In Vivo Thrombus Formation in Murine Models

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Abstract—Platelets play a central role in hemostasis, but also in atherothrombosis, as they rapidly adhere to tissue and to one another as a response to any vascular injury. This process involves a large number of surface receptors, signaling pathways, and enzymatic cascades as well as their complex interplay. Although in vitro experiments proved successful in both identifying new receptors and pathways and developing potent and selective antithrombotic drugs, in vitro research cannot mimic the myriad hemodynamic and spatiotemporal cellular and molecular interactions that occur during the generation and propagation of thrombi in vivo. Animal models, and, with the availability of genetically modified mouse strains and of modern intravital imaging techniques, mouse models in particular, have opened new ways to identify both individual roles and the interplay of platelet proteins in complex in vivo settings. In vivo models revealed the important role of, eg, Gas6 or blood coagulation factor XII in thrombus formation, and results obtained in vivo models raised the interesting possibility that (physiologic) hemostasis and (pathologic) thrombosis might represent 2 mechanistically different processes. This review summarizes in vivo findings that contributed significantly to our understanding of hemostatic and thrombotic processes and which may help to guide future research. (Circ Res. 2007;100:979-991.)

Key Words: thrombosis ■ in vivo models ■ platelets

Platelets, anucleated cells that originate from bone marrow megakaryocytes, circulate in the blood, surveying the integrity of the vascular system. As a response to vascular injury, platelets rapidly adhere to tissue and to one another to form a platelet plug, which, in combination with the coagulation system, allows the reestablishment of normal blood flow in the disrupted vasculature. Neither platelets nor other components of the hemostatic process can distinguish between traumatic wounds and lesions that occur in diseased vessels, eg, on rupture of an atherosclerotic plaque. Therefore, uncontrolled platelet activation in diseased vessels may lead to arterial occlusion and infarction of vital organs. Increasing knowledge of the mechanisms of platelet function has triggered the development of potent and selective antithrombotic drugs, and first-generation antiplatelet compounds that specifically block distinct activation pathways or effector functions have already proved beneficial in the clinic. Despite these important developments, acute ischemic cardiovascular and cerebrovascular syndromes are still a major cause of death or serious pathological complications in Western soci-
eties. To inhibit the thrombotic and proinflammatory activity of platelets, while preserving their hemostatic function, a more detailed understanding of both the hemostatic cascade and pathological thrombus formation is required.

Both hemostasis and arterial thrombosis are thought to be initiated by the disruption of the endothelial layer and exposure of the subendothelial extracellular matrix (ECM). These processes trigger a complex series of events, including platelet adhesion, activation, aggregation, granule release, and coagulant activity, finally leading to the formation of fibrin-rich thrombi at the site of injury within minutes. A large number of surface receptors, activatory and inhibitory signaling pathways, and enzymatic cascades and their complex interplay are involved. In vitro studies on platelet function have been and still are of paramount importance in both basic and clinical research as well as clinical diagnostics. Studies performed on material from patients with bleeding disorders considerably contributed to our understanding of the underlying mechanisms relevant to normal hemostasis and, subsequently, thrombosis. Bernard–Soulier syndrome, described in 1948, and Glanzmann’s disease, described back in 1918, were the first disorders discovered involving platelet receptors. A detailed analysis of platelets from these patients in the 1970s and 1980s identified the affected receptors and provided evidence for their function. When animal models of these diseases were established later, they basically proved what was known from humans already. However, it was a pioneering finding that physiology and pathology of hemostasis and thrombosis can be mimicked in animals.

The ability to isolate one factor and study the mechanisms by which that factor contributes to thrombosis is a strong argument for performing in vitro experiments in which individual components can be studied in detail. These approaches were not only successful in studying patients lacking certain molecules but also in identifying important new receptors, such as collagen receptors and ADP receptors. Although these approaches give evidence what the principal function of the molecules is, it occurs that in vitro findings are difficult to correlate with their true in vivo relevance. They cannot mimic the myriad hemodynamic and localized cellular and molecular interactions that occur during the generation and propagation of thrombi in vivo. These limitations can be overcome by studies on thrombus formation in the intact living organism which are, however, excluded in humans. Much effort has been spent on the development of animal models for platelet-related physiological and pathophysiological processes, such as hemostasis, arterial thrombosis, atherosclerosis, and wound healing. During the last decade, the quality of these models has improved enormously by the advent of intravital imaging techniques that allow the detection of individual cell–cell interactions or spatiotemporal activation processes within a growing thrombus at sites of injury in the living animal.

