Animal Models of Thrombosis From Zebrafish to Nonhuman Primates

Use in the Elucidation of New Pathologic Pathways and the Development of Antithrombotic Drugs

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Abstract: **Thrombosis is a leading cause of morbidity and mortality worldwide. Animal models are used to understand the pathological pathways involved in thrombosis and to test the efficacy and safety of new antithrombotic drugs. In this review, we will first describe the central role a variety of animal models of thrombosis and hemostasis has played in the development of new antiplatelet and anticoagulant drugs. These include the widely used $P2Y_{12}$ antagonists and the recently developed orally available anticoagulants that directly target factor Xa or thrombin. Next, we will describe the new players, such as polyphosphate, neutrophil extracellular traps, and microparticles, which have been shown to contribute to thrombosis in mouse models, particularly venous thrombosis models. Other mouse studies have demonstrated roles for the factor XIIa and factor XIa in thrombosis. This has spurred the development of strategies to reduce their levels or activities as a new approach for preventing thrombosis. Finally, we will discuss the emergence of zebrafish as a model to study thrombosis and its potential use in the discovery of novel factors involved in thrombosis and hemostasis. Animal models of thrombosis from zebrafish to nonhuman primates are vital in identifying pathological pathways of thrombosis that can be safely targeted with a minimal effect on hemostasis. Future studies should focus on understanding the different triggers of thrombosis and the best drugs to prevent each type of thrombotic event. (*Circ Res*. 2016;118:1363-1379. DOI: 10.1161/CIRCRESAHA.115.306823.)**

Key Words: animal models ■ anticoagulants ■ hemostasis ■ thrombosis ■ zebrafish

Hemostasis is a physiological process that involves formation of a hemostatic clot at the site of vessel injury to prevent blood loss. In contrast, thrombosis is a pathological process, where thrombotic clots are formed within blood vessels and obstruct the flow of blood in the circulatory system.[1–4] Thrombotic clots contain platelets as the major cellular component and crosslinked fibrin as the main protein component. In addition, clots contain red blood cells and leukocytes. However, arterial and venous clots differ in the relative amounts of platelets and fibrin. Arterial thrombi most often form rapidly after rupture of atherosclerotic plaques and are platelet rich; these clots cause myocardial infarction and

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Nonstandard Abbreviations and Acronyms	
FDA	Food and Drug Administration
DVT	deep vein thrombosis
IVC	inferior vena cava
NDA	New Drug Applications
PAR	protease-activated receptor
TF	tissue factor
TTO	time to occlusion
vWF	von Willebrand factor

stroke.[5] Venous clots form in large veins, particularly in the legs, and are fibrin-rich with high numbers of red blood cells and develop over hours to days.[5–7]

Vessel injury leads to rapid platelet activation by binding of exposed collagen and deposited von Willebrand factor (vWF) to platelet receptors. In parallel, the clotting system is activated by exposure of tissue factor (TF) in the vessel wall and formation of the TF/factor VIIa complex (Figure 1). Factor XII of the intrinsic pathway has also been shown to contribute to thrombosis. Figure 2 shows simplified versions of the platelet and coagulation cascades. Platelets are activated by primary stimuli, such as collagen, vWF, and thrombin, and this leads to the release of secondary stimuli, such as thromboxane A2 and ADP. Full activation of platelets ultimately leads to a change in the conformation of the integrin GPIIb/IIIa ($\alpha II_b\beta_3$) that allows binding of ligands, such as fibrinogen, that mediate platelet aggregation. The coagulation cascade is composed of the extrinsic pathway (TF/factor VIIa), the intrinsic pathway (factors XIIa, XIa, IXa, and VIIIa) and the common pathway (factors Va, Xa, and thrombin). Thrombin cleaves fibrinogen into fibrin monomers that self-polymerize and are subsequently crosslinked by the transglutaminase factor XIIIa. Importantly, there is cross talk between the platelet and coagulation cascades (Figures 1 and 2). For instance, activated platelets provide a thrombogenic surface for the assembly of various coagulation complexes, such as the tenase complex (factor VIIIa/factor IXa) and the prothrombinase complex (factor Va/factor Xa). In addition, thrombin is a potent activator of human platelets via cleavage of protease-activated receptors 1 and 4 (PAR1 and PAR4). Figure 2 also shows the targets of currently approved antithrombotic drugs that are used to prevent and treat both arterial and venous thrombosis.

Animal models have played a central role in the identification of factors and pathways that drive thrombosis and in evaluating the efficacy and safety of antithrombotic drugs. There are many reviews that have described in detail animal models of thrombosis and hemostasis, including zebrafish, rodents, and larger animals, such as nonhuman primates.[8–20]

In this review, we chose to focus on the use of animal models of thrombosis in the development of antithrombotic drugs, and in the identification of new pathological pathways of thrombosis. The efficacy and safety of antithrombotic drugs is evaluated in a variety of animal models of thrombosis and hemostasis before initiating phase I clinical trials. More basic studies of the thrombotic process are performed in mouse models because of the availability of different transgenic mouse strains. These studies, particularly with venous thrombosis models, have identified new players in thrombosis, such as polyphosphates, neutrophil extracellular traps, and microparticles. Finally, we will describe the emergence of zebrafish as a model of thrombosis and hemostasis and its potential to identify new factors involved in thrombosis.

Development of Animal Models of Thrombosis

There are several general models of thrombosis originally developed in species larger than mice. In 1952, Wessler[21] described a vein stasis model in which vein segments in dogs were clamped for >20 minutes to induce clot formation. In 1976, Folts et al[22] described an arterial injury model that involved a 60% to 80% stenosis of the coronary artery of dogs. The coronary artery exhibited cyclic reductions in blood flow that were proposed to mimic changes that occur in a stenosed coronary vessel. Administration of aspirin abolished the cyclic reductions in blood flow and reduced platelet aggregation. Variants of these models have been subsequently applied in a number species, including rabbits, rats, and mice. Other models use a form of intraluminal injury, most often created by opening the vessel (eg, creating an arteriotomy) and mechanically scratching or removing the intima.[23] In addition, thrombosis can be initiated by placing a synthetic material inside the vessel, such as a suture.[24] Another common model is to create an arteriovenous shunt using a synthetic material that collects thrombus on its surface.[25,26] This model offers several advantages because it can be performed in a broad range or species—rats, rabbits, dogs, and nonhuman primates. In addition, it can be used for acute or more chronic treatment. There are variations in the vessels cannulated—carotid or femoral artery, jugular or femoral vein. A graft with an artificial surface can be added. A disadvantage of such a model is the limitations of clinical relevance in testing efficacy of the antithrombotic drugs targeting indications that this model does not simulate. For anticoagulants, these would include stroke prevention in atrial fibrillation and treatment and prevention of venous thrombosis. These various animal models of thrombosis have been used for many preclinical studies evaluating antithrombotic drugs.

There has been a lack of uniformity in the application of these various models, both within and across species, which has hindered standardization of any one model or group of models. The Folts model is arguably the one closest to simulating clinical arterial thrombosis but its application in various species and anatomic vessels leads to variability. Other models have even greater variability and questionable relevance. For instance, the arteriovenous shunt model uses a synthetic surface to simulate thrombus formation (eg, polyethylene or Dacron) in a circuit going from arterial to venous pressure, that has little relevance to most forms of pathophysiologic thrombus formation. The main value in these models is in their ability to demonstrate thrombus inhibition in a vessel of comparable size to clinically thrombosing vessels.

