were injected (100 μL) into the retroorbital sinus. Four weeks after irradiation, recipient mice underwent $FeCl_3$ injury to the saphenous vein to determine vascular occlusion times. At termination, recipient mouse bone marrow was genotyped to verify successful repopulation of donor cells by polymerase chain reaction. Expression of $p16^{INK4a}$ was compared with an interleukin-2 loading control.

Statistics

All statistical analyses were performed with GraphPad Prism. All measurements are represented as the mean±SEM. One-way ANOVA or Student *t* test was performed where indicated. Values of $P<0.05$ were considered statistically significant.

Results

$p16^{INK4a}$ Transgenic and Wild-Type Mice Respond Similarly in a Hemostasis Model

To determine the contribution of $p16^{INK4a}$ overexpression to potential hemostatic defects, mice initially underwent a model of saphenous vein hemostasis. No difference was observed in the number of hemostatic clots formed over 20 minutes between transgenic mice (25.8±3.4) and wild-type mice (25.8±2.1, Figure 1A) or in the average time to hemostasis (33.5±3.7 and 36.6±2.6 seconds, respectively, Figure 1B). Furthermore, no significant differences were observed in body weight, venous blood flow velocity, plasma prothrombin time, and complete blood count between the 2 groups of mice (Supplemental Table I). These results suggest that there is no obvious physical or hematologic phenotype in the $p16^{INK4a}$ transgenic mice at the ages studied.

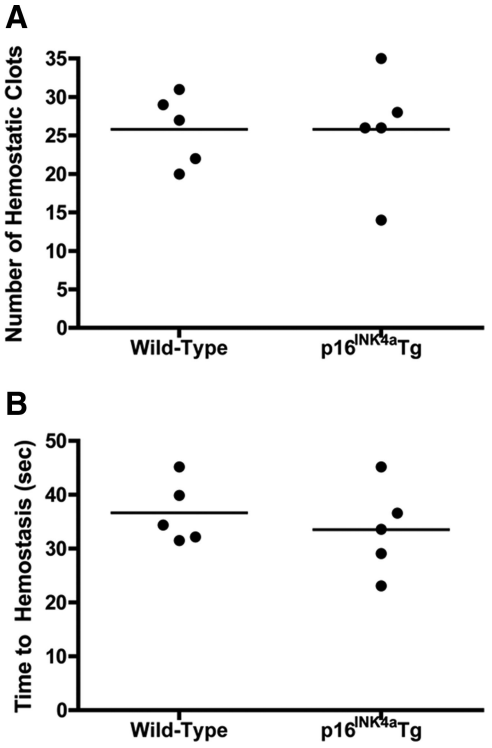

Figure 1. Hemostatic parameters in saphenous vein hemostasis model. Hemostatic measurements were compared between wild-type and p16^{INK4a} transgenic (Tg) mice following blunt injury to the saphenous vein. A, The number of hemostatic clots formed. B, The average time to stoppage of bleeding following serial clot disruption over 30 minutes. Data are not statistically significant.