Most of the findings of in vivo thrombus formation summarized and discussed in this review were generated in mice. Because of its small size, high fertility, and exceptional reproductive capacity, the common laboratory mouse has become the most frequently used inbred animal species for biological research purposes. The advent of genetic methods that allow targeted manipulations in the mouse genome has opened new ways to study protein function and has been enormously successful in unraveling signal transduction pathways in platelets both in vitro and in vivo. Mutations introduced into the germline can range from null mutations to subtle changes in coding or noncoding sequences of genes, chromosomal translocations, and spatially and temporally restricted gene deletions using the Cre/loxP system, techniques that theoretically open unlimited thrombosis research in vivo. Extrapolation of data from mouse to human platelets, however, requires a thorough determination of murine hemostasis with regard to coagulation and fibrinolytic systems, platelet structure, and platelet receptor/enzyme systems. Differences between the 2 species must be acknowledged and appreciated in the interpretation of the data. Platelet counts in mice on average are four times those of humans and platelets are only approximately one-half of the volume of human platelets. Receptors and signaling pathways in mouse platelets show striking similarities to the human system, with virtually every protein represented and with every cascade appearing to serve similar functions in both species. Nonetheless, differences exist in the expression of individual proteins (eg, protease-activated receptors [PARs], FcγRIIα), at the molecular structural level (varying degrees of homology of proteins) and, therefore, potentially also at the functional level. In addition, there is evidence that genetic differences between mouse strains determine differences in hemostasis and thrombosis. The inbred mouse strains C57BL/6J and A/J have been reported to differ in thrombus occlusion time, and Li et al have described a genetic variation in integrin α2 expression that accounts for differences in platelet responses to collagen. An excellent review of the murine hemostatic system has been provided by Tsakiris et al, and aspects of murine platelet receptor functions have been addressed recently by Ware.

Besides mice, other animal models play an important role in thrombosis research as well. Historically, rabbit models have been used extensively since the introduction of the Wessler model in 1952. Because of their size, rabbits, but also rats, are valuable models for studies involving transmural angioplasty and thrombotic events and restenosis associated therewith. Guinea pigs are used because their platelets share, to some extent, similarity with human platelets, including a close structural homology of glycoprotein (GP) IIb and von Willebrand factor (vWF); in addition, in contrast to mouse platelets, guinea pig platelets express PAR-1 and PAR-4. More advanced experimental models include porcine, canine, and nonhuman primate thrombosis models, but they are usually reserved for advanced stages of drug or biocompatibility testing, as they require special facilities, and their maintenance is difficult and expensive. Pigs are prone to develop atherosclerosis even on a normal diet and thus are useful animals in studying atherothrombosis. They are also valuable for studies involving angioplasty. In addition, a pig model of von Willebrand disease was established demonstrating that vWF in plasma, but not in platelets, is essential for the development of arterial thrombi. A famous example for the use of the canine model is when Coller and Scudder...
demonstrated that F(ab′)2 fragment of the monoclonal antibody 7E3 against GPIb/IIa inhibited platelet function in dogs,24 and dogs are still used for interventional studies.

Because of high similarity between human and nonhuman primate hemostasis, the nonhuman primate thrombosis model is thought to be the best model to evaluate novel thrombotic agents. One of the more commonly used models is the baboon arteriovenous shunt model, in which a thrombogenic segment is placed in an exteriorized chronic arteriovenous shunt. Blood is pumped at a specific shear rate through the segment, and the thrombus is quantitated by deposition of radiolabeled platelets or fibrin on the segment.25 In such a model, the therapeutic use of anti-human FXI antibodies was evaluated, demonstrating that they interfere with thrombus formation without prolonging bleeding time or affecting the prothrombin time.26 Inhibition of the vWF–collagen interaction by an antihuman vWF monoclonal antibody results in abolishment of in vivo arterial platelet thrombus formation in baboons,27,28 and GPIb blocking monoclonal antibody Fab fragments displayed antithrombotic effects in the same model as well.28