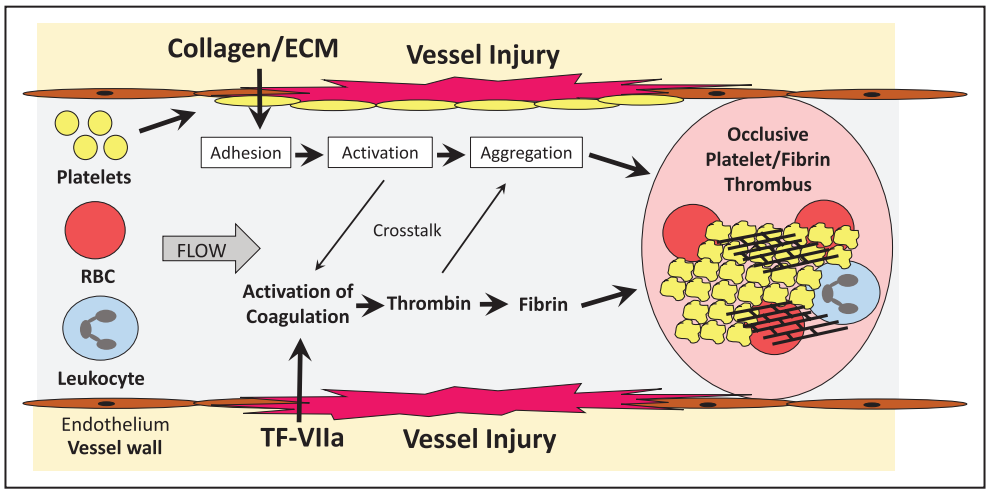

Figure 1. Formation of an occlusive thrombus. After vessel injury platelets rapidly adhere to collagen and deposited von Willebrand Factor. The adhered platelets are activated by primary and secondary activators that lead to platelet aggregation mediated by various ligands, including fibrinogen. In parallel to platelet activation, the clotting system is activated by exposure of tissue factor (TF) in the vessel wall. In addition, factor XII may contribute to the activation of coagulation. Thrombin is the central protease of the coagulation cascade and cleaves fibrinogen to fibrin monomers that are crosslinked into a network by factor XIIIa. There is cross talk between the platelet and coagulation cascades. For instance, activated platelets provide a thrombogenic surface for the assembly of various coagulation protease complexes and thrombin is a potent activator of platelets by cleavage of protease activated receptors. Formation of an occlusive thrombosis will block blood flow.

Use of Animal Models for the Approval of New Antithrombotic Drugs

In the first part of this review, we will discuss the use of animal models of thrombosis for the approval of antithrombotic drugs. Clearly, this is a large topic so we have focused on the use of models of thrombosis for approved Food and Drug Administration (FDA) applications of antithrombotic drugs (anticoagulants and antiplatelets) since 1997 at http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm. The FDA evaluates submissions on a compound for data from animal studies for 3 criteria: mechanism of action, efficacy on targeted biology, and safety pharmacology. For some drugs, the target is specific for a human protein, and thus nonhuman primates are the only suitable model for evaluating the effect.

We will summarize the thrombosis models used in the New Drug Applications (NDA) to demonstrate mechanisms of action and efficacy on targeted biology to obtain NDA approval for the various drugs. Although nonhuman primates

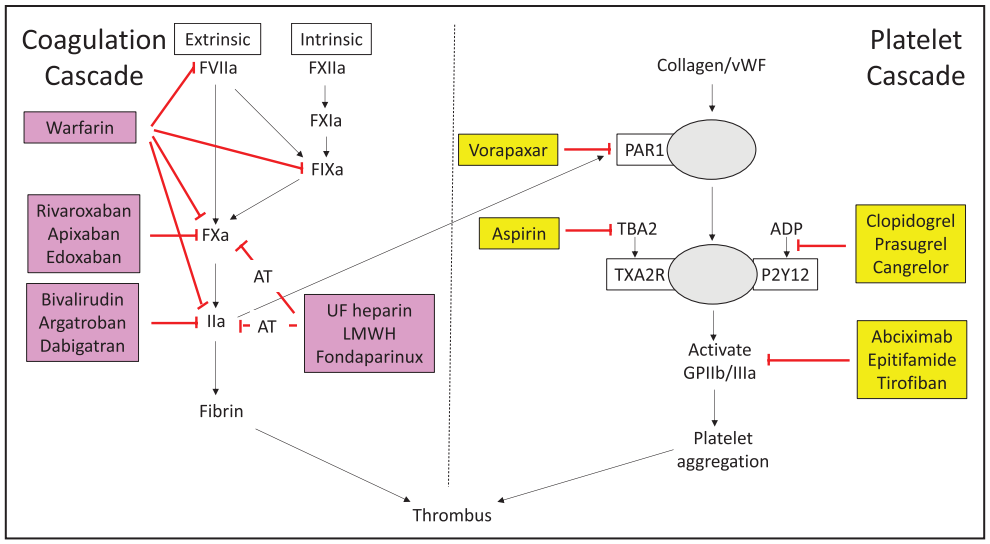

Figure 2. Animal models of thrombosis are used in the development of antithrombotic drugs. The targets of both anticoagulant and antiplatelet drugs are shown. Platelet inhibitors include inhibitor of the primary activator and secondary activators. The protease-activated receptor 1 (PAR1) inhibitor vorapaxar blocks thrombin activation of platelets. Aspirin inhibits the generation of the secondary activator thromboxane A2. There are several inhibitors of the ADP receptor P2Y12 that are used clinically. Finally, inhibitors of integrin GPIIb/IIIa prevent platelet aggregation. Warfarin acts by inhibiting the generation of γ-carboxylation domains on several coagulation proteases. Unfractionated heparin (UF heparin) acts in an antithrombin-dependent manner to inhibit both thrombin (IIa) and factor Xa (FXa). Low-molecular-weight heparins are more selective for thrombin and the synthetic pentasaccharide fondaparinux only inhibits FXa. Direct thrombin inhibitor include parenteral drugs (bivalirudin and argatroban) and the oral inhibitor dabigatran etexilate (dabigatran). Three oral, direct FXa inhibitors have been developed. vWF indicates von Willebrand factor.

and pigs are often held up as the most human-like in their physiology, use of dogs, rabbits, and rats has been an acceptable practice for many NDA submissions. There are several caveats with using NDA to review animal models of thrombosis. One caveat is that we will not describe the extensive pharmacokinetic and off-target safety studies that have been performed in many species, including nonhuman primates. In addition, published studies that were not included in the regulatory filing will not be discussed. Finally, we have limited our presentation to NDAs for drugs that have been approved because this is what is accessible.

The drugs are divided into antiplatelet drugs and anticoagulants, and each group is presented in chronological order of submission because this gives some context to the choice of models. The primary reference is the FDA accessible NDA. The references cited are for the publications of the data in the NDA and are provided to add experimental detail. However, not all data in the NDAs are published but this can be found on the Website. The references also may contain additional thrombosis models that were not submitted in the NDA. There are also additional references of animal thrombosis models using the drugs, but if they are not part of the NDA then these are not reviewed. Antithrombotic drugs must be also assessed for safety using a variety of animal models of bleeding.[27–29] The most common rodent bleeding model is the tail transection model.[30] Use of a template to standardize the diameter of the tail that is cut improves the reproducibility of the model. Another mouse bleeding model is the saphenous vein model.[31] The template skin bleeding test uses a standard skin cut and is used in nonhuman primates to assess bleeding.[28] Other bleeding tests used to assess antithrombotic drugs include the rabbit ear, cuticle bleeding, and the rat renal cortex template models.

