Role of Coagulation Factors in Cerebral Venous Sinus and Cerebral Microvascular Thrombosis

Mutsumi Nagai, MD, PhD
Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana

Cigdem Erkuran Yilmaz, MD, PhD
Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana

Daniel Kirchhofer, PhD
Department of Protein Engineering, Genentech, Inc, South San Francisco, California

Charles T. Esmon, PhD
Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation; Departments of Pathology and Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center; and Howard Hughes Medical Institute, Oklahoma City, Oklahoma

Nigel Mackman, PhD
Division of Hematology/Oncology, Department of Medicine, University of North Carolina, Chapel Hill, North Carolina

D. Neil Granger, PhD
Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana

Reprint requests:
D. Neil Granger, PhD, Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130.
E-mail:dgrang@lsuhsc.edu

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OBJECTIVE: The objective of this study was to define the relative contributions of three major pro- and anti-coagulation pathways (heparin-antithrombin, protein C, and tissue factor (TF)) in the thrombogenic responses that occur in large and small vessels of the brain.

METHODS: Cerebral venous sinus thrombosis was induced by topical application of $FeCl_3$ on the superior sagittal sinus, while photoactivation of fluorescein was used to induce thrombus formation in cerebral microvessels. Heparin, activated protein C (APC), and antibodies to either APC or TF were used to assess thrombogenesis in wild-type mice. Mutant mice that overexpress the endothelial protein C receptor (EPCR-tg) or with TF deficiency in Tie2-expressing endothelial cells (LTFE) were also used.

RESULTS: Thrombus formation in the superior sagittal sinus of wild-type mice was attenuated by heparin and in EPCR-tg mice, while treatment with the APC antibodies enhanced thrombogenesis. Arteriolar thrombosis was largely unresponsive to the interventions studied. However, in cerebral venules, thrombosis was inhibited by heparin and in EPCR-tg mice. TF antibody treatment also inhibited venular thrombosis, with a similar attenuation noted in LTFE mice.

CONCLUSION: Thrombin promotes while the APC pathway blunts thrombus formation in an experimental model of cerebral venous sinus thrombosis. TF involvement is more evident in cerebral microvascular thrombogenesis, with endothelial cell–associated TF mediating this response in venules, but not arterioles.

KEY WORDS: Activated protein C, Brain, Heparin, Thrombosis, Tissue factor

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Cerebrovascular occlusion attributable to thrombosis is a major cause of morbidity and mortality.[1] Consequently, thrombus formation in large (cerebral venous sinus) and small vessels (cerebral venule and arteriole) of the brain has been studied to better understand the underlying pathophysiology and to improve treatment options for conditions such as thrombotic stroke, focal cortical infarctions, and cerebral venous sinus thrombosis (CVST). A variety of different experimental approaches have been used to elicit thrombus formation in large and small blood vessels.[2-4] Two widely used methods are based on producing vessel injury by topical application of a potent oxidizing agent ($FeCl_3$) on the vessel surface or by focal activation of a photosensitive dye or fluorochrome within the vessel lumen.[5,6] These models have been used to demonstrate the efficacy of tissue plasminogen activator and other drugs (eg, aspirin) in the dissolution of existing thrombi or in the inhibition of clot formation. However, the relative contributions of different components of the coagulation and anticoagulation pathways to thrombus formation in the cerebral vasculature remain poorly understood.

ABBREVIATIONS: Ab, antibody; **APC**, activated protein C; **CVST**, cerebral venous sinus thrombosis; **EPCR**, endothelial protein C receptor; **FITC**, fluorescein isothiocyanate; **ICV**, inferior cerebral vein; **LTFE**, low-tissue factor-expressing; **SSS**, superior sagittal sinus; **TF**, tissue factor; **tg**, transgenic; **TM**, thrombomodulin; **WI**, water immersion; **WT**, wild-type

The heparin-antithrombin system, the protein C pathway, and the tissue factor (TF) pathway inhibitor system represent the 3 major anticoagulant mechanisms that function to prevent thrombosis.[7,8] Down-regulation of these natural anticoagulant mechanisms has been invoked to explain the thrombus formation that accompanies different disease states, including

CVST and stroke.[9,10] For example, activated protein C (APC) resistance may explain the increased risk for thrombus formation in both CVST and stroke in children.[9,11] Although heparin is widely used in the treatment of CVST, the contribution of this anticoagulant, as well as APC and TF, to thrombus formation in either large or small blood vessels of the brain has not been previously evaluated.

The objective of this study was to define the relative contributions of 3 major pro- and anticoagulation pathways (heparin-antithrombin system, protein C pathway, and TF pathway) in the thrombogenic responses that occur in large and small vessels of the brain. $FeCl_3$ and fluorescein isothiocyanate (FITC) photoactivation were used to induce thrombus formation in the superior sagittal sinus (SSS) and cerebral microvessels, respectively. The findings of this study reveal differing roles for coagulation-anticoagulation mechanisms in mediating thrombosis between large and small vessels and between arterioles and venules.