Based on ethical, experimental, and economic considerations, thrombosis research in vivo will most likely continue to focus on the use of murine models. Nonetheless, other models are being established that allow even faster genetic approaches to study the principal function of certain molecules in blood clotting. Only recently, the zebrafish system has been proposed as an additional model to study hemostasis and thrombosis.29 It appears that many pathways in zebrafish are comparable to those we currently know from mammals. Two important advantages of zebrafish are easy knockdown of individual genes with antisense morpholino oligonucleotides and the transparency of embryos, which will allow studies of cell–cell interaction not simply in vivo but in the entirely intact organism. Currently, it remains speculative whether this model will be more than a quick screening method for the contribution of newly discovered genes in the future.

A comprehensive analysis of all aspects of platelet function in thrombus formation is far beyond the scope of this review. Rather, we summarize data derived from in vivo studies on thrombus formation that have been performed in the mouse and have used intravital imaging techniques. A short summary of the key findings made in these studies is shown in the Table.

**Thrombus Induction**

A large number of experimental models have been established to allow in vivo observation of thrombus formation in mice. Arterial lesions can be induced in different branches of the vascular system and by different methods (reviewed by Day et al30). One procedure to induce injury is direct application of ferric chloride (FeCl3) to the adventitial surface of an artery which results in the formation of occluding platelet-rich thrombi.31 The exact mechanism by which thrombus formation is triggered in this model is not clear, but it has been shown that the morphology of the thrombi is similar to those found in humans.32 This model is widely used with direct blood vessel visualization by intravital microscopy or modified with a Doppler flow probe to monitor vessel occlusion. Another chemical method to trigger thrombosis involves the intravenous injection of the photoactive substance Rose Bengal, which rapidly accumulates in the membranes of endothelial and other cells. The subsequent exposure of an arterial segment to green light (540 nm) locally triggers a photochemical reaction, resulting in the formation of reactive oxygen species that damage the endothelium and induce the formation of occlusive thrombi within 30 to 40 minutes.33,34 More recently, various groups have started to use lasers to induce endothelial injury in the microcirculation,35 an injury that typically triggers the formation of nonoccluding thrombi within a few minutes. The laser pulse, applied through the microscope optics, is targeted at the vessel wall, with intensity and exposure time of the laser being variable. This allows the production of lesions of different type and severity. It has been proposed that the severity of laser-induced injury determines the pathway of platelet activation at the site of injury.36 A superficial injury has been proposed to induce thrombus formation predominantly via collagen fibers, whereas a deep injury is supposed to induce thrombosis primarily via thrombin. Although more data are required, these findings may help to explain why partly contradictory data on the involvement of collagen and/or thrombin as the major trigger of thrombus formation have been obtained with this model.

Endothelial denudation can also be induced mechanically in large arteries (aorta, carotid artery, femoral artery), either by direct damage of the luminal side of the vessel using a guide wire57,38 or indirectly by compression or ligation of the vessel using a forceps or a filament59,40 or by a combination of these methods.41

Optimized visualization of the thrombotic event with integrated intravital widefield and confocal digital microscopy has further improved the analysis of hemostatic and thrombotic mechanisms in the living animal. Single steps of the complex process, such as calcium mobilization in platelets and translocation of P-selectin as indicators of platelet activation or uptake of tissue factor and fibrin generation in the developing thrombus, can now be elegantly dissected in vivo under real-time conditions.35,42,43

It is important to note that each model has its specific advantages and limitations. The laser injury model appears to be more similar to inflammatory reactions associated with thrombin-induced thrombosis, whereas the FeCl3 model may induce thrombosis primarily via the collagen/GPVI axis.41,44,45 It is yet unclear which model (if any) is closest to the relevant pathomechanism in humans, as each might only be loosely linked to distinguishable thrombotic events in humans. All models remain artificial because for technical reasons experiments are performed in mice, an animal model not prone to atherothrombosis, and many of them are performed in arterioles rather than large arteries. Most importantly, thrombus formation is triggered in normal healthy blood vessels, whereas arterial thrombosis in humans, eg, in myocardial infarction, occurs in diseased areas of the vascular system. Nonetheless, studies of thrombus formation in vivo offer important steps toward an understanding of events associated with atherothrombosis in humans.
### Phenotype of Mice With Defined Platelet/Coagulation Defects Studied In Vivo for Thrombus Formation