Antiplatelets

Aspirin has been used for many years as an antiplatelet drug. More recently, several parenteral inhibitors of GPIIb/IIIa ($\alpha_{IIb}\beta_3$) have been developed (Figure 2).[32] The first GPIIb/IIIa inhibitor approved by the FDA was abciximab in 1994 followed by tirofiban and eptifibatide in 1998. The most recent developments in antiplatelet drugs include 2 new classes: $P2Y_{12}$ inhibitors (clopidogrel, prasugrel, and ticagrelor) and a PAR-1 inhibitor (vorapaxar; Figure 2). These antiplatelet drugs are approved for acute treatment of myocardial infarction, including with percutaneous coronary interventions, and, in patients with previous stroke, myocardial infarction, or peripheral vascular disease, for prevention of recurrent thrombosis. For their registration trials, they were superior when added to aspirin, when compared with previously approved drugs in the same class or added to standard of care.

Abciximab and Tirofiban

The FDA NDA submissions for these 2 drugs are not available on the accessible Website.

Eptifibitide (1987; NDA 20–718)

The efficacy of eptifibitide was demonstrated in dogs where it reduced cyclic flow reductions in a major artery. In addition in dogs, coronary artery thrombi produced by an external electric injury then lysed with tissue-type plasminogen activator reoccluded less frequently after eptifibitide infusion, and even less frequently when lower doses of eptifibitide were administered with hirudin. Finally, eptifibitide improved platelet count and platelet function after a hypothermic cardiopulmonary bypass in dogs. In baboons, eptifibitide reduced the accumulation of ^{111}In-oxine–labeled platelets on a Dacron graft in a femoral arteriovenous shunt model. This effect was not increased by heparin or aspirin. The drug worked best when administered before graft placement. The effect of eptifibitide on template bleeding was also evaluated in dogs and baboons.

Clopidogrel (1997; NDA 20–839)

The efficacy of clopidogrel was demonstrated in rats using thrombosis models induced by inserting a metallic coil or an electric injury to arteries, and by venous stasis. These same models were used to demonstrate efficacy in rabbits. In addition, clopidogrel reduced platelet accumulation in de-endothelialization carotid arteries in rabbits.[33] Clopidogrel reduced cyclic flow reductions in the left anterior descending artery induced by brief pinch injury to the artery with forceps and placing a constrictor around the injured portion of the vessel.[34] The effect of clopidogrel on bleeding was evaluated in a rat tail model and a rabbit template model.

Prasugrel (2007; NDA 22–307)

Prasugrel reduced thrombosis in a rat model consisting of a carotid to contralateral jugular arteriovenous shunt model using a polyethylene catheter with a silk thread inside.[35] Prasugrel also delayed time to occlusion (TTO) after electric injury to the rat carotid artery.[36] Finally, prasugrel inhibited aggregation of platelets that were isolated from the blood of cynomolgus monkeys dosed orally with prasugrel. The effect of prasugrel on bleeding was evaluated in a rat tail model; it is notable that the dose that caused 50% increase in bleeding was lower than clopidogrel.

Ticagrelor (2010; NDA 22–433)

Efficacy of ticagrelor was demonstrated in rats using a ferric chloride injury to the carotid artery.[25] In dogs, ticagrelor restored normal blood flow after injury of the femoral artery by squeezing it and partly obstructing it with an occluder (modified Folts model).[37] In addition, ticagrelor was evaluated in combination with aspirin or the direct thrombin inhibitor melagatran. The effect of ticagrelor on bleeding was evaluated in dogs using a template model. In addition, the submission also presented data on antithrombotic-bleeding efficacy by calculating the ratio of the IC_{50} of each effect and comparing this with other antiplatelet drugs.

Vorapaxar (2013; NDA 204886)

Vorapaxar is a molecule that binds to PAR-1 and blocks the binding of the tethered ligand released by thrombin cleavage. It has no activity in rodents. The FDA submission of vorapaxar did not include any efficacy data on the antithrombotic effects of vorapaxar. Instead, data on a similar compound, SCH602539, was presented and accepted as a bioequivalent. The efficacy of SCH602539 was demonstrated in cynomolgus monkeys using a carotid artery modified Folts model.[38] SCH602539 reduced cyclic flow reductions as well as a $P2Y_{12}$ inhibitor, and the effect of combining the 2 drugs was more than synergistic.

Anticoagulants

Warfarin and heparin have been the mainstay of anticoagulant therapy for the past 50 years. Warfarin is administered orally and inhibits the formation of a Gla domain on several different coagulation proteases (factors VIIa, IXa, Xa, and thrombin; Figure 2). It is most often used for long-term anticoagulant therapy. Heparins are administered parenterally. Unfractionated heparin inhibits both factor Xa and thrombin in an antithrombin-dependent manner (Figure 2). Low-molecular-weight heparins are more selective for factor Xa. The synthetic pentasaccharide fondaparinux is selective for factor Xa. Newer anticoagulants have been designed to directly inhibit either factor Xa or thrombin. The first generation of these inhibitors was the thrombin inhibitor bivalirudin, which is administered parenterally to treat patients undergoing percutaneous coronary angioplasty. The next generation of these inhibitors included orally available inhibitors of either factor Xa (rivaroxaban, apixaban, and edoxaban) or thrombin (dabigatran etexilate; Figure 2). These drugs are approved for the prevention and treatment of venous thrombosis, and for the prevention of stroke in patients with atrial fibrillation.

Bivalirudin (1999; NDA 20–873)

Bivalirudin inhibited platelet and fibrin deposition as measured in electron microscopy images of the thrombi in a rat carotid endarterectomy model.[39] Bivalirudin also decreased reperfusion time when given with tissue-type plasminogen activator as a thrombolytic to aortic thrombi in rats. Bivalirudin also decreased ^{111}In-oxine–labeled platelet accumulation in the brain after intracarotid injection of thrombin to rabbits. In this model, it was compared with aspirin and with heparin.[40] Bivalirudin inhibited thrombosis and the frequency of subsequent occlusions in a pig model where the carotid artery was repeatedly occluded with a clamp. In baboons (*Papio anubis*), bivalirudin reduced platelet and fibrin deposition in various versions of an exteriorized femoral arteriovenous access shunt model, including those with endarterectomized aortae, collagen-coated Gortex, Dacron, a 2-chambered device, and a chronic arteriovenous shunt.[26] Bleeding was evaluated in the same animals using a template bleeding model.