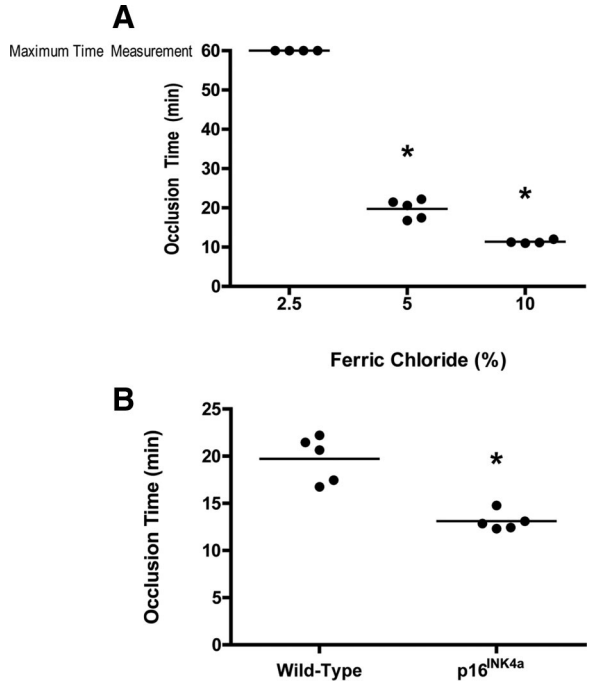

Figure 2. Overexpression of p16^{INK4a} decreases time to occlusion in $FeCl_3$ vascular injury model. A, Wild-type mice were subjected to increasing doses of $FeCl_3$ to the saphenous vein to determine a dose-dependent effect on the occlusion time, as described in Materials and Methods. *$P<0.01$ versus 2.5% $FeCl_3$. B, Vascular occlusion times were compared between wild-type and p16^{INK4a} transgenic mice after 5% $FeCl_3$ injury to the saphenous vein. The occlusion time represents the amount of time required to form an occlusive thrombus, as described in Materials and Methods. *$P<0.01$ versus wild-type control by the Student *t* test.

p16^{INK4a} Transgenic Mice Display a Prothrombotic Phenotype in an $FeCl_3$ Injury Model

$FeCl_3$ injury is a well-established mechanism for inducing thrombus formation in vivo.[22–24] We first demonstrated a dose-dependent effect of $FeCl_3$ on the occlusion time in the saphenous vein, exposing wild-type mice to 2.5%, 5%, and 10% $FeCl_3$ injuries to the saphenous vein (Figure 2A).

Wild-type and p16^{INK4a} transgenic mice were then subjected to $FeCl_3$ (5%) injury to the saphenous vein. The p16^{INK4a} transgenic mice showed a significantly shorter time to occlusion (13.1±0.4 minutes) compared with wild-type mice (19.7±1.1 minute, Figure 2B). Furthermore, the time to flow restriction was also measured. The p16^{INK4a} transgenic mice demonstrated shorter times to flow restriction (6.4±0.91) compared with wild-type controls (8.7±0.54, $P<0.05$, data not shown). These results indicate that overexpression of p16^{INK4a} results in a prothrombotic phenotype following vascular injury.

p16^{INK4a} Transgenic Mice Display a Prothrombotic Phenotype in a Photochemical Injury Model

The excitation of Rose Bengal to induce photochemical injury is another well-established mechanism for inducing thrombus formation in vivo.[25–27] On photochemical injury to the saphenous vein, p16^{INK4a} transgenic mice displayed a significantly shorter time to occlusion (12.7±2.0 minutes) compared with wild-type mice (18.6±1.9 minutes, Figure 3). These results suggest that the prothrombotic phenotype in mice overexpressing p16^{INK4a} can be recapitulated in other vascular injury models.

p16^{INK4a} Transgenic Mice Exhibit Delayed Thrombus Resolution

Venous thrombosis is characterized by the presence of unresolved thrombi in the lower extremities. To study the effect of

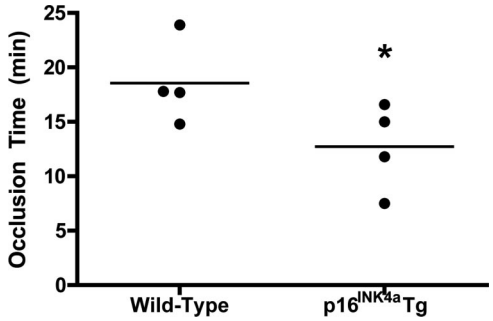

Figure 3. p16INK4a transgenic (Tg) mice have decreased time to occlusion in Rose Bengal photochemical vascular injury model. Vascular occlusion times between wild-type and p16^{INK4a} transgenic mice were compared following saphenous vein injury with 75 mg/kg Rose Bengal excited with 1.75 mW green light at 540 nm. Occlusion times represent the amount of time required to form an occlusive thrombus. *$P<0.05$ versus wild-type control by the Student *t* test.