<table>
<thead>
<tr>
<th>Type of Defect</th>
<th>Impact on Hemostasis*</th>
<th>Impact on Thrombus Formation</th>
<th>Characteristics of In Vivo Thrombus Formation</th>
<th>Reference</th>
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<td>Receptors</td>
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<tr>
<td>$\alpha_2$-/-</td>
<td>No</td>
<td>No</td>
<td>Unchanged (probably delayed)</td>
<td>Holtkotter et al; Gruner et al; He et al</td>
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<tr>
<td>$\beta_3$-/-</td>
<td>Yes</td>
<td>Yes</td>
<td>No thrombus formation</td>
<td>Massberg et al</td>
</tr>
<tr>
<td>blockage of GP Ibalpha</td>
<td>NT</td>
<td>Yes</td>
<td>No thrombus formation</td>
<td>Massberg et al</td>
</tr>
<tr>
<td>IL4Ralpha/GP Ibalpha-tg</td>
<td>Yes</td>
<td>Yes</td>
<td>No adhesion, no thrombus formation</td>
<td>Bergmeier et al</td>
</tr>
<tr>
<td>GPV-/-</td>
<td>No (probably decreased bleeding)</td>
<td>Yes</td>
<td>Accelerated (but decreased tendency to form occlusive thrombi)</td>
<td>Kahn et al; Ni et al; Moog et al</td>
</tr>
<tr>
<td>Depletion of GPVI</td>
<td>NT</td>
<td>Yes</td>
<td>Defective adhesion and thrombus formation</td>
<td>Massberg et al</td>
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<td>FcRy-/-</td>
<td>NT</td>
<td>Yes</td>
<td>Defective adhesion and thrombus formation</td>
<td>Gruner et al; Konishi et al</td>
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<tr>
<td>P2Y1-/-</td>
<td>Yes (moderate)</td>
<td>Yes</td>
<td>Protected from thromboembolism</td>
<td>Fabre et al; Leon et al</td>
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<tr>
<td>P2Y12-/-</td>
<td>Yes</td>
<td>Yes</td>
<td>Delayed, small, unstable thrombi</td>
<td>Andre et al</td>
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<td>P2X1-/-</td>
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<td>Yes</td>
<td>Decreased thrombus size, protected from thromboembolism</td>
<td>Hechler et al</td>
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<td>PAR-3-/-</td>
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<td>Yes</td>
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<td>Weiss et al</td>
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<td>PAR-4-/-</td>
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<td>Yes</td>
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<td>Weiss et al</td>
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<td>$\alpha_2$-/-</td>
<td>Yes (moderate)</td>
<td>Yes</td>
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<td>Pozgajova et al</td>
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<td>Mer-/-</td>
<td>Yes (rebleeding)</td>
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<td>Small thrombi, partially protected from thromboembolism</td>
<td>Chen et al; Angelillo-Scherrer et al</td>
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<td>Axl-/-</td>
<td>Yes (rebleeding)</td>
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<td>Small thrombi, protected from thromboembolism</td>
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<tr>
<td>Tyro3-/-</td>
<td>Yes (rebleeding)</td>
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<td>CD150</td>
<td>No</td>
<td>Yes</td>
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<td>Nanda et al</td>
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<td>Fgr/Δ5</td>
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<td>Yes</td>
<td>Unstable thrombi</td>
<td>Holmback et al; Ni et al</td>
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<td>vWF-/-</td>
<td>Yes</td>
<td>Yes</td>
<td>Defective thrombus formation</td>
<td>Denis et al</td>
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<tr>
<td>Compound fibrinogen-/-, vWF-/-</td>
<td>NT</td>
<td>Yes</td>
<td>Defective thrombus formation (highly fragile thrombi)</td>
<td>Ni et al</td>
</tr>
<tr>
<td>Low plasma fibronectin</td>
<td>No</td>
<td>Yes</td>
<td>Delayed thrombus formation</td>
<td>Sakai et al; Ni et al</td>
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<tr>
<td>Vitronec-/-</td>
<td>No</td>
<td>?</td>
<td>Enhanced formation or unstable thrombi</td>
<td>Foy et al; Konstantinides et al</td>
</tr>
<tr>
<td>Thrombospondin-1-/-</td>
<td>NT</td>
<td>No</td>
<td>Unchanged</td>
<td>Ni et al; Chou et al; Parry et al</td>
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<td>Low tissue factor</td>
<td>No</td>
<td>Yes</td>
<td>Small thrombi</td>
<td>Andre et al</td>
</tr>
<tr>
<td>CD40L-/-</td>
<td>No</td>
<td>Yes</td>
<td>Unstable, loosely packed thrombi</td>
<td>Angelillo-Scherrer et al</td>
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<tr>
<td>Gas6-/-</td>
<td>No</td>
<td>Yes</td>
<td>Small thrombi, protected from thromboembolism</td>
<td>Angelillo-Scherrer et al</td>
</tr>
<tr>
<td>G proteins</td>
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<td></td>
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<tr>
<td>Gq-/-</td>
<td>Yes</td>
<td>Yes</td>
<td>No thrombus formation</td>
<td>Offermanns et al; B. Nieswandt, M. Pozgajova, S. Offermanns, unpublished data, 2004</td>
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<td>Gq13-/-</td>
<td>No</td>
<td>No</td>
<td>Unchanged</td>
<td>Moers et al</td>
</tr>
<tr>
<td>Gq15/fl/fl Cre-</td>
<td>Yes</td>
<td>Yes</td>
<td>No thrombus formation</td>
<td>Moers et al</td>
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(Continued)
Platelet Tethering and Adhesion

