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FORMATION OF THE CLOT

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Thrombosis is the leading cause of death in the Western world, with more than half of the population dying of myocardial infarction, stroke, and cancer-associated thrombosis including pulmonary emboli and deep vein thrombosis. Given the medical importance of hemostasis and thrombosis, and the major advances in understanding its mechanisms, diagnosis, and therapeutics, this field is now an essential component of hematology, cardiology, pulmonology, neurology, pediatrics, vascular medicine, and surgery. Hemostasis and thrombosis are complex physiologic and pathologic processes, respectively, that involve the vessel wall, circulating blood cells, with their receptors and their ligands, soluble blood coagulation and regulatory proteins, and blood cell-derived microparticles [1]. Although we and others have worked using purified system to parse this complexity and then to develop models for the workings of these processes, it has become increasingly important to analyze hemostasis and thrombosis in a living animal where all of the components are present simultaneously. Furthermore, the availability of genetically altered mice that lack the ability to synthesize a specific protein offers opportunity to study these processes in a mouse model of thrombosis. The confluence of the development of knockout technology in the mouse, improved microscopic imaging instrumentation and high speed computing machines has supported the development of a novel system for high speed intravital digital videomicroscopy in the microcirculation of a living mouse [2]. The goal has been to visualize thrombus formation following vessel wall injury in the microcirculation of a living mouse using an intravital confocal and widefield microscopy imaging system. Using this system we have studied P-selectin/PSGL-1-mediated tissue factor-bearing microparticle accumulation in the developing thrombus [3], the role of tissue factor-bearing microparticles in fibrin formation [4], platelet dynamics during thrombus formation [5], calcium mobilization during platelet activation [6], the role of PAR (thrombin receptor) [7] and GPVI (collagen receptor) [8] in platelet activation in a live mouse, microparticle and leukocyte accumulation in the developing arteriolar thrombus [9], the role of PECAM-1 [10] and bile salt-dependent lipase [11] in thrombus formation, anti-$\beta_2$glycoprotein-1 in the anti-phospholipid syndrome and its amplification of thrombus size [12], activation of the

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CONFLICTS OF INTEREST

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endothelium and its generation of fibrin [13], the role of thiol isomerases in thrombus formation [14–16] and new approaches to antithrombotic therapy using small molecule inhibitors of thiol isomerases [17]. By revisiting and testing the models and hypotheses that have been developed from in vitro studies, we have been able to determine that many of these models and hypotheses are correct whereas some need some significant modification. The study of biochemistry and cell biology in a living animal offers opportunities to understand physiology and pathology in complex biological systems.

Perhaps the most unanticipated discovery to emerge from our application of intravital microscopy to thrombus formation was the finding that extracellular thiol isomerases are required for platelet thrombus accumulation and fibrin generation. The oxidation state of labile disulfide bonds in certain hemostatic proteins and receptors has been implicated in regulating thrombus formation following tissue injury [18]. The oxidation state of these bonds is regulated by enzyme of the thiol isomerase family, of which there are now twenty members. Thiol isomerases, including protein disulfide isomerase (PDI), while containing endoplasmic reticulum retention signals, are found extracellularly as well as outside of the endoplasmic reticulum. Among the cells that secrete PDI and express these enzymes on their surface are platelets and endothelial cells.

Thiol isomerases catalyze disulfide oxidation, reduction and isomerization, playing an important role during protein synthesis. To determine whether extracellular protein disulfide isomerase played a role in thrombus formation in a mouse model, protein disulfide isomerase expression, platelet accumulation, and fibrin generation were monitored in mouse arterioles within the cremaster muscle microcirculation using intravital fluorescence microscopy following laser-induced injury. A time-dependent increase in protein disulfide isomerase antigen was observed in the thrombus following injury [14]. Protein disulfide isomerase remained associated with the developing thrombus despite the significant shear in the circulation. Infusion of bacitracin, a non-specific inhibitor of thiol isomerases, or a blocking antibody to protein disulfide isomerase into the circulation inhibited platelet thrombus formation and fibrin generation [14]. Fibrin deposition is normal in mice lacking Par4 although there is no stable accumulation of platelets [7]. Infusion of monoclonal antibodies against protein disulfide isomerase into the circulation of a Par4 null mouse prior to vessel wall injury inhibited fibrin generation. These results indicated that protein disulfide isomerase is absolutely required in vivo for both fibrin generation and platelet thrombus formation.