MATERIALS AND METHODS

Mice

Male C57BL/6 (wild-type [WT] control strain) mice (Jackson Laboratory, Bar Harbor, ME), transgenic mice overexpressing the endothelial protein C receptor (EPCR-tg) (Oklahoma Medical Research Foundation, Oklahoma City, OK), and mutant mice that were TF-deficient in Tie2-expressing cells (LTFE) and that did not contain TF in endothelium and hematopoietic cells (not published), or TF floxed control ($Cre^{-/-}$) mice were used. The EPCR-tg and the LTFE were backcrossed onto C57BL/6 mice. A total of 76 WT (weight, 25.0 ± 0.3 g), 19 EPCR-tg (weight, 25.8 ± 0.8 g), 7 LTFE-tg (weight, 30.6 ± 1.0 g), and 6 $Cre^{-/-}$ (weight, 30.1 ± 0.8 g) mice were used. All mice were housed under specific pathogen-free conditions in standard cages and fed standard laboratory chow and water. The experimental procedures used were reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center and performed according to the criteria outlined by the National Institutes of Health.

Animal Preparation

Mice were anesthetized with intraperitoneally administered pentobarbital (50 mg/kg), with supplemental doses (12.5 mg/kg) given as needed. The left femoral vein was cannulated for intravenous administration of FITC-dextran. Body temperature was maintained at 36.5° to 37.0°C during the experiment with a homeothermic blanket and monitored with a rectal temperature probe. The head of each mouse was fixed on the acrylic frame before the cranial window was created. After skull fixation, a circular skin incision was made, and a craniectomy was created 3 mm lateral and 2 mm posterior to the bregma. The exposed brain tissue was immersed in artificial cerebrospinal fluid and covered with a glass slide. Cerebral vessels were observed through the dura mater.

Intravital Videomicroscopy

The mouse was moved onto the stage of an upright fluorescent microscope (Optiphot; Nikon, Tokyo, Japan) with a ×20 water immersion objective lens (20 × 0.40 WI [water immersion]; Nikon). The microscopic image was projected onto a monitor (PVM-2030; Sony, Tokyo, Japan) through a 3–charge-coupled device video camera (DXC-390; Sony) and recorded using a digital video disk recorder (SR-MV50; JVC, Wayne, NJ). A video timer (Time-Date Generator WJ-810; Panasonic, Tokyo, Japan) was connected to the monitor to record time and date. The diameters of the brain vessels were measured by video analysis software (Image J 1.37v; National Institutes of Health, public domain software) on a personal computer (G5 Macintosh; Apple, Cupertino, CA).

Light/Dye–Induced Thrombosis

The procedure used to induce microvascular thrombosis by the light/dye method is described elsewhere.[3,12,13] Briefly, after the preparation was stabilized, 10 mL/kg of 5% FITC-dextran (excitation, 495 nm; emission, 519 nm; 150 000 molecular weight; Sigma Chemical Co, St Louis, MO) was slowly injected intravenously. It was allowed to circulate for 10 minutes, and then venules and arterioles with diameters ranging between 30 to 45 μm were selected for study. A 100-μm length of vessel was epi-illuminated using a 175-W xenon lamp (Lambda LS, Sutter, CA) and a fluorescein filter cube (DM510 B-2A; Nikon). The average excitation power density was 0.5 W/cm^2. Epi-illumination was continuously applied to the vessels, and thrombus formation was quantified by determining the time required for complete flow cessation for more than 60 seconds (flow cessation time). One venule and 1 arteriole were subjected to thrombosis using this method.

SSS Thrombosis

After anesthesia and fixation of the skull (as described above), an elliptical skin incision was made to produce a narrow rectangular cranial window along the SSS (8 mm) from the confluence of sinuses to a point just posterior to the inferior cerebral vein (ICV). Care was taken to avoid injury to the SSS and to minimize overheating brain tissue while drilling through the cranium. The exposed SSS was kept moist with phosphate-buffered saline. Next, 50 μL of 1% FITC-dextran (150 000 molecular weight; Sigma Chemical Co) was administered intravenously. Ten minutes later, a 1-mm^2 square filter paper soaked with 40% $FeCl_3$ was placed (using an operating microscope) on the anterior part of the SSS, where it runs anterior to the ICV. The filter paper was covered with a plastic film to avoid evaporation of the $FeCl_3$ solution. Upon placing the $FeCl_3$ on the SSS, the flow of blood in this vessel was observed using fluorescence intravital videomicroscopy to ensure occlusion of the anterior segment of the SSS. This procedure was needed because the ICV is well developed in mice, and blood flow from the SSS drains into the ICV only when the posterior part of the SSS is occluded. The microscope, video monitor, and recording system used to observe the SSS were identical to those described above except for the objective lens (10 × 0.25, Spencer, Buffalo, NY), light path (including neutral density filter), and video camera (C2400; Hamamatsu, Hamamatsu City, Japan). Once the anterior SSS was occluded, a 1-mm^2 square filter paper soaked with 10% $FeCl_3$ was placed on the posterior part of SSS, ie, anterior to the confluence of the cerebral venous sinuses. A timer was activated to measure the time to flow cessation, which could be discerned from the SSS at the edges of the filter paper. The time to flow cessation was determined when flow in the SSS ceased for a period of more than 60 seconds.