Dabigatran Etexilate (2010; NDA 22–512)

Dabigatran was given intravenously to rats where it reduced thrombus weight in a thromboplastin/stasis inferior vena cava (IVC) model.[41,42] Rats were also dosed orally with dabigatran etexilate. In rabbits, the weight of clots in the jugular vein induced by stenosis and polidocanol, a sclerosant, was also reduced by dabigatran.[43] Bleeding was evaluated in rats using a template tail bleeding model[42]; the efficacy of an activated prothrombin complex concentrate and recombinant factor VIIa to reverse dabigatran bleeding was also demonstrated.[44]

Rivaroxaban (2011; NDA 202439 and 22406)

Rivaroxaban reduced thrombosis in a mouse model of ferric chloride injury to the carotid artery and prevented death after injection of thromboplastin. In rats, rivaroxaban reduced thrombosis in a carotid to contralateral jugular[45] arteriovenous shunt model with a polyethylene catheter with a nylon thread inside. This evaluation also included combining rivaroxaban with heparin, with low-molecular-weight heparin, with aspirin, with various nonsteroidal anti-inflammatory drugs, with clopidogrel, with clopidogrel and aspirin, and with warfarin. Studies in rats showed that rivaroxaban reduced the size of thrombi induced by an electrolytic or ferric chloride injury of the carotid artery.[46] The drug was also shown to be effective in an IVC stenosis and thromboplastin-induced hypercoagulability model.[47] Rivaroxaban also reduced thrombosis in rabbits in a similar carotid to contralateral jugular arteriovenous shunt model, but with a polyurethane catheter with a larger nylon thread inside. Baboons (template), rats (tail bleeding), and rabbits (ear bleeding)[47] were used to evaluate the effect of rivaroxaban on bleeding. The efficacy of recombinant factor VIIa and prothrombin complex concentrates and activated prothrombin concentrates on bleeding was evaluated in rats only.[48]

Apixaban (2011; NDA 202155)

Clot weights were reduced by apixaban in rats in an arteriovenous shunt model, and after ferric chloride injury to the carotid artery and to the IVC. Apixaban also reduced clot weight in an arteriovenous shunt model and maintained flow in an electrolytical injury carotid model[49]; the latter was also combined with antiplatelet drugs. In dogs, apixaban reduced thrombus weight in an arteriovenous shunt model and delayed TTO after electric injury to the femoral artery.[50] The effect of apixaban on bleeding was evaluated in rats with a renal cortex template model and in rabbits with a cuticle bleeding model.

Edoxaban (2014; NDA 206316)

In the FDA NDA, the antithrombotic effectiveness of edoxaban was only demonstrated in rats. The models included an arteriovenous shunt model, and 2 IVC ligation models, one with double ligation and the other with partial ligation.[51] In the arteriovenous shunt model, edoxaban reduced thrombus protein content, whereas in the IVC models edoxaban reduced thrombus weight. Edoxaban also reduced thrombus weight in a venous thrombosis model induced by placing a platinum wire into the IVC. This was also compared with treatments with enoxaparin, fondaparinux, and warfarin. In a disseminated intravascular coagulation model where thromboplastin is injected into the femoral vein, edoxaban normalized the amount of thrombin–antithrombin complexes and the platelet counts and the fibrinogen concentration. A subsequent publication also included a study in rabbits using a model of thromboplastin and jugular vein stenosis (modified Wessler model) to cause thrombosis.[52] The effect of edoxaban on bleeding was also only evaluated in rats using tail and plantar template bleeding models where it was compared with low-molecular-weight heparin.

Summation of Findings From NDAs

Table 1 summarizes the different thrombosis models that have been used in the development of various antithrombotic drugs for NDA submission. Over the past 18 years several trends are apparent in the use of nonmurine animal models in FDA submissions for antithrombotic drugs. Over time, the use of nonhuman primates is becoming limited to cases where close similarity to humans is required. The use of dogs is also

Table 1. Types of Thrombosis Models Used to Support New Drug Application Submissions of Approved Antithrombotic Drugs

Year of Approval/Drug	Arterial	Venous	Arteriovenous Shunt	Systemic Activation	Folts Type
1997/Eptifibatide	X		X		X
1997/Clopidogrel	X	X			X
1999/Bivalirudin	X		X	X	
2007/Prasugrel	X		X		
2010/Dabigatran	X				
2010/Ticagrelor	X				X
2011/Rivaroxaban	X	X	X	X	
2011/Apixaban	X	X	X		
2013/Vorapaxar					X
2014/Edoxaban		X	X	X	

becoming less prevalent (Table 2). The only mouse models used to demonstrate efficacy in the NDAs were applied for evaluating rivaroxaban in 2011. There also seems to be a drug class-effect in the animal models selected where later drugs in the same class have less testing in higher-order species. This is evident with edoxaban, the third factor Xa inhibitor approved, which only had efficacy data in rats in the NDA.

In these NDAs, the efficacies of antiplatelet drugs have been largely demonstrated in arterial models, such as variants on the Folts model. This model mimics many features important in preventing thrombosis in coronary interventions where the antiplatelet drugs that have been approved have shown efficacy. Interestingly, clopidogrel was also shown to reduce venous thrombosis in a mouse model.[53] The most prevalent model used to show efficacy of anticoagulants in the NDAs is the arteriovenous shunt model. Table 2 summarizes the different animal models that have been used in the evaluation of antithrombotic drugs.

In these NDAs, the efficacy of the direct factor Xa inhibitors were demonstrated in both arterial and venous models (Table 1). The efficacy of dabigatran was not demonstrated in an artery-specific model in its NDA, whereas the efficacy of bivalirudin was not demonstrated in a vein-specific model (Table 1). In conclusion, the nonmurine thrombosis models used in FDA NDAs for antithrombotic drugs that were eventually approved used a variety of animal models of thrombosis to demonstrate efficacy. It should be noted that these models lack close mimicry to clinical scenarios, and are not necessarily closely correlated to the clinical indication for which the antithrombotic drug is later approved for.

Murine Models of Thrombosis

Mice are the most common animal species utilized as a research tool in thrombosis for several reasons. They are a mammalian system with many physiological similarities to humans with a wealth of biological information available for research, and they are economical to house and to manipulate. Currently, the most far-reaching advantage of mice, over other mammalian species, is the ease with which their genome can be manipulated and the availability of numerous transgenic, knockout, and knockin lines for a multitude of genes. Use of inbred mouse lines also reduces variability in experiments compared with outbred animals. In addition, sequencing of the mouse genome has created a vast knowledge

Table 2. Animal Models Used to Support New Drug Application Submissions of Approved Antithrombotic Drugs

Year of Approval/Drug	Rat	Rabbit	Dog	Pig	Nonhuman Primate
1997/Eptifibatide			X		X
1997/Clopidogrel	X	X	X		
1999/Bivalirudin	X	X		X	X
2007/Prasugrel	X				X
2010/Dabigatran	X	X			
2010/Ticagrelor	X		X		
2011/Rivaroxaban	X	X			X
2011/Apixaban	X	X	X		
2013/Vorapaxar					X
2014/Edoxaban	X				

Table 3. Murine Models of Thrombosis by Vessel Type and Mechanism of Injury

Vessel	Injury/Thrombosis Induction	Specific Mechanism	References
Arterial			
Carotid	Free-radical injury	Ferric chloride	Farrehi et al[54]
Carotid	Free-radical injury	Rose bengal+light	Eitzman et al[55]
Carotid	Free-radical injury	Electrolysis with iron ions	Cooley[56]
Carotid	Mechanical	Pinch	Mangin et al[57]
Carotid	Mechanical	Hard, brief ligation	Schulz et al[58]
Carotid	Mechanical	Endothelial wire/needle injury	Cornelissen et al[59]
Carotid	Mechanical	Ultrasound (plaque disruption)	Kuijpers et al[60]
Carotid	Intraluminal collagen	Adventitial collagen surface	Cooley[61]
Carotid	Heat	Cautery	Carmeliet et al[62]
Carotid	Anastomosis	Sutured repair	Cooley and Daley[63]
Venous			
IVC	Stasis	IVC ligation	Myers et al[64]
IVC	Low flow	IVC stenosis	Singh et al[65]
IVC	Low flow+mild mechanical	IVC stenosis+brief clamp injury	Singh et al[66]
IVC, jugular, saphenous	Free-radical injury	Ferric chloride	Wang et al[67]
Femoral, IVC	Free-radical injury	Electrolysis with iron ions	Cooley[56] and Cooley et al[68]
Femoral	Mechanical	Pinch	Pierangeli et al[69]
Femoral	Intraluminal collagen	Adventitial collagen surface	Cooley[61]
Femoral	Anastomosis	Sutured repair	Cooley and Daley[63]
Microvessels			
Cremasteric or mesenteric	Free-radical injury	Ferric chloride	Denis et al[70]
Cremasteric or mesenteric	Laser	Heat/photochemical	Falati et al[71]

IVC indicates inferior vena cava.

base for this species. Therefore, mice have played a prominent role in research in hemostasis and thrombosis. Table 3 shows the variety of vessels that have been used in the different thrombosis models.