Extending these studies, we showed that endothelial cells are a critical cellular source of secreted PDI, important for fibrin generation and platelet accumulation in vivo. Functional PDI is rapidly secreted from human umbilical vein endothelial cells in culture upon activation with thrombin or after laser-induced stimulation [15]. PDI was localized in different cellular compartments in activated and quiescent endothelial cells, and then redistributed to the plasma membrane after cell activation. Weibel-Palade bodies in particular did not contain PDI. If platelet thrombus formation was inhibited by the infusion of eptifibatide into the circulation, PDI was detected after vessel wall injury, and fibrin deposition was normal. Treatment of mice with a function blocking PDI antibody completely inhibited fibrin generation in eptifibatide-treated mice. These results indicate

that, although both platelets and endothelial cells secrete PDI after laser-induced injury, PDI from endothelial cells is required for fibrin generation in vivo.

When PDI is secreted from platelets and endothelial cells upon cellular activation, secreted PDI is captured within the injured vasculature during thrombus formation. We establish that the platelet integrin αIIbβ3 binds PDI, and recombinant β3 also binds PDI [16]. Using intravital microscopy, we demonstrated that PDI accumulation at the site of laser-induced arteriolar wall injury was markedly reduced in β3−/− mice, and neither a platelet thrombus nor fibrin was generated at the vessel injury site [16], The absence of fibrin following vascular injury in β3−/− mice was due to the absence of extracellular PDI. To evaluate the relative importance of endothelial αVβ3 versus platelet αIIbβ3 or αVβ3, we performed reciprocal bone marrow transplants on wild-type and β3−/− mice. PDI accumulation and platelet thrombus formation were decreased after vessel injury in wild type mice transplanted with β3−/− bone marrow or in β3−/− mice transplanted with wild type bone marrow. However, the presence of either intact αVβ3 or αIIbβ3 on the endothelium or platelet, respectively, allowed for capture of PDI [16], These results indicate that both endothelial and platelet β3 integrins contribute to extracellular PDI binding at the vascular injury site.

While currently available antithrombotic agents inhibit either platelet aggregation or fibrin generation, inhibition of secreted PDI blocks the earliest stages of thrombus formation, suppressing both pathways. We explored extracellular PDI as an alternative target of antithrombotic therapy [17], A high-throughput screen identified quercetin-3-rutinoside as an inhibitor of PDI reductase activity in vitro. Inhibition of PDI was selective, as quercetin-3-rutinoside failed to inhibit the reductase activity of other thiol isomerases. Quercetin-3-rutinoside inhibited aggregation of human and mouse platelets and endothelial cell–mediated fibrin generation in human endothelial cells in vitro. Using intravital microscopy in mice, we demonstrated that quercetin-3-rutinoside blocks thrombus formation by inhibiting PDI. Infusion of recombinant PDI reversed the antithrombotic effect of quercetin-3-rutinoside. Thus, PDI is a viable target for small molecule inhibition of thrombus formation, and its inhibition may prove to be a useful adjunct in refractory thrombotic diseases that are not controlled with conventional antithrombotic agents.

Conclusion

The development of technology to permit highspeed digitial widefield and confocal intravital videomicroscopy has contributed to better understanding of the molecular and cellular basis of thrombus formation. With this technology, the discovery of an electron transport system that regulates the initiation of thrombus formation has broad implications. This system appears to regulate and activate a series of critical receptors important for thrombus formation and is required for the generation of thrombin. The components of this system and the pathways that define electron transport provide challenges for the future. Moreover, the opportunity to inhibit these pathways, including the inhibition of specific thiol isomerases such as PDI, offers a new target to explore for antithrombotic therapy.