Experimental Protocols

To determine whether heparin, APC, and TF contribute to small and large vessel thrombosis in the brain, the following experimental groups were studied using the photoactivation (arterioles and venules) and $FeCl_3$ (SSS) thrombosis models: (1) control WT mice; (2) WT mice receiving

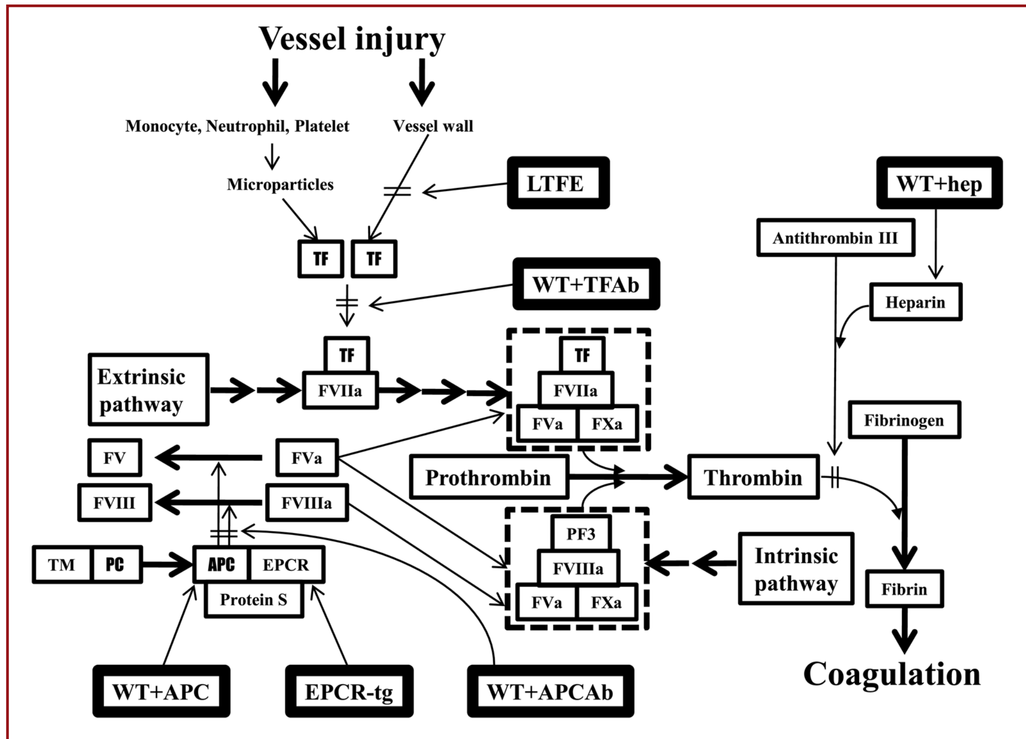

FIGURE 1. *Diagram showing the targets of action of genetic and pharmacologic interventions used to probe the role of components of the coagulation and anticoagulation pathways. Tissue factor (TF), activated factor VII (VIIa), activated factor V (FVa), and activated factor X (FXa) complex is derived from the extrinsic pathway, while platelet factor 3 (PF3), activated factor VIII (FVIIIa), FVa, and FXa complex are derived from the intrinsic pathway. These complexes (prothombin-activating complex [PAC], surrounded by broken-line square) generate thrombin from prothrombin. TF acts as an initial activator after vessel injury after binding FVIIa. Low-TF-expressing (LTFE) mice do not contain TF in endothelium and hematopoietic cells. Protein C (PC) is activated through the interactions of thrombomodulin (TM), protein S, and endothelial protein C receptor (EPCR) binding. Activated protein C (APC) inactivates FVa and FVIIIa, which is a component of PAC. Heparin (hep) acts as an enhancer of antithrombin, which blocks both thrombin activity and other coagulation factors to generate fibrin. WT, wild-type; tg, transgenic; Ab, antibody.*

100 IU/kg[14] of heparin (heparin sodium; Abraxis Bioscience, Melrose Park, IL) 10 minutes before thrombus induction (WT + heparin); (3) WT mice receiving 10 μg/mouse[15] of murine APC (Oklahoma Medical Research Foundation) 10 minutes before thrombus induction (WT + APC); (4) WT mice receiving 120 μg/mouse[15] of rat antimouse APC monoclonal antibody (Ab) (MPC1609; Oklahoma Medical Research Foundation) 20 minutes before thrombus induction (WT + APC Ab); (5) EPCR-tg mice; (6) WT mice receiving 20 mg/kg[13] of a rat antimouse TF monoclonal Ab 1H1 (TF Ab)[16] 20 minutes before thrombus induction (WT + TF Ab); (7) LTFE mice; and (8) $Cre^{-/-}$ mice. In some experiments, we confirmed that blood pressure and blood gases were not affected by each procedure. Figure 1 illustrates the specific sites within the coagulation/anticoagulation pathways that were targeted by our pharmacologic and genetic interventions.