Arterial Thrombosis Models

The majority of thrombosis experiments in mice use the common carotid artery because of the ease of access, dissection, and manipulation of a long, unbranched vessel segment. Transferring techniques learnt in rats,[72] the models use either ferric chloride[54] or Rose bengal-plus-light[55] to induce thrombus formation. These models are relatively easy to learn and apply, have modest equipment needs, and yield similar end point outcomes that have shown strong discriminative use in thrombosis research. Both models rely on a free-radical–based mechanism of injury: ferric chloride presents an initially brief but persistent vessel wall injury via outer surface application at a defined time and concentration, with what seems to be a multimodal mechanism of injury to the blood vessel[73–75]; the Rose bengal model requires continuous light activation of circulating Rose bengal at the site of laser illumination on the carotid artery, which confers free-radical–localized injury from the inside of the vessel, again by an incompletely understood mechanism. The time to flow cessation or flow below a chosen cutoff (TTO) is determined with flow monitoring. Thus, acute thrombosis that leads to thrombotic occlusion is the operative clinical simulation. A more recent free-radical–based model uses electrolytic injury applied to the arterial surface,[56,76] with intravital fluorescence microscopy[56] for image acquisition and off-line quantitation of thrombus formation. Other models of thrombus use mechanical injuries[14,58,77] including a Folts-like model, which includes a stenosis site,[57] direct electric injury,[62] intraluminal collagen,[61] microvascular anastomosis,[63,78] or ultrasound to cause disruption of atherosclerotic lesions.[60] However, outcome measures for these models have shown more variability, lowering their use and selection for research studies.

Uses of Arterial Thrombosis Models

These models have been used both for in vivo characterization of antithrombotic drugs (as described above) and for the contribution of individual gene–based influences on thrombotic responses. The acute nature and rapid onset of clinical arterial thrombosis leaves the murine arterial simulations as reasonable analogs for evaluating the acute clinical response. The murine models are of particular value for studying platelet responses under in vivo conditions, to evaluate agonists and inhibitors of platelet receptor responses, platelet activation, and the subsequent aggregatory response.

The initial characterization of the ferric chloride model was described by Farrehi et al[54] and was used to show a reduction in thrombus formation in plasminogen activator inhibitor-1 knockout mice compared with wild-type mice. Several versions of the model have been used, with descriptions for optimizing the methodology.[17,79] Importantly, the degree of injury can be modulated by the concentration of the ferric chloride and the time of exposure. The model has been applied to demonstrate the roles of platelet aggregation inhibitors, such as aspirin, clopidogrel, ticagrelor, and other clinically approved or experimental compounds,[56,58,80–82] on preventing arterial thrombosis. In parallel to these studies, transgenic/knockout mouse lines have been used to demonstrate the critical role of different platelet receptors using this model. For example, PAR-3, PAR-4, or platelet $P2Y_{12}$ receptor knockout mice have shown reduced thrombotic responses to ferric chloride–induced thrombosis.[59] Interestingly, combining PAR-3 or PAR-4 deficiency with a deficiency in $P2Y_{12}$ mimic pharmaceutical inhibition of PAR-1 and $P2Y_{12}$ in human platelets. Platelet adhesion receptor function in thrombosis has also been revealed,[83,84] as has the critical role of the GPIIb/IIIa receptor in formation of an occlusive thrombus.[85,86]

Coagulation factors have also been evaluated with the ferric chloride model to demonstrate a role in arterial thrombosis. For example, deletion of vascular smooth muscle cell TF was associated with a prolonged occlusion time.[87] In addition, mice with deficiencies in different components of the intrinsic pathway factors (IXa, XIa, or XIIa) had reduced thrombosis in this model.[88–90] Importantly, the protective effect of a deficiency of either factor XI or factor IX was only revealed at a low dose of ferric chloride. These studies on the role of the intrinsic pathway in thrombosis, particularly factor XIIa, has spurred the development of new anticoagulant drugs that decrease levels or block activity of factor XIa or factor XIIa.[91,92] In contrast, increasing levels of factor VIII or fibrinogen shortened the occlusion times in the ferric chloride model.[93,94] The Rose bengal model has shown similar use for understanding arterial thrombotic responses under various platelet- and coagulation-inhibited conditions.[55,95,96]

Whereas the models that generate TTO data are easy to apply in many laboratories, the outcome measures are limited with no information about thrombodynamics. Using fluorescence imaging provides an enhanced understanding of temporal and spatial responses to various injury mechanisms, as exemplified by a recent report showing this response in 9 different injury mechanisms to the mouse carotid artery, showing more rapid responses to abrupt mechanical injury, and with slower development but more sustained response after free-radical–based injuries.[97] How this understanding translates to clinical arterial thrombosis will need further investigation.

Venous Thrombosis Models

Several murine venous thrombosis models have been developed; however, their analogy to clinical deep vein thrombosis (DVT) is unclear, due in part to the slower development of venous thrombi, to the lack of knowledge for the clinical scenario of DVT and how best to simulate it in the much smaller species, and to the more fragile and variable anatomy of mouse veins. Venous models of thrombotic induction fall into 2 general categories: those that use a low-flow or no-flow state to impart slow thrombus development, and those that use an acute injury to induce more rapid clot formation. Most models have been created in the IVC; this is the largest easily accessible vein in the mouse, yet has inherent problems in its manipulation, and variable side- and back-branch anatomy that can influence thrombotic outcomes.[98] The jugular, femoral, and saphenous veins are other choices for model sites, used for acute thrombosis studies.

The IVC is large enough to generate clots of sufficient size for weight and length measurements and for Western blot–based characterization of clotting components.[11,99] Smaller veins, such as the femoral or saphenous, are more suited to intravital microscopic evaluations, documenting and quantitating acute thrombotic phenomena and responses via fluorophore-labeled thrombus-targeting molecules and cells. Evaluation of thrombus-targeting fluorophore accrual at the injury site is better suited to acute thrombogenesis not exceeding 3 to 4 hours with intravital imaging. In contrast, the low-flow models developed primarily in the IVC form a thrombus gradually, over hours to days, which may have better parallels to clinical DVT.