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REFERENCES

[1]. Furie B , Furie BC . Mechanisms of thrombus formation. N Engl J Med. 2008;359:938–49.18753650

[2]. Falati S , Gross P , Merrill-Skoloff G , Furie BC , Furie B . Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. Nat Med. 2002;8:1175–81.12244306

[3]. Falati S , Liu Q , Gross P , Merrill-Skoloff G , Chou J , Vandendries E , et al. Accumulation of Tissue Factor into Developing Thrombi In Vivo Is Dependent upon Microparticle P-Selectin Glycoprotein Ligand 1 and Platelet P-Selectin. J Exp Med. 2003;197:1585–98.12782720

[4]. Chou J , Mackman N , Merrill-Skoloff G , Pedersen B , Furie BC , Furie B . Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. Blood. 2004;104:3190–7.15280200

[5]. Sim DS , Merrill-Skoloff G , Furie BC , Furie B , Flaumenhaft R . Initial accumulation of platelets during arterial thrombus formation in vivo is inhibited by elevation of basal cAMP levels. Blood. 2004;103:2127–34.14645013

[6]. Dubois C , Panicot-Dubois L , Gainor JF , Furie BC , Furie B . Thrombin-initiated platelet activation in vivo is vWF independent during thrombus formation in a laser injury model. J Clin Invest. 2007;117:953–60.17380206

[7]. Vandendries ER , Hamilton JR , Coughlin SR , Furie B , Furie BC . Par4 is required for platelet thrombus propagation but not fibrin generation in a mouse model of thrombosis. Proc Natl Acad Sci U S A. 2007;104:288–92.17190826

[8]. Dubois C , Panicot-Dubois L , Merrill-Skoloff G , Furie B , Furie BC . Glycoprotein VI-dependent and - independent pathways of thrombus formation in vivo. Blood. 2006;107:3902–6.16455953

[9]. Gross PL , Furie BC , Merrill-Skoloff G , Chou J , Furie B . Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development. J Leukoc Biol. 2005;78:1318–26.16204641

[10]. Falati S , Patil S , Gross PL , Stapleton M , Merrill-Skoloff G , Barrett NE , et al. Platelet PECAM-1 inhibits thrombus formation in vivo. Blood. 2006;107:535–41.16166583

[11]. Panicot-Dubois L , Thomas GM , Furie BC , Furie B , Lombardo D , Dubois C . Bile salt-dependent lipase interacts with platelet CXCR4 and modulates thrombus formation in mice and humans. J Clin Invest. 2007;117:3708–19.18037996

[12]. Arad A , Proulle V , Furie RA , Furie BC , Furie B . {beta}2 glycoprotein-1 autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. Blood. 2011.

[13]. Atkinson BT , Jasuja R , Chen VM , Nandivada P , Furie B , Furie BC . Laser-induced endothelial cell activation supports fibrin formation. Blood. 2010;116:4675–83.20675401

[14]. Cho J , Furie BC , Coughlin SR , Furie B . A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. J Clin Invest. 2008;118:1123–31.18292814

[15]. Jasuja R , Furie B , Furie BC . Endothelium-derived but not platelet-derived protein disulfide isomerase is required for thrombus formation in vivo. Blood. 2010.

[16]. Cho J , Kennedy DR , Lin L , Huang M , Merrill-Skoloff G , Furie BC , et al. Protein disulfide isomerase capture during thrombus formation in vivo depends on the presence of beta3 integrins. Blood. 2012.

[17]. Jasuja R , Passam FH , Kennedy DR , Kim SH , van Hessem L , Lin L , et al. Protein disulfide isomerase inhibitors constitute a new class of antithrombotic agents. J Clin Invest. 2012;122:2104–13.22565308

[18]. Chen VM , Hogg PJ . Allosteric disulfide bonds in thrombosis and thrombolysis. J Thromb Haemost. 2006;4:2533–41.17002656