Statistical Analysis

Each group was compared with its WT control counterpart. Statistical difference was determined by a 2-tailed *t* test. All analyses were performed using Statview software 4.5 (Abacus Concepts, Inc, Piscataway, NJ). All values are expressed as means ± standard error, and statistical significance was set at $P < .05$.

RESULTS

Figure 2A presents an image of a cranial window preparation, illustrating the location of the filter paper used to occlude the anterior and posterior segments of the SSS. Angiograms derived from each vessel segment are shown in Figure 2, B and C. Flow cessation in the SSS was readily apparent in this experimental model of CVST. Similarly, photoactivation-induced thrombus formation in cerebral arterioles and venules was readily discerned using intravital videomicroscopy. The average diameters of the vessels exposed to thrombus formation in this study were: 453.2 ± 4.8 μm, 32.7 ± 0.6 μm, and 35.1 ± 0.4 μm for SSS, arterioles, and venules, respectively.

Figure 3 summarizes the effects of heparin treatment on thrombus formation in the SSS and in cerebral venules. Heparin significantly prolonged the time to flow cessation in both the macroscopic and microscopic venous vessels. Arterioles were not influenced by heparin treatment (data not shown).

Figure 4 shows the responses of the SSS and cerebral venules to thrombus formation under conditions of altered APC availability. Administration of exogenous murine APC did not alter thrombus formation in the SSS (Fig. 4A), cerebral venules (Fig. 4D), or arterioles (data not shown). Immunoblockade of endogenous APC with an anti-murine APC Ab enhanced the thrombosis response in the SSS (Fig. 4B) but had no effect on either venules (Fig. 4E) or arterioles. Overexpression of EPCR, which enhances the activation of endogenous protein C to APC, significantly prolonged the time to flow cessation in the SSS (Fig. 4C) and cerebral venules (Fig. 4F), but not in arterioles.

The responses of the SSS, venules, and arterioles to thrombosis after treatment with a TF blocking Ab are summarized in Figure 5. The TF Ab significantly prolonged the time to flow cessation in arterioles and venules, but not in the SSS. To assess the contribution of endothelial vs peripheral blood cell–associated TF to the thrombosis response in arterioles and venules, we compared the time to flow cessation between mutant mice expressing low TF in Tie2-positive cells and their control counterparts (Fig. 6).

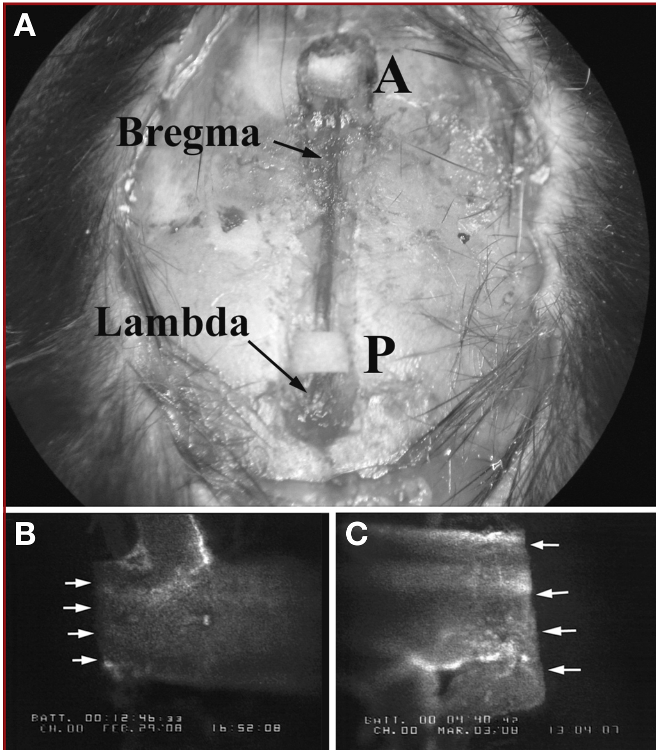

FIGURE 2. *Measurement of thrombosis in the superior sagittal sinus (SSS).* **A**, *intraoperative photograph showing view through the cranial window. P, the $FeCl_3$-soaked filter paper lying on the posterior segment of the SSS; A, the filter paper lying on the anterior segment.* **B**, *angiogram of a flow cessation determination in the posterior segment of the SSS.* **C**, *angiogram of the plugging in the anterior segment of SSS after exposure to 40% $FeCl_3$.*

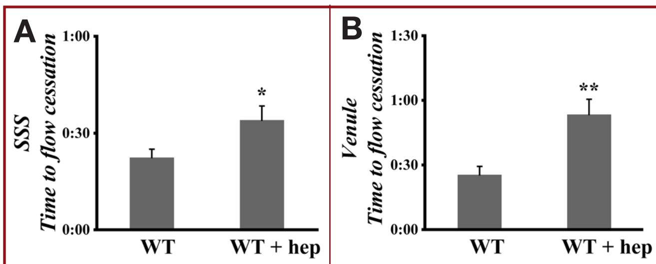

FIGURE 3. *Graphs showing the effects of hep on thrombin formation in the SSS and cerebral venules.* **A**, *WT (n = 10) and WT + hep (n = 9).* **B**, *WT (n = 10) and WT + hep (n = 7). *, P < .05 vs WT; **, P < .01 vs WT.*

These experiments revealed that TF deficiency in endothelial or hematopoietic cells prolonged thrombus formation in venules, but not in arterioles.