For IVC thrombosis models, the most common approach is to place a ligature around the IVC just distal to the left renal vein, either tying it completely to cause stasis[64] or tying it over a spacer (0.1–0.36 mm diameter) that is immediately withdrawn, leaving a stenosed lumen with 80% to 90% flow reduction.[65,66,100,101] Subsequent clot growth occurs upstream of the ligation site, generally peaking in growth at ≈48 hours. Other modifications include side- or back-branch ligation or cauterization,[98,101] or combinations of side- and back-branch occlusion, or brief application of a mildly traumatic clamp to the IVC wall as originally developed,[65,66] as an augmenting factor to thrombus initiation. These various manipulations lack direct analogy to clinical DVT in a few important ways: (1) the stenosis/stasis site is downstream of the clot, whereas clinical DVTs seem to have an upstream source; (2) the stasis model is used to form a clot, whereas clinical DVTs precede and progress to stasis, a reversed scenario; (3) the compromised or immediate abruption of flow can alter or prevent thrombolytic processes that are deemed critical to DVT formation and resolution; (4) experiments are performed on young, healthy mice (unlike clinical DVTs which are more common in older/elderly patients); (5) the IVC is a central and critical vein in the mouse and its manipulation may alter the systemic state, unlike some low-flow risk factors of DVT, such as long-haul travel; and (6) the IVC does not contain a valve and, therefore, cannot mimic the initiating events that are thought to take place in hypoxic valve pockets in human valves.[102]

Free-radical injury applied to the outer surface or intraluminally to the endothelial surface is a general approach for thrombus induction in mouse veins, using either ferric chloride[31,67] or electrolytic injury mechanisms.[56] The latter have shown more consistency when applied to the femoral vein[68,75] or IVC[103] to generate both acute and more chronic thrombi. Other models of acute thrombosis have included pinch injury,[69] intraluminal insertion of collagen-dominated surfaces,[61] and microsurgical anastomosis.[63,78] These models induce rapid clot formation, in seconds or minutes, and yield various

outcome measures, from fluorescence detection of thrombotic markers to occlusion times. Under conditions of more severe injury and low-flow induction, thrombus in smaller veins like the femoral can be shown to resolve more slowly.[104]

Use of Venous Thrombosis Models

These vein-based thrombosis models have been used to confirm the effects of various risk factors for DVT on development of larger thrombi. The IVC stenosis models[65,66,100,101] are designed to simulate low venous flow or disturbed flow that occurs in valve pockets, which is assumed to promote DVT in nonambulatory patients. A direct comparison of normal versus low flow in the mouse femoral vein demonstrated larger and more sustained thrombus presence under low-flow conditions.[68] The genetic risk factors, factor V Leiden and prothrombin G21210A, have been modeled in mice either by transgenic lines (for the V Leiden gene) or by infusion of exogenous protein (prothrombin for G21210A). These studies have demonstrated enhanced venous thrombosis over arterial thrombosis in the femoral vein electrolytic injury models.[105,106] For instance, elevated levels of prothrombin increase thrombus size in these models. Microparticles (also known as microvesicles or extracellular vesicles) are small membrane vesicles released from activated and apoptotic cells. TF-bearing microparticles associated with pancreatic tumors have been shown to increase venous thrombus size after IVC stenosis.[107,108] A deficiency of vWF was shown to dramatically reduce the size of the thrombus in an IVC stenosis model.[100] Polyphosphates, stored and released from platelet granules upon activation, have also been found to augment venous thrombosis,[109] indicating a prominent role for platelets in venous thrombus development, which is supported by findings that inhibition of platelets with clopidogrel[81] and other agents[56] reduces venous thrombogenesis.

The role of inflammatory cells on venous thrombosis has been demonstrated with murine models. In recent work, it was found that neutrophils, monocytes, and platelets interact in the developing venous thrombus to promote clot formation,[110] a finding confirming early work in a similar rat version of the IVC stasis model.[110] Neutrophil extracellular traps, extruded nucleic acids and histones from localized neutrophils, have been shown to promote venous thrombus growth in the IVC stenosis model,[111] with histone modification influencing this process.[112] P- and E-selectins were found to have a role in modulating thrombosis in the IVC stasis model, with single- or double-knockouts for these genes having reduced thrombus size at 2 days.[113,114] P-selectin has also been shown to enhance the formation of neutrophil extracellular traps in an IVC stenosis model.[115] Late remodeling of the vein wall has been shown to be influenced by matrix metalloproteinases[114] and other factors.[116,117]

Microvessel Thrombosis Models

Several models have been developed using translucent murine tissues for imaging their microvessels under thrombogenic conditions: the hairless ear,[118] the cremaster muscle,[71,119] and the connective fascia attached to mesenteric structures.[70,120] Both arterioles and venules can be targeted for highly localized laser injury.[71] This model involves use of a precise pulse of laser light induces heat injury to the vessel with subsequent monitoring of the site at high magnification over time using fluorescence microscopy for image acquisition of thrombus-targeting fluorophore accrual. Quantitation of the relative fluorescence of multiple labels over time provides data revealing thrombodynamic responses and interactions within a microvascular environment. An alternative thrombus induction mechanism is to superfuse the microvascular bed with a ferric chloride solution, or to apply a piece of ferric chloride–saturated filter paper, to instill free-radical injury to a large region of microvessels under flow[70]; this approach generally uses microvessel occlusion time as an outcome measure.

Use of Microvessel Thrombosis Models

Early studies with these models revealed findings corroborated by large-vessel models above, such as the role of neutrophils, P-selectin, and the inflammatory response in venous thrombosis[119,121] and the interaction of platelets with vWF in injured venules.[70] TF was found to be a key initiator of arteriolar thrombus formation,[74] with subsequent identification of protein disulfide isomerase as another critical early-response thrombotic element.[122,123] The influence of many other clotting factors on thrombus development has been studied with these microvessel models, such as fibrinogen, vWF, fibronectin, and vitronectin.[124–126] Another important finding was the influence of ADAMTS13 on cleaving VWF multimers and greatly curtailing thrombus formation.[127] The fundamental structure of a clot has been defined in these microvessels as having an inner core of resistant thrombus with an outer shell that has a more transient presence.[128] The capacity of stimulatory molecules to diffusively transport through these regions of the clot has also been shown to regulate thrombus structure and stability.[129]

Summary

Murine models mimic many conditions relevant to clinical thrombosis. These models can be matched to specific vessel types, such as vein, artery, and venule/arteriole, to evaluate thrombotic conditions specific to thrombosis in these vascular structures. Current trends are to create analogy with a particular clinical problem, such as simulating DVT risk factors or inducing arterial thrombosis by plaque rupture in atherosclerotic mice using ultrasound.[60] Interestingly, 2 studies showed that inhibition of the TF/factor VIIa complex reduced early thrombus formation after rupture of the plaques, whereas inhibition of factor XIIa or a reduction of factor XI levels reduced thrombus at later times.[130,131] This suggests that targeting the intrinsic pathway would be safer strategy.[132] Because of the imprecision of these models to directly simulate clinical thrombosis, it is recommended that more than one model of thrombosis is used to assess the role of a particular factor or antithrombotic drug. Future efforts should focus on refining our understanding of how these established models and future developed models fit into the evaluation of thrombogenesis, thrombus resolution, and the development of new therapeutic strategies.