The initial tethering of platelets at sites of vascular injury requires the action of a receptor that functions irrespective of cellular activation and thereby facilitates rapid interactions that resist shear forces. This process is mediated by GPIb-V-IX, a platelet-specific receptor encoded by 4 different genes, the α and β subunits of GPIb, GPIX, and GPV.46,47 Mice deficient in GPIbα or GPIbβ lack the entire receptor complex and reflect human Bernard–Soulier syndrome, as they display a severe bleeding phenotype. Increased bleeding is attributed to the lack of the extracellular domain of GPIb and the loss of the binding ability of the receptor to several ligands including vWF or thrombin.48 However, Bernard–Soulier platelets are also large and abnormally shaped and have been shown to possess unusually deformable membranes and a decreased negative charge.49,50 Inhibition of the vWF-binding site on the receptor with Fab fragments of the antibody p0p/B in wild-type mice abrogates platelet tethering and adhesion at the injured arterial wall,39 reflecting a mandatory role of GP Ib-V-IX in this process. This was only very recently confirmed by an elegant study by Bergmeier et al, who demonstrated that arterial thrombus formation is completely abolished in transgenic mice expressing GPIbα in which the extracellular domain was replaced by that of the human interleukin-4 receptor.51

Absence of the specific GP Ib-V-IX ligand vWF leads to prolonged bleeding times and spontaneous bleeding in ~10% of newborn vWF−/− mice. In addition, vWF−/− mice display defective thrombus formation in FeCl3-injured arterioles.52 In contrast to GPIb, vWF is not essential for thrombus formation, as much delayed adhesion still occurs even under arterial flow conditions. This indicates that GPIb can initiate adhesion by interacting with other ligands, and one of the strongest candidates is thrombospondin-1, which has been shown to interact with GPIb under high shear flow conditions in vitro.53