DISCUSSION

The goal of this study was to determine whether interventions that target some of the major pro- and anticoagulation pathways alter thrombus formation in large and small blood vessels of the

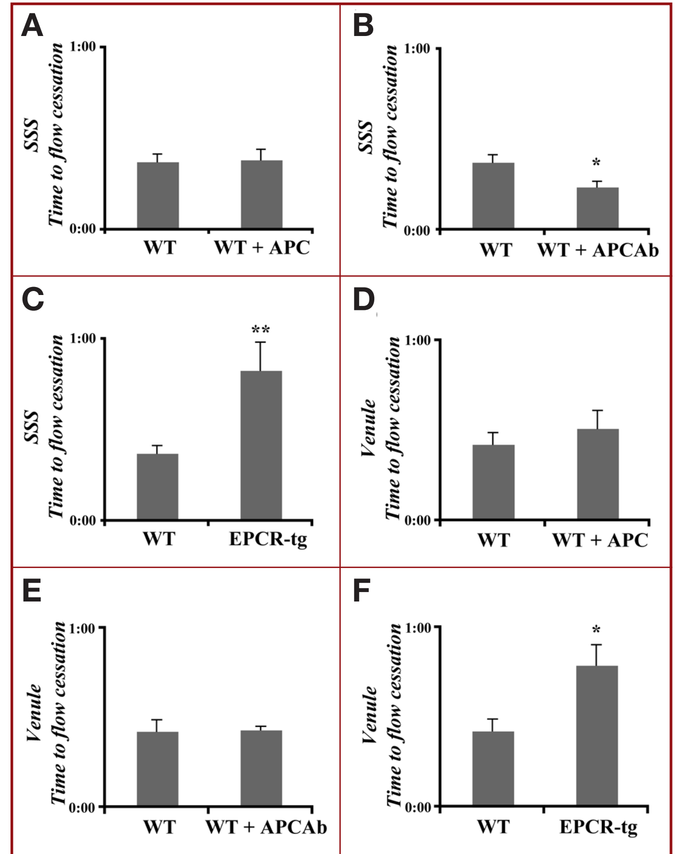

FIGURE 4. *Graphs showing the role of the protein C pathway in thrombus formation within the SSS and cerebral venules.* **A**, **B**, *and* **C**, *WT (n = 10), WT + APC (n = 10), WT + APC Ab (n = 7), and EPCR-tg (n = 8).* **D**, **E**, *and* **F**, *WT (n = 10), WT + APC (n = 7), WT + APC Ab (n = 5), EPCR-tg (n = 11). *, P < .05 vs WT; **, P < .01 vs WT.*

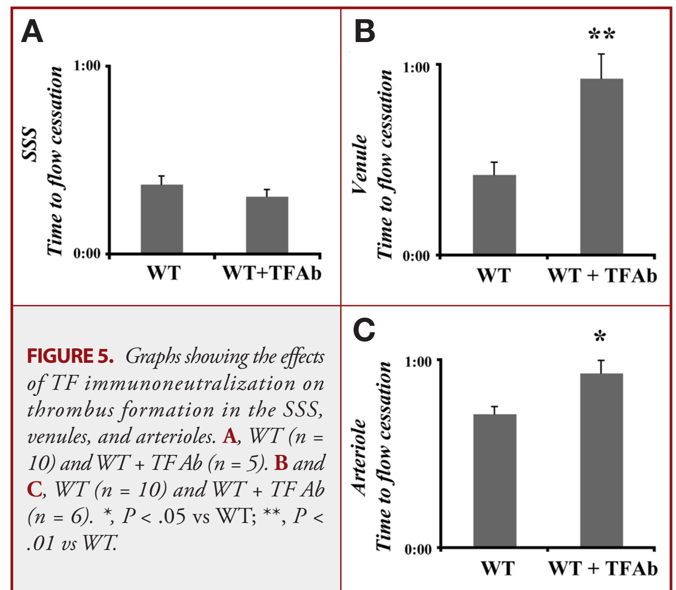

FIGURE 5. *Graphs showing the effects of TF immunoneutralization on thrombus formation in the SSS, venules, and arterioles.* **A**, *WT (n = 10) and WT + TF Ab (n = 5).* **B** *and* **C**, *WT (n = 10) and WT + TF Ab (n = 6). *, P < .05 vs WT; **, P < .01 vs WT.*