Nonmurine Models

The use of nonmurine models of thrombosis in the investigation of the pathophysiology of thrombosis is far less common than murine models. Usually nonmurine models are used after

the underlying biology of a pathway has been elucidated in other models, such as mice. There are both advantages and disadvantages of large animals of thrombosis (rabbits, dogs, baboons, etc). The advantages include the larger vessel size, as well as blood flow and physiology more similar to humans, including studies of valve function, and they offer a means to evaluate a drug or therapeutic target in a biological setting that is closer to the human patient. The disadvantages include the expense of housing larger animals, the need for infrastructure, animals are outbred, small group sizes because of the expense, a general absence of gene-deleted animals, and there is less cultural acceptance of using nonrodent models, such as dogs and baboons. Indeed, as discussed above there has been a shift away from using dogs for NDA submissions. One large animal of thrombosis that we would like to highlight is the baboon model of venous thrombosis developed by Dr Wakefield and colleagues.[133] This model has been used to study the efficacy of different antithrombotic drugs, including inhibitors of P-selectin, on valve function and recanalization.[133]

The Zebrafish Model

The zebrafish model was introduced to study the genetics of development in the early 1980s by Streisinger.[134,135] The advantages of the model have been noted in an earlier review.[136] Briefly, these advantages are ease of laboratory maintenance because of small size, an ability to study vertebrate-specific functions, and the transparency of embryos.[136] Furthermore, technological advancements, such as mutant generation and complete genome sequencing, have enhanced the genetic capabilities of this model system.[137] Below, is a summary of the advancement of these technologies and their applications to thrombosis and hemostasis.

Modeling Thrombosis and Hemostasis in Zebrafish

It has been shown that zebrafish have human orthologs for the majority of the genes encoding proteins with roles in coagulation, anticoagulation, and platelet signaling pathways.[138–142] Not only are these zebrafish genes syntenic to human genes, functional assays have also shown that extrinsic and intrinsic coagulation factors and several platelet surface receptors are also present in zebrafish blood or thrombocytes.[143,144] Zebrafish have nucleated thrombocytes which are functionally equivalent to enucleated platelets in mammals.[145] Similarly, the vascular endothelium in zebrafish possesses several factors found in human endothelial cells.[146] Therefore, the overall machinery responsible for hemostasis and thrombus formation seems to have evolved in earlier vertebrates and seems to be conserved throughout evolution. This conservation is especially important in identifying novel factors in thrombosis so that factors identified in zebrafish can be studied in murine and nonmurine species and eventually be translated into targets for antithrombotic drugs. Below, is a description of vessel injury–based thrombosis models that give similar results as mammalian models.

Ferric chloride and laser injury methods, which are used in mammalian models, have both been developed for use in the zebrafish.[147] In these models, zebrafish larvae are immobilized in agarose at 3 to 5 days post fertilization and thrombus formation examined under a microscope. In the ferric chloride method, the larvae are first immobilized and ferric chloride is layered on top of the agarose. Because the larvae tails are thinner than the rest of the body the only injuries in the caudal vessels can be observed. This creates a thrombus at the tail and the TTO is measured. It should be noted that this method also generates cellular clumps in the circulation, which may potentially compromise measurement of TTO. A phenylhydrazine-induced thrombosis model has also been developed.[147] In this model, phenylhydrazine is layered onto the larvae immobilized in agarose. Phenylhydrazine is thought to activate flippase, which would externalize phosphatidylserine on red cells and thrombocytes. Thrombocytes rapidly adhere to the endothelial surface after phenylhydrazine treatment and vessel occlusion occurs in the caudal area.

A laser-induced injury model was introduced to address some of the shortcomings of the ferric chloride model. TTO is measured after a nitrogen pulsed laser beam is used to injure larval blood vessels. Two additional parameters can be measured in this model: (1) time to adhesion of the first cells in the vessel and (2) time to dissolution of the thrombus after thrombus formation by laser injury. Shortened TTO, shortened time to adhesion, and prolonged time to dissolution would all be indicators of thrombotic conditions. Because these times are applicable to either arteries or veins, there are 6 different measurements that allow for assaying the strength of thrombosis: arterial TTO, time to adhesion, and time to dissolution and venous TTO, time to adhesion, and time to dissolution.[148]

Mechanisms of Thrombus Formation in Zebrafish

Because thrombosis has not been well characterized in fish, it was important to first understand the basic physiology of thrombus formation. To demonstrate coagulation and fibrin deposition, fluorescein isothiocyanate–labeled fibrinogen was injected intravenously into larvae that were then subjected to vessel injury.[147] This study demonstrated that fibrin formed at the site of injury. In the laser-induced venous thrombosis model, fibrin formed in a half-moon–shaped structure from the endothelial surface of the vessel toward the lumen, whereas in the ferric chloride thrombosis model, fibrin formed in clusters within the caudal vessel.

Without labeled thrombocytes, it was a challenge to show the presence of thrombocytes in the laser-induced arterial thrombosis model. However, specific labeling of thrombocytes with DiI-C18 demonstrated that in arterial thrombosis DiI-C18–labeled thrombocytes accumulated at the site of injury.[149] Subsequently, the use of transgenic zebrafish expressing GFP from the *αIIb* promoter confirmed the participation of thrombocytes in arterial thrombosis.[150] In the DiI-C18–labeled larvae, although thrombocytes accumulated in the arterial thrombus, gaps were observed in the thrombus area. Further studies used mepacrine (green fluorescence) to label the thrombocytes. It seems that mepacrine labels both young and mature thrombocytes, whereas DiI-C18 (red fluorescence) labels only young thrombocytes. Thus, when a DiI-C18/mepacrine mixture was used, young thrombocytes gave an orange

fluorescence (red and green combined) and mature thrombocytes gave a green fluorescence. By using this labeling method, young thrombocytes were found to cluster and initiated the thrombus, whereas mature thrombocytes filled the gaps.[145] Closer examination revealed that the thrombus contained a mosaic of clusters in the following order: initiating clusters of young thrombocytes, clusters of more mature thrombocytes, and alternating clusters of young and mature thrombocytes.

The next development in the zebrafish model was the generation of transgenic fish expressing GFP from the endothelial-specific *Fli1* promotor, which allowed GFP-labeling of thrombocytes.[151,152] In contrast to *αIIb GFP* transgenic fish, laser injury showed endothelial damage as well as thrombocyte aggregation. Interestingly, 2 thrombocyte populations were found in *Fli1 GFP* transgenic fish: intensely labeled thrombocytes and less intensely labeled thrombocytes. The less intensely GFP-labeled thrombocytes seemed to be similar to the population of thrombocytes labeled with DiI-C18.[152] Subsequent to these findings, similar experiments were performed in mice with intravital microscopy, and the thrombus was found to have an initial core of highly activated platelets together with a shell of less activated platelets.[128] Whether the core constitutes young platelets followed by a shell of mature platelets remains to be explored.

Similar to platelet microparticles in mammals, thrombocyte microparticles are present in fish.[153] These microparticles are slightly larger than platelet microparticles, whose functions in hemostasis and thrombosis remain to be defined. However, zebrafish thrombocyte microparticles agglutinate in response to ristocetin.[153] Similarly, microparticles seem to aggregate on the endothelial surface in zebrafish larvae injected with DDAVP.[153] In the laser injury arterial thrombosis model, microparticles seemed to be the first responder to this injury. Taken together, the above results suggest that thrombocyte microparticles act like glue, facilitating thrombocyte adhesion to the subendothelial matrix. Interestingly, G6fl seems to be the collagen receptor in thrombocytes, while the platelet collagen receptor GPVI is not present in fish thrombocytes.[154] It is possible that thrombocyte G6fl may be weaker than platelet GPVI, thus necessitating microparticle facilitation of thrombocyte adhesion. Alternatively, it is possible that platelet microparticles play a similar role in mammalian thrombosis, but that the data supporting such a role are not yet available because it is difficult to distinguish microparticles from platelets with the current technology.