In contrast to vWF, subendothelial fibrillar collagen is a highly efficient substrate in supporting firm platelet adhesion and thrombus formation, because it directly activates the cells. Besides GPIb-IX-V and αIIbβ3, which indirectly interact with collagen via vWF, several collagen receptors have been identified on platelets, including integrin α5β1 and GPVI.54 The immunoglobulin superfamily member GPVI is a platelet/megakaryocyte-specific low-affinity collagen receptor that noncovalently associates with the FcRγ chain, which serves as the signal-transducing subunit of the receptor. Platelets in which GPVI has been depleted by in vivo administration of antibodies against the receptor55 do not respond to collagen. These platelets fail to firmly adhere to the immobilized protein under high or low shear flow conditions because of defective activation of integrins αIIbβ3 and α5β1.56 Similar observations were also made with platelets from FcRγ-chain knockout mice, which fail to express GPVI, or with wild-type platelets, in which the ligand binding site of GPVI had been blocked by the addition of JAQ1 in vitro. The generation of GPVI germline knockout mice was reported in 2003 by Kato et al and confirmed the essential role of this receptor for collagen-induced platelet activation and spreading on the matrix protein.57 Mice lacking the GPVI/FcRγ chain complex show no major bleeding phenotype, but they are profoundly protected from arterial thrombosis. Konishi et al found markedly reduced platelet attachment and subsequent neointimal hyperplasia at sites of guide wire-induced arterial injury in FcRγ-chain-deficient mice.57 Similarly, Massberg et al observed dramatically reduced thrombus formation in FeCl3-, or mechanically injured arteries of GPVI-depleted mice. As shown by in vivo fluorescence microscopy, platelet tethering/slow surface translocation at sites of arterial injury, as well as firm adhesion, is impaired in those animals.58 This surprising observation indicates that GPIb and GPVI/FcRγ chain may act in concert to recruit platelets to the exposed subendothelial matrix under conditions of high shear.

In contrast to these findings, FcRγ chain–deficient mice were reported to display normal thrombus formation in a laser injury model.44 Normalized for thrombus size, thrombus-associated tissue factor was 5-fold higher in laser-induced thrombi than in FeCl3-induced thrombi,44 indicating that thrombin-mediated thrombus formation is more important after laser injury than platelet activation via collagen/GPVI. An important role for thrombin in overcoming GPVI deficiency has also been reported by Mangin et al in a Folts-type stenosis-injury model and a model of full thickness vascular injury induced by electrical current. These authors conclude that GPVI has a greater functional redundancy in arterial thrombogenesis than previously recognized. However, as outlined above, it is currently difficult to judge which animal model (if any) is closest to the relevant pathomechanism in humans. In contrast to the findings by Mangin, an in vivo model of carotid artery balloon injury in rabbits (mimicking thromboembolic complications after coronary interventions) demonstrated that local delivery of soluble GPVI efficiently inhibits thrombus formation.59 Again, these data are not without controversy as others have found no inhibitory effect of soluble GPVI, even at extremely high concentrations, whereas anti-GPVI antibodies were fully protective.59 Together, these observations indicate that the type of

<table>
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<th>Characteristics of In Vivo Thrombus Formation</th>
<th>Reference</th>
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<td>FIX−/−</td>
<td>Yes</td>
<td>Yes</td>
<td>No thrombus formation</td>
<td>Wang et al118</td>
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<tr>
<td>FXI−/−</td>
<td>No</td>
<td>Yes</td>
<td>No thrombus formation</td>
<td>Wang et al118</td>
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<td>FXII−/−</td>
<td>No</td>
<td>Yes</td>
<td>Unstable, small thrombi, protected from thromboembolism</td>
<td>Renne et al115; Kleinschnitz et al112</td>
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*As assessed by bleeding time assays. NT indicates not tested.
injury determines whether collagen or thrombin acts as the major stimulus to initiate platelet adhesion and subsequent thrombus growth in these animal models (Figure 1). Recently, Penz et al provided evidence that atheromatous plaques material derived from human carotid arteries induces thrombus formation in anticoagulated blood only in the presence, but not in the absence of functional GPVI. As tissue factor activity was absent, platelet GPVI was essential for mediating plaque-induced thrombus formation in these studies. Additional in vivo data obtained in higher species will be required to evaluate the antithrombotic potential of inhibition of the GPVI–collagen axis in arterial thrombosis.