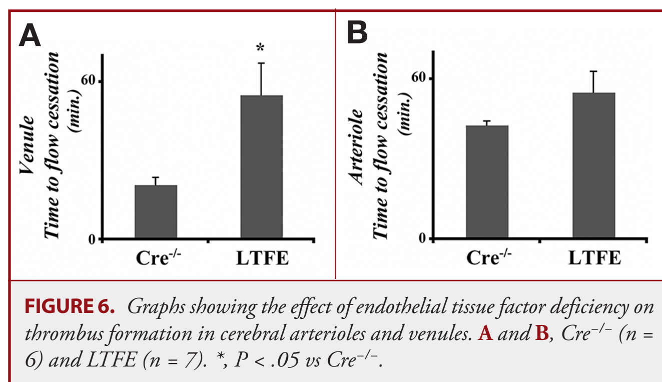

FIGURE 6. *Graphs showing the effect of endothelial tissue factor deficiency on thrombus formation in cerebral arterioles and venules.* **A** *and* **B**, *Cre$^{-/-}$ (n = 6) and LTFE (n = 7). *, P < .05 vs Cre$^{-/-}$.*

TABLE 1. Summary of the Results[a]

Pro- or Anticoagulant System and Model	Time to Flow Cessation		
	Light/dye Model		$FeCl_3$ Model
	Venule	Arteriole	SSS
Heparin-antithrombin system			
WT + hep	↑	→	↑
Protein C pathway			
WT + APC	→	→	→
WT + APC Ab	→	→	↓
EPCR-tg	↑	→	↑
TF pathway			
WT + TF Ab	↑	↑	→
LTFE	↑	→	NE

[a] SSS, superior sagittal sinus; WT, wild-type; hep, heparin; APC, activated protein C; Ab, antibody; EPCR, endothelial protein C receptor; tg, transgenic; TF, tissue factor; LTFE, low-tissue factor-expressing; ↑, prolongation; →, no change; ↓, shortening of time to flow cessation; NE, not examined.

brain. A novel method was developed to simulate the clinical problem of CVST, which involved the assessment of $FeCl_3$-induced thrombus formation in the SSS of WT and mutant mice. This model is relatively easy to perform and allows for in vivo quantification of thrombus formation in veins with a narrow range of diameters. CVST is associated with mixed cell thrombi that are composed of platelets, leukocytes, fibrin, and red blood cells,[17] which can be recapitulated in experimental animals after exposure of vessels to $FeCl_3$.[2,18]

Our findings with the CVST model (summarized in Table 1) reveal that thrombus formation can be significantly delayed by treatment of WT mice with heparin. Heparin is used clinically not only to treat CVST to prevent expansion of the thrombus afterward, but also as prophylaxis against relapse.[10,19-22] Although some reports describe the effect of heparin on recanalization of CVST, no effort has previously been made to assess its effectiveness on thrombus formation in an experimental model of CVST.[14,23] Our positive findings with heparin in experimental CVST support its clinical use and confirm the central role of thrombin in producing the mixed thrombi that are characteristic of CVST- and $FeCl_3$-induced thrombosis.

Our study also provides novel insights into the protective role of the protein C anticoagulant pathway in experimental CVST. This pathway is initiated by thrombin binding to thrombomodulin, with the subsequent activation of protein C by the thrombin- thrombomodulin complex and the EPCR.[7,24] APC interacts with protein S to inactivate factors Va and VIIIa, thereby exerting an anticoagulant effect. Our findings in the experimental CVST model indicate that treatment of WT mice with an APC blocking Ab enhances SSS thrombosis, whereas EPCR-tg mice exhibit an attenuated thrombogenic response. Hence, the availability of endogenous APC appears to be a significant determinant of the rate of thrombus formation in the SSS. This observation may be relevant to clinical CVST in view of reports that describe an association between an impaired protein C pathway and the risk for development of CVST.[10] Our analysis of thrombus formation in microvessels revealed a somewhat different pattern of involvement of the different coagulant/anticoagulant pathways. Cerebral arterioles appeared to be largely unresponsive to the different interventions evaluated, including heparin. Since the APC blocking Ab and overexpression of EPCR have been shown to effectively alter light/dye–induced thrombus formation in arterioles of cremaster muscle,[15] the absence of any change in thrombus formation with these interventions in the present study suggests that cerebral arterioles may not express a robust protein C pathway. This is supported by reports describing low thrombomodulin levels in the brain.[25,26]

Although 2 different methods ($FeCl_3$ and photoactivation) were used to promote thrombus formation in the SSS and cerebral venules, the responses to the different anticoagulant interventions exhibited some significant similarities, ie, both heparin and EPCR overexpression delayed the thrombus development in large and microscopic veins. However, some differences were noted, including an unresponsiveness of cerebral venules to the APC blocking Ab. A similar unresponsiveness of light/dye–induced thrombus formation to APC blocking Ab has been previously described in cremaster muscle venules.[15]