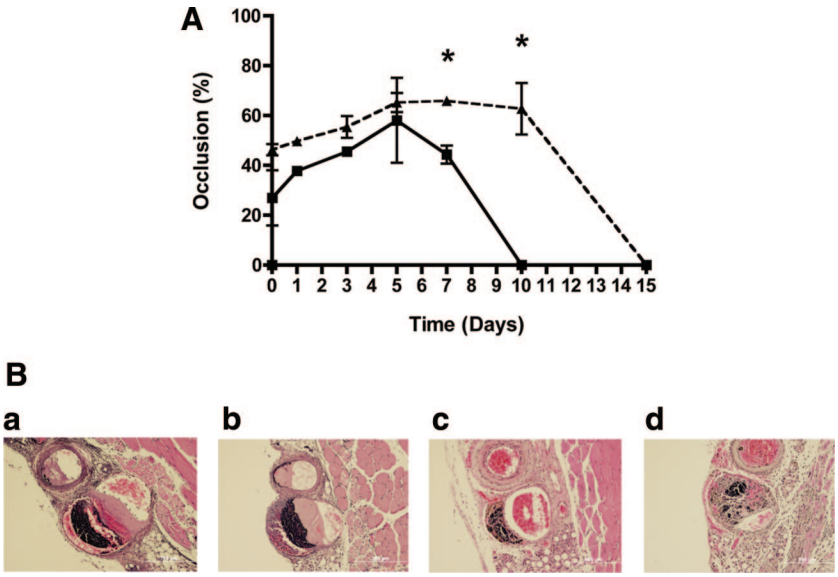

Figure 4. p16^{INK4a} transgenic mice display defective thrombus resolution. A, Thrombus resolution was measured over time after 10% $FeCl_3$ injury to the saphenous vein and stasis induced by ligation as described in the Materials and Methods. *$P<0.05$ versus the respective wild-type control by the Student *t* test. ■, wild-type; ▲, p16^{INK4a} transgenic. B, Representative histological images were analyzed using ImageJ software to determine the percentage of occlusion (plotted in A). a, Wild-type at 1 day; b, p16 transgenic at 1 day; c, wild-type at 10 days; d, p16 transgenic at 10 days.

overexpressing p16^{INK4a} on thrombus resolution, thrombi formed post-$FeCl_3$ injury in wild-type and p16^{INK4a} transgenic mice were monitored over time. Mice were euthanized from 1 hour to 15 days post-$FeCl_3$ injury and vascular ligation. No significant differences in thrombus resolution were observed until 7 days after vascular injury. By 10 days postinjury, all wild-type mice exhibited complete thrombus resolution, whereas p16^{INK4a} transgenic mice maintained an average of 60% vessel occlusion. p16^{INK4a} transgenic mice required additional time postinjury for thrombus resolution relative to wild-type controls (Figure 4A). Representative images show little difference in percentage of occlusion at 1 day (Figure 4B) between p16^{INK4a} transgenic and wild-type mice. Black staining represents $FeCl_3$ trapped within the thrombus. At 10 days, we observed that residual $FeCl_3$ was mostly contained within inflammatory macrophages and was present in the perivascular space of wild-type mice. However, residual $FeCl_3$ contained within macrophages was still present in the intravascular space of p16^{INK4a} transgenic mice at 10 days (Figure 4B). These results demonstrate a defect in thrombus resolution with p16^{INK4a} overexpression.

p16^{INK4a} Transgenic Mice Display Enhanced Thrombin Generation in Response to LPS Challenge

Chronic inflammation and endothelial dysfunction have been linked to enhanced thrombin generation and the risk of venous thrombosis.[28–30] LPS is known to activate the vascular endothelium and promote the formation of spontaneous thrombi.[31–34] To study the effects of inflammation-induced coagulation, we exposed p16^{INK4a} transgenic and wild-type mice to low-dose LPS. When analyzed by calibrated automated thrombography, plasma from the p16^{INK4a} transgenic mice showed a significantly shorter lag time to initiation of thrombin generation and time to peak amount of thrombin generated at all time points post–LPS treatment (Table). The peak amount of thrombin generated was significantly higher in p16^{INK4a} transgenic mice 3 and 5 hours after LPS treatment (Table). The observed differences in thrombin generation demonstrates p16^{INK4a} transgenic mice are able to generate more thrombin and have a prothrombotic phenotype when challenged with LPS.