Figure 1. Model for platelet adhesion to the subendothelial matrix at sites of vascular injury. The initial contact (tethering) to the ECM is mediated predominantly by GPIb–vWF interactions. The GPIb–vWF interaction is essential at high shear rates (>500 sec⁻¹) but may not be required at lower-shear rates. In a second step, GPVI–collagen interactions initiate cellular activation followed by shifting of integrins to high-affinity state and the release of second-wave agonists, most importantly ADP, ATP, and TxA₂. GPIb-mediated signaling may amplify GPVI-induced activation pathways. In parallel, exposed tissue factor (TF) locally triggers the formation of thrombin, which in addition to GPVI, mediates cellular activation. Depending on the nature of the injury, 1 of the 2 activation pathways may prevail and sufficient to arrest and activate platelets. Finally, firm adhesion of platelets to collagen through activated α2β1 (directly) and α5β3 (indirectly via vWF or other ligands) results in sustained GPVI signaling, enhanced release, and procoagulant activity. In this process, α2β1 and α5β3 have partially redundant roles. Released ADP, ATP, and TxA₂ amplify integrin activation on adherent platelets and mediate thrombus growth by activating additional platelets. This scheme does not exclude the involvement of other receptor-ligand interactions.

Figure 2. Involvement of FXII in pathological thrombus formation. a, Mice received fluorescently labeled platelets and thrombosis was monitored on mesenteric arterioles after topical application of 20% FeCl₃ by in vivo fluorescence microscopy. Note the severe defect in thrombus formation in both FXI⁻/⁻ and FXII⁻/⁻ mice. Representative pictures of 1 experiment. b, A model of thrombin formation at sites of vascular injury. The initiation of thrombus formation at sites of vascular lesions is predominantly attributable to tissue factor (TF) and collagens exposed in the subendothelial matrix. Tissue factor in complex with FVII initiates thrombin formation, which promotes fibrin formation and platelet activation. The contribution of FXII in this early phase of thrombus formation is minor. On platelet adhesion and activation, FXII activity on the exposed surface of the thrombus contributes to thrombin generation and additional platelet activation, propagating thrombus growth. Accordingly, FXII, as well as factor XI, deficiency severely impairs thrombus formation. This model does not exclude the possibility that the 2 pathways of thrombin generation are linked by unidentified mechanisms.
The second collagen receptor on the platelet surface, integrin \(\alpha_{IIb}\beta_3\), plays a significant, but not an essential, role for the adhesion process of platelets.\(^6\) Two \(\alpha\)-deficient mouse lines were independently reported in 2002.\(^{62,63}\) These mice have normal tail-bleeding times and display only minor defects in their adhesion and aggregation response to native fibrillar collagen.\(^6\) In line with this, these mice form occlusive thrombi in the injured carotid artery, although, in one study, this was found to be delayed.\(^{61,64}\) This rather mild defect can be explained by the fact that multiple integrin–ligand interactions contribute to platelet adhesion at sites of injury. As demonstrated by Gruner et al, unaltered platelet adhesion and thrombus formation in the injured carotid artery was found even in mice with a Cre/loxP-mediated loss of the integrin \(\beta_3\) subunit in platelets, which, besides \(\alpha_{IIb}\beta_3\), also lack \(\alpha_{IIa}\beta_1\) (fibronectin receptor) and \(\alpha_{IIa}\beta_1\) (laminin receptor). In those mice, platelet adhesion at the site of injury was mediated exclusively by integrin \(\alpha_{IIa}\beta_1\), which interacts with various ligands, including vWF and fibronectin.\(^6\) Inhibition of \(\alpha_{IIb}\beta_3\) in wild-type mice reduces platelet adhesion by \(\approx 60\%\) suggesting that this integrin is the principal receptor not only for aggregation (see below) but also for adhesion to the ECM in vivo. Recent studies indicate that \(\alpha_{IIb}\beta_3\) may require strong activation signals, such as provided by GPVI, to mediate shear-resistant adhesion, whereas \(\alpha_{IIa}\beta_1\) can arrest the cells also under conditions of weak integrin activation.\(^40\)