TF, a key mediator of the initiation phase of thrombogenesis,[27,28] activates coagulation by binding to and activating factor VII. TF is mainly produced and expressed by monocytes, neutrophils, platelets, and the vessel wall, including adventitia or media. Microparticles (0.05–1.0 μm) shed from monocytes and platelets are also a rich source of TF that can initiate coagulation. We noted a significant delay in thrombus formation after TF Ab treatment in cerebral arterioles and venules, but not in the SSS. Whether the different role of TF in large and small vessels reflects the levels of expression in the different vessel populations or is the result of the different models ($FeCl_3$ vs photoactivation) used to elicit thrombus formation is unclear. However, a novel finding was the significant difference in thrombus formation between mice that genetically express low levels of TF

in vascular endothelial and hematopoietic cells (LTFE) and their control counterparts with venules, but not with arterioles exhibiting a prolongation of thrombus formation. These results suggest that endothelial cell and/or hematopoietic cell TF likely accounts for the protective effect of the TF immunoneutralization against thrombosis in cerebral venules, while blood cell and/or microparticle–associated TF mainly contributes to the arteriolar thrombosis response. These responses may be unique to the cerebral circulation, since previous reports ascribe a dominant role for vessel wall–associated TF in arterial thrombosis, while blood cell (or microparticle)–associated TF plays a major role in venous thrombosis.[28] Additional work is needed to define the specific cell populations that account for TF-mediated thrombosis in cerebral arterioles.

This study provides the first quantitative estimates of thrombus formation in an experimental model of CVST and provides novel insights into the coagulant/anticoagulant mechanisms that modulate thrombus formation in the SSS and in cerebral arterioles and venules. Our findings reveal differing roles for heparin, APC, and TF in mediating/attenuating thrombosis between large and small vessels, and between arterioles and venules of the brain. These observations may bear on the clinical utility of targeting specific components of the coagulation/anticoagulation pathways for treatment of CVST or the cerebral microvascular thrombosis associated with ischemic stroke.

Disclosures

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REFERENCES

1. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. *Lancet.* 2008;371(9624):1612-1623.
2. Pinel C, Wice SM, Hiebert LM. Orally administered heparins prevent arterial thrombosis in a rat model. *Thromb Haemost.* 2004;91(5):919-926.
3. Rumbaut RE, Slaff DW, Burns AR. Microvascular thrombosis models in venules and arterioles in vivo. *Microcirculation.* 2005;12(3):259-274.
4. Maxwell KA, Dyck RH. Induction of reproducible focal ischemic lesions in neonatal mice by photothrombosis. *Dev Neurosci.* 2005;27(2-4):121-126.
5. Kurz KD, Main BW, Sandusky GE. Rat model of arterial thrombosis induced by ferric chloride. *Thromb Res.* 1990;60(4):269-280.
6. Rosenblum WI, El-Sabban F. Platelet aggregation in the cerebral microcirculation: effect of aspirin and other agents. *Circ Res.* 1977;40(3):320-328.
7. Esmon CT. The interactions between inflammation and coagulation. *Br J Haematol.* 2005;131(4):417-430.
8. Esmon CT. The impact of the inflammatory response on coagulation. *Thromb Res.* 2004;114(5-6):321-327.
9. Boekholdt SM, Kramer MH. Arterial thrombosis and the role of thrombophilia. *Semin Thromb Hemost.* 2007;33(6):588-596.
10. Masuhr F, Mehraein S, Einhäupl K. Cerebral venous and sinus thrombosis. *J Neurol.* 2004;251(1):11-23.
11. Rahemtullah A, Van Cott EM. Hypercoagulation testing in ischemic stroke. *Arch Pathol Lab Med.* 2007;131(6):890-901.
12. Rumbaut RE, Randhawa JK, Smith CW, Burns AR. Mouse cremaster venules are predisposed to light/dye-induced thrombosis independent of wall shear rate, CD18, ICAM-1, or P-selectin. *Microcirculation.* 2004;11(3):239-247.
13. Anthoni C, Russell J, Wood KC, et al. Tissue factor: a mediator of inflammatory cell recruitment, tissue injury, and thrombus formation in experimental colitis. *J Exp Med.* 2007;204(7):1595-1601.
14. Kim DE, Schellingerhout D, Jaffer FA, Weissleder R, Tung CH. Near-infrared fluorescent imaging of cerebral thrombi and blood-brain barrier disruption in a mouse model of cerebral venous sinus thrombosis. J Cereb Blood Flow Metab. 2005;5(2):226-233.
15. Yoshida H, Russell J, Stokes KY, Yilmaz CE, Esmon CT, Granger DN. Role of the protein C pathway in the extraintestinal thrombosis associated with murine colitis. Gastroenterology. 2008;135(3):882-888.
16. Kirchhofer D, Moran P, Bullens S, Peale F, Bunting S. A monoclonal antibody that inhibits mouse tissue factor function. *J Thromb Haemost.* 2005;3(5):1098-1099.
17. Mackman N. Triggers, targets and treatments for thrombosis. *Nature.* 2008; 451(7181):914-918.
18. Lockyer S, Kambayashi J. Demonstration of flow and platelet dependency in a ferric chloride-induced model of thrombosis. *J Cardiovasc Pharmacol.* 1999;33(5):718-725.
19. Masuhr F, Einhäupl K. Treatment of cerebral venous and sinus thrombosis. *Front Neurol Neurosci.* 2008;23:132-143.
20. Ferro JM, Canhão P. Acute treatment of cerebral venous and dural sinus thrombosis. *Curr Treat Options Neurol.* 2008;10(2):126-137.
21. Ferro JM, Canhão P. Complications of cerebral vein and sinus thrombosis. *Front Neurol Neurosci.* 2008;23:161-171.
22. Azin H, Ashjazadeh N. Cerebral venous sinus thrombosis—clinical features, predisposing and prognostic factors. *Acta Neurol Taiwan.* 2008;17(2):82-87.
23. Röttger C, Madlener K, Heil M, et al. Is heparin treatment the optimal management for cerebral venous thrombosis? Effect of abciximab, recombinant tissue plasminogen activator, and enoxaparin in experimentally induced superior sagittal sinus thrombosis. *Stroke.* 2005;36(4):841-846.
24. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. *Blood.* 2007;109(8):3161-3172.
25. Ishii H, Salem HH, Bell CE, Laposata EA, Majerus PW. Thrombomodulin, an endothelial anticoagulant protein, is absent from the human brain. *Blood.* 1986;67(2):362-365.
26. Wong VL, Hofman FM, Ishii H, Fisher M. Regional distribution of thrombomodulin in human brain. *Brain Res.* 1991;556(1):1-5.
27. Mackman N. Role of tissue factor in hemostasis and thrombosis. *Blood Cells Mol Dis.* 2006;36(2):104-107.
28. Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arterioscler Thromb Vasc Biol.* 2007;27(8):1687-1693.