Genetics of Thrombosis

To date, ≈300 factors have been found to participate in hemostasis and thrombosis. The zebrafish model is ideally suited to discover novel factors because it has the power to combine forward and reverse genetics approaches with unbiased screening using the thrombosis models. In forward genetics of thrombosis, zebrafish were subjected to saturated mutagenesis by ethylnitrosourea, and the resulting mutants were screened using the laser-induced thrombosis model. One mutant called *Victoria* was identified that had prolonged TTO, and it was determined to be associated with prothrombin gene by using linkage analysis.[147] Knockdown methods were used in reverse genetics of thrombosis, and proof of principle was provided by knocking down prothrombin gene in zebrafish larvae.[155] Similarly, the Vivo-Morpholino knockdown method was introduced to knockdown different genes, such as *vWF* and *factor VII* in adult zebrafish.[146,156] Recently, the zinc finger nuclease knockout method was used to mutate the *antithrombin III* gene, which modeled disseminated intravascular coagulation in zebrafish.[157] With all these available tools, it should be possible to discover more novel factors that participate in thrombosis. To date, the laser injury model was used in conjunction with knockdown methods to analyze the role of prothrombin, factor VII, factor VIIi, hepsin, FSAP, Mlck1a, protein kinase c α, protein kinase c β, G6fl, and fibrinogen in hemostasis.[147,154–156,158–160] In addition, genome-wide association studies have identified several genes associated with thrombotic disorders, which were then validated with knockdown methods followed by laser-induced thrombosis using the zebrafish model.[161] Table 4 shows the different zebrafish models that have been generated and evaluated in the laser injury thrombosis model.

Future Directions

To date, only zinc finger nuclease knockouts have been performed in zebrafish.[157,162] The recent introduction of the

Table 4. Zebrafish Models of Thrombosis and Hemostasis Subjected to Laser Injury Thrombosis

Genetic Method	Genes Targeted	Phenotype	References
Transgenic	*αIIb promoter-GFP*	Labeled thrombocytes seen with TTO assay	Lin et al[150]
Transgenic	*Fli1 promoter-GFP*	Labeled endothelium and thrombocytes seen with TTO assay	Lawson and Weinstein[151]
Ethylnitrosourea mutagenesis	*Prothrombin*	Altered TTO/thrombus formation	Gregory et al[147]
Morpholino knockdown (MO)	*f7, f7i, prothrombin, vWF, αIIb,G6fl, mlck1a, bambi, lrrc32, dcbld2, esam, PKCα, PKCβ, hepsin*	Altered TTO/thrombus formation	Carrillo et al,[146] Gregory et al,[147] Hughes et al,[154] Day et al,[155] Khandekar and Jagadeeswaran,[156] Tournoij et al,[158] Williams et al,[159] and O'Connor et al[161]
	FSAP	Lack of altered TTO	Khandekar and Jagadeeswaran[156]
Zinc-finger nuclease knockout	*ATIII*	Altered TTO/thrombus formation	Liu et al[157]

TTO indicates time to occlusion.

CRISPR/Cas9 knockout technology makes it possible not only to knockout known genes but also to create a knockout bank and then screen for defects in thrombosis and hemostasis.[163] However, applying the CRISPR/Cas9 technology to a genome-wide search, though feasible, would be an ominous undertaking because of the need for a large amount of fish husbandry. In a newly developed piggyback knockdown technology, an antisense deoxyoligonucleotide can be piggybacked onto a nongene-specific Vivo-Morpholino, usually used as a control.[164] Because this technology only requires a simple injection followed by assaying for the phenotype, it is more practical for conducting genome-wide knockdowns to identify novel genes. The CRISPR/Cas9 technology also allows for the possibility of creating knockin models.[165] Furthermore, the kinetics of thrombus formation could be imaged using injected fluorescent substrates. Thus, the zebrafish model is and will continue to be an asset for the thrombosis field to understand the fundamental aspects of thrombus formation.

Natural Selection and Thrombosis

The evolution of coagulation factors has been discussed in several thoughtful reviews.[166,167] It should be noted that although fish have an extrinsic coagulation pathway and some intrinsic pathway components, the upstream intrinsic pathway components, namely factor XI and factor XII, are not present because they evolved in amphibians.[166] Similar to fish, birds also have thrombocytes. In mice, a deficiency of factor XII does not result in bleeding but protects against arterial thrombosis.[168] Because such thrombosis is an age- and a lifestyle-dependent disease, at least in humans, the contact pathway evolution may not have a role in natural selection as thrombosis would occur past the reproductive age of an organism. Unfortunately, the inferences about platelet evolution have not taken into account the conditions under which mammals evolved. Mammalian evolution occurred in the Triassic period, during which oxygen levels first were abruptly reduced.[169,170] Interestingly, the first mammals are thought to have evolved with constricted blood vessels because of this lack of oxygen,[171,172] and this may have driven the generation of small, enucleated platelets to accommodate the narrow vessels.

The increased blood pressure in mammals means that platelets should be more efficient than thrombocytes in preventing bleeding. It has been argued that the smaller platelets increase surface area for efficient coagulation.[173–175] In fact, the total surface area per microliter of blood for 2-μm diameter human platelets (at least 150 000/μL) is ≈1.5× higher than 6-μm diameter fish thrombocytes (at least 10 000/μL), assuming that they are spherical.[176,177] These data are consistent with the previous arguments; however, when the same calculations are applied to cell volume, the total volume of thrombocytes is almost 2× greater than that of platelets per microliter. In fact, when zebrafish larvae are subjected to arterial laser thrombosis the sheer size of thrombocytes allows them to efficiently fill the lumen, forming an occlusive thrombus.[147] However, under similar conditions, platelets in a mouse do not generate an occlusive thrombus.[128] Therefore, despite the increased platelet activity required for effective hemostasis, their small size limits their ability to fatally occlude the vessel. Interestingly, although most of those species with thrombocytes have become extinct, birds retained thrombocytes, most likely because they evolved a few million years later during a time with higher oxygen levels.[178] However, flight under hypoxic conditions, either from flying at high altitudes or from the high oxygen demands of flying, may have been a selective pressure for losing the contact activation pathway in birds.[166] Although the loss of this pathway may limit thrombocyte activation, fatal thrombosis would be prevented at high altitudes. In addition to the small platelet volume limiting thrombus growth, shear forces may also play a role in inhibiting arterial platelet thrombi. However, there is limited data comparing blood velocity and shear stress among vertebrates. Interestingly, although birds have high blood pressure, their thrombocytes are still able to prevent blood loss. Therefore, it has been suggested that either small thrombocyte aggregates are sufficient to stop bleeding or that birds may have additional hemostatic mechanisms, such as thrombocyte microparticles.[179]

The evolution of megakaryocytes from thrombocytes during the Triassic period suggests that thrombocytes must have had the machinery to evolve into megakaryocytes, which have features of endomitosis and polyploidy.[180] However, it is unknown whether there is an intermediate between thrombocytes and megakaryocytes after mammals radiated from reptiles. Interestingly, a cell line derived from blood cells of the hibernating (hypoxic conditions) tree frog recently demonstrated that thrombocytes develop polyploidy and megakaryocyte-like features.[181] This finding supports the notion that the hypoxic conditions of the Triassic period contributed to the evolution of megakaryocytes.

Conclusions

In this review, we have described the use of animal models ranging from zebrafish to baboons for the study of mechanisms of thrombosis. In addition, mammalian models of thrombosis have been used to evaluate the efficacy and safety of new antithrombotic drugs. Future studies will continue to optimize these animal models of thrombosis and determine the role of potentially new players in thrombosis.

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