p16^{INK4a} Expression in Hematopoietic Cells Contributes to the Observed Prothrombotic Phenotype

To determine the relative contribution of p16^{INK4a} expression in the hematopoietic cell compartment to the observed prothrombotic phenotype, bone marrow transplants were performed between transgenic and wild-type mice. Following transplantation and recovery, mice were subjected to 10% $FeCl_3$ injury to the saphenous vein. Consistent with our previous results (Figure 2B), transgenic mice receiving transgenic bone marrow had retained their significantly reduced occlusion time (8.4±0.48 minutes) when compared with

Table. Thrombin Generation in Wild-Type vs p16^{INK4a} Transgenic Mouse Plasma after LPS Treatment

	Lag Time (minutes)		Peak Height (nM)		Time to Peak (minutes)	
Time (hours)	WT	p16^{INK4a} Tg	WT	p16^{INK4a} Tg	WT	p16^{INK4a} Tg
0	2.17±0.19	2.33±0.24	46.16±0.53	49.15±1.98	5.33±0.87	5.21±0.62
1	3.4±0.16	1.85±0.12*	45.44±9.45	44.21±1.3	6.19±0.27	4.52±0.14*
3	2.85±0.11	1.85±0.09*	36.44±0.43	51.26±0.63*	6.07±0.16	4.41±0.16*
5	2.01±0.17	2.51±0.19*	32.06±0.46	39.55±0.67*	5.18±0.23	5.52±0.11*

Thrombin generation in plasma from mice treated with 2 mg/kg LPS was measured using calibrated automated thrombography, as described in the Supplemental Materials and Methods. Data represent experiments performed in duplicate with 5 mice per group per time point. WT indicates wild-type; Tg, transgenic.

*$P<0.05$.

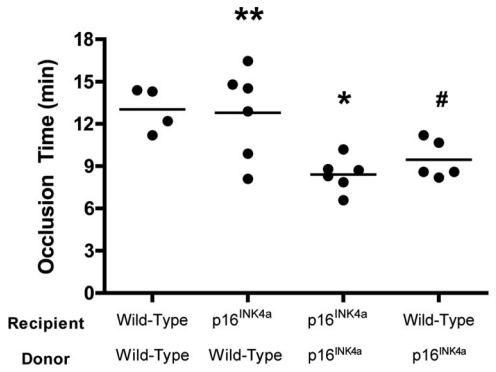

Figure 5. Vascular occlusion times are altered by bone marrow transplantation. Vascular occlusion times were compared between cohorts of bone marrow–transplanted mice following injury of the saphenous vein with 10% $FeCl_3$. Occlusion time represents the amount of time required to form an occlusive thrombus. *$P<0.05$ versus wild-type control cohort; **$P<0.05$ versus transgenic control cohort; #$P=0.08$ versus wild-type control cohort. Statistical relevance was determined by 1-way ANOVA with the Tukey post hoc analysis.

wild-type mice receiving wild-type bone marrow (13.0±0.79 minutes, Figure 5). Interestingly, transgenic mice receiving wild-type bone marrow displayed occlusion times similar to those of wild-type mice (12.8±1.3 minutes), whereas wild-type mice receiving transgenic bone marrow displayed occlusion times similar to those of transgenic mice (9.5±0.61 minutes, Figure 5). Polymerase chain reaction results confirmed successful reconstitution by donor bone marrow cells (Supplemental Figure III). These results demonstrate that the effects of $p16^{INK4a}$ overexpression are, at least in part, mediated by hematopoietic cells.