### Platelet Activation

Following initial adhesion of platelets to the ECM, extension of the thrombus requires a rapid response of platelets to locally produced and released soluble agonists, including thrombin, ADP, thromboxane A\(_2\) (TXA\(_2\)), ATP, and epinephrine, which amplify and sustain the initial platelet response, recruit circulating platelets from the flowing blood, and thereby promote thrombus growth and stability. Thrombin is rapidly generated at sites of vascular injury and represents the most potent platelet activator. Platelet activation by thrombin induces shape change, secretion, and aggregation. Platelet responses to thrombin are largely mediated by members of the PAR family, with PAR-1 and -4 being expressed in human platelets and PAR-3 and -4 in mouse platelets.\(^65\) The fact that mice express a different set of PARs in platelets has somewhat hampered in vivo studies on these receptors that might be of direct relevance for humans. Nevertheless, studies in mice lacking PAR-3 or PAR-4 have provided insight into a 2-receptor-mediated activation mechanism that may be of basic interest. PAR-3 in mice was shown to mediate effects of low thrombin concentrations. Absence of PAR-3 causes a markedly delayed and reduced, but not absent, response to thrombin\(^66\) and a protection against FeCl\(_3\)-induced thrombosis of mesenteric arterioles.\(^67\) PAR-4-deficient platelets show a similar degree of protection in comparable in vivo studies.\(^67\) However, these cells are completely resistant to thrombin,\(^68\) indicating that PAR-4 is the sole signaling protease-activated thrombin receptor in mouse platelets, but fully intact thrombin responses are required for normal platelet activation.

ADP is released from internal stores of activated cells and potentiates many platelet responses, including integrin \(\alpha_{IIb}\beta_3\)-mediated \(\alpha_{IIb}\beta_3\)-dependent procoagulant activity. Platelets express 2 different ADP receptors, P2Y\(_1\) and P2Y\(_12\). In vivo, P2Y\(_1\)-null mice have moderately increased bleeding times and are resistant against collagen/epinephrine or ADP-induced thromboembolism.\(^69\) In contrast, transgenic mice overexpressing P2Y\(_1\) in their platelets have a shortened bleeding time and are more susceptible to ADP- and collagen-induced thromboembolism and to arterial thrombosis triggered by FeCl\(_3\).\(^70\) P2Y\(_12\)-null animals have a prolonged bleeding time and are protected from arterial thrombosis as measured in the FeCl\(_3\) model.\(^71\) These in vivo observations identify the P2Y\(_12\) receptor as the major ADP receptor to amplify and sustain platelet activation.

TXA\(_2\) is produced by platelets from arachidonic acid via the cyclooxygenase pathway. Once formed, it can diffuse across the platelet membrane and activate other platelets. In platelets, activation of the TXA\(_2\) receptors TP\(_\alpha\) and TP\(_\beta\) triggers shape change, secretion, hydrolysis of phosphoinositides, increment in cytosolic calcium, and protein phosphorylation, but does not influence cAMP synthesis. TP-null mice have prolonged bleeding times and do not respond to TXA\(_2\).\(^72\) Furthermore, these platelets display delayed aggregation in response to collagen.

In contrast to the abovementioned agonists, ATP and epinephrine are not full platelet activators per se. However, they contribute significantly to thrombus growth and stability, as absence of their receptors has been demonstrated to severely impair thrombus formation. Mice lacking the fast ATP-gated P2X\(_3\) cation channel are partially protected from collagen/adrenaline-induced thromboembolism. The size of the thrombus after a laser-induced vessel wall injury in small arteries is decreased in these mice.\(^73\) This can be explained by impaired aggregation and secretion of these platelets in response to collagen and by a reduced ability to adhere to the matrix protein under high shear conditions.

Adrenaline is able to potentiate the effect of other platelet stimuli by activating the G\(_i\)-coupled adrenergic \(\alpha_{2A}\) receptor, resulting in the inhibition of adenyl cyclase. Recent data from our group show that mice lacking the \(\alpha_{2A}\) receptor have a moderate hemostatic defect and display enhanced embolus formation in both the FeCl\(_3\) and the aorta occlusion model. These results demonstrate a significant and previously not anticipated role for adrenaline in thrombus stabilization in vivo.\(^74\)

The \(\alpha_{2A}\) receptor belongs to the family of G protein–coupled receptors, a family of molecules that on binding soluble platelet activators, including thrombin, ADP, TXA\(_