COMMENTS

This study by Nagai et al investigates the relative contributions of 3 major pro- and anticoagulation pathways, including heparin-antithrombin, protein C, and tissue factor (TF), and the thrombogenic responses that occur in large and small vessels in the brain. Thrombosis was induced in the superior sagittal sinus by topical application of $FeCl_3$ or by fluorescein isothiocyanate photoactivation within the vessel lumen. The authors' objective was to elucidate the relative contributions of the above-stated pathways in the cerebral vasculature.

In their experiment, the 3 pathways were studied by using the $FeCl_3$ method on the superior sagittal sinus and the photoactivation method on arterioles and venules by comparing mice in the following groups: (1) control wild-type (WT) vs WT mice treated with heparin before thrombus induction; (2) control WT vs WT mice receiving activated protein C; control WT mice vs WT mice receiving rat antimouse activated protein C monoclonal antibody, and control WT vs transgenic mice overexpressing endothelial protein C receptor; (3) control WT vs WT mice receiving TF monoclonal antibody; (4) TF-deficient mutant mice vs TF floxed control mice.

Comparisons between groups were made by quantifying time required for complete flow cessation. This was done via observation through videomicroscopy and epi-illumination. However, the explanation of their quantification method needs to be further clarified. It is unclear whether "flow cessation time" means complete flow cessation for a total of 60 seconds or 60 seconds from first observed flow cessation. Since the Discussion section states, "this model is relatively easy to perform and allows for in vivo quantification of thrombus formation," the explanation of the quantification method should be more clearly expressed to assess feasibility of its reproducibility.

This is an interesting and potentially useful article that provides a novel experimental model. This may provide valuable insight into defining differing roles of pro- and anticoagulation pathways in cerebral vasculature. This may have future implications on clinical treatment targets not only for cerebral venous sinus thrombosis, but also for any area related to ischemic stroke.

Amy Lee
Ralph G. Dacey, Jr
St Louis, Missouri

The authors carry out a series of cleverly designed experiments that seek to delineate, perhaps for the first time, the role of 3 antithrombotic pathways in human cerebral venous thrombotic disease. The pathways are the heparin-antithrombin 3, protein C-thrombin-thrombomodulin, and TF pathways. Mouse transgenic and other molecular manipulations are used to alter these pathways and determine the effects on superior sagittal sinus thrombosis in mice and on photothrombosis of mouse arterioles and venules.

The results suggest that the antithrombin 3 and protein C pathways are involved in large venous sinus thrombosis, whereas the TF pathway is less important. This fits with clinical data showing that the former are associated with cerebral venous thrombosis. The results with TF are complicated, however, by differences between brain and systemic vessels.

The conclusions here could be that the mouse models reflect human disease, which seems supported by some of the results. More specific treatments could perhaps be designed on the basis of these results, with the goal to increase efficacy and decrease side effects such as iatrogenic hemorrhaging. The limitations, however, are that one does not know the relative contributions of these pathways to thrombosis and antithrombosis in mice and humans. Second, to what extent the manipulations inhibited the pathways involved is uncertain. Several methods were used to inhibit the protein C and TF pathways, and the results were not the same for each method. Is this because of incomplete inhibition of the pathway? Finally, activating thrombosis by light or oxidant stress, as done here, may not be the same as precipitating factors in human cerebral venous thrombosis. Thus, more work is needed, and one has to consider whether some fundamental advance is going to be made in a lower species or whether advances can only be made by studying humans or closely allied species.

R. Loch Macdonald
Toronto, Canada

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