Discussion

Senescence is a complex process that is thought to contribute to cardiovascular pathologies associated with aging. Despite several reviews describing prothrombotic changes in senescent vascular endothelial cells,[35,36] no studies have described a venous thrombotic phenotype in mice overexpressing senescence-promoting genes. In the current study, we examined parameters that define venous thrombotic potential in a mouse model of premature senescence through transgenic overexpression of the cell cycle inhibitor $p16^{INK4a}$. As expected, the $p16^{INK4a}$ transgenic mouse exhibited increased expression of $p16^{INK4a}$ mRNA by real-time polymerase chain reaction analysis in all tissues tested. Mice overexpressing $p16^{INK4a}$ exhibit normal basal hemostatic parameters as tested by complete blood count, prothrombin time, and an in vivo hemostasis model. This suggests that in the absence of vascular injury, overexpression of $p16^{INK4a}$ has no overt hemostatic consequences. However, on challenge in various vascular injury models, the $p16^{INK4a}$ transgenic mice displayed an obvious prothrombotic response.

We have demonstrated a prothrombotic phenotype using 2 different vascular injury models. Exposure of the vessel to $FeCl_3$ is a type of biochemical injury that results in endothelial denudation and exposure of the subendothelium following lipid peroxidation.[37] This type of oxidative damage produces thrombi that are rich in platelets but also contain red blood cells both encased in a dense fibrin meshwork, indicating a role for soluble plasma factors driving thrombus formation.[38–40] Rose Bengal is a fluorescein-based chemical that is excited to produce reactive oxygen species when exposed to green light at 540 nm. This results in endothelial activation, although there is very little denudation, and is accompanied by rapid platelet adhesion. Thrombi in this model are composed primarily of platelets and contain less fibrin, implying that this process is mostly platelet driven.[38] The observation that $p16^{INK4a}$ transgenic mice exhibit shorter occlusion times in both of these models suggests that there is likely a contribution by both soluble plasma factors and circulating cells. The differences observed in the time to flow restriction may reflect altered rates of thrombus growth between wild-type and $p16^{INK4a}$ transgenic mice, which could be indicative of the potential to produce larger thrombi.

In addition to more rapid rates of venous occlusion, $p16^{INK4a}$ transgenic mice also display impaired thrombus resolution. The percentage of occlusion appears to be correlated with the sustaining of inflammatory infiltration. It is possible that the inability to clear residual $FeCl_3$ from the intravascular space could be involved in further promoting thrombus formation. The increased production of PAI-1 observed in $p16^{INK4a}$ transgenic mice could also partly explain the thrombus resolution defect (Supplemental Figure II). Evidence in the literature suggests increased circulating PAI-1 could have a negative impact on wound healing and fibrinolysis. Originally, Farrehi et al demonstrated enhanced fibrinolysis in PAI-1-deficient mice.[41] Eitzman et al found that transgenic mice overexpressing PAI-1 have more severe fibrosis following bleomycin-induced lung injury.[42] Zaman et al showed a profibrotic effect of PAI-1 overexpression in the heart following myocardial infarction.[43] Recently, McDonald et al demonstrated that aged mice display impaired thrombus resolution following stasis induced by inferior vena cava ligation.[44] In addition, they reported differences in various plasma and venous endothelium-associated proteins between aged and young wild-type mice.[44] Although an exact mechanism to account for the observed thrombus resolution defect in the aged mice is not yet known,[44] it is possible that changes in both the vessel wall and soluble plasma factors contribute, which may also be true of $p16^{INK4a}$ overexpressing mice.

To better understand differences in thrombus formation between $p16^{INK4a}$ transgenic and wild-type mice, coagulation parameters in mouse plasma samples were analyzed by calibrated automated thrombography after inducing endothelial dysfunction with LPS. Plasma analysis by calibrated automated thrombography is sensitive to changes in coagulation factor levels[45] and able to detect differences in thrombin generation parameters following a thrombotic event in human patients.[46] Our results show that $p16^{INK4a}$ transgenic mice are able to initiate thrombin generation faster, achieve a higher peak amount of thrombin, and peak at a faster rate than wild-type controls. Therefore, $p16^{INK4a}$ transgenic mice exhibit greater thrombin generation after LPS challenge compared with wild-type controls. To complement these data, $p16^{INK4a}$ transgenic mice also showed elevated plasma levels of thrombin-antithrombin and PAI-1 following LPS challenge. These markers are commonly used to measure activa-

tion of coagulation (thrombin-antithrombin)[33,47] and endothelial activation (PAI-1).[48,49] Yamamoto et al showed that aged mice had elevated induction of PAI-1 compared with young mice after LPS treatment, suggesting that PAI-1 is important in endotoxin-induced thrombosis.[31] Because PAI-1 is both a marker of endothelial cell senescence and a potent fibrinolysis inhibitor,[50] it could also participate in the delayed thrombus resolution seen in $p16^{INK4a}$ transgenic mice.

To begin establishing a mechanism for the observed differences between wild-type and $p16^{INK4a}$ transgenic mice, bone marrow transplants were performed to determine the contribution of hematopoietic cells to the prothrombotic phenotype. We found that wild-type mice given $p16^{INK4a}$ transgenic bone marrow had occlusion times very similar to those of transgenic controls. Similarly, $p16^{INK4a}$ transgenic mice given wild-type bone marrow had occlusion times very similar to those of wild-type controls. These data show that the prothrombotic phenotype observed in mice overexpressing this gene is attributed to $p16^{INK4a}$ expression in hematopoietic cells.

A growing body of in vitro evidence suggests that senescence in the vascular endothelium may also participate in the transition to a procoagulant state during aging. Senescence in the vascular endothelium is associated with an array of phenotypic changes with pathological consequences.[7,8] These changes include upregulation of PAI-1, inflammatory cytokines (including interleukin-1α and interleukin-6), matrix metalloproteinases, and the downregulation of endothelial nitric oxide synthase.[35,36,51] Thus, a role for the endothelium cannot be discounted and may warrant further investigation in this model.

In contradistinction to the present data supporting a role for $p16^{INK4a}$ in venous thrombosis, a differing role for the expression of $p16^{INK4a}$ in arterial vascular diseases has been suggested. Through genome-wide association studies, several groups have found a link between single-nucleotide polymorphisms on chromosome 9p21.3 close to the $p16^{INK4a}$ open-reading frame and several atherosclerotic diseases (coronary artery disease, ischemic stroke, abdominal aortic aneurysm).[52] Liu et al have recently shown that individuals harboring the single-nucleotide polymorphism genotypes associated with increased atherosclerotic risk exhibit decreased expression of $p16^{INK4a}$ and other *INK4/ARF* transcripts.[53] Individuals at increased risk appear to differ in the expression and splicing of linear and circular forms of *ANRIL*, a long, noncoding RNA emanating from the *INK4a/ARF* locus thought to participate in *INK4a/ARF* expression.[54] This observation suggests that decreased production of $p16^{INK4a}$ is associated with an increased risk of atherosclerosis, likely through limiting aberrant or excess proliferation of cellular components of atheromatous plaques. This suggests that expression of antiproliferative molecules at the *INK4a/ARF* locus protects individuals from atherosclerosis.[52,55] In accord with this view, mice lacking $p16^{INK4a}$ have been shown to be more prone to vessel occlusion in a carotid artery injury model.[56] Our current data, combined with prior work in the venous system,[35,36] suggest the intriguing possibility that age-induced $p16^{INK4a}$ expression and cellular senescence might play opposing roles with regard to thrombosis and atherosclerosis in the venous and arterial systems, respectively.

Characterizing the link between age-related genetic changes and age-related cardiovascular diseases, such as venous thrombosis, is of paramount importance. Overexpression of proteins such as $p16^{INK4a}$, which promote senescence and vascular dysfunction, could be the key age-related genetic change explaining cardiovascular maladies. Together, our results demonstrate that $p16^{INK4a}$ overexpression and cellular senescence contribute to a prothrombotic phenotype and defective thrombus resolution. The results of this study provide the foundation for research on the effects of vascular senescence on venous thrombosis.

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Disclosures

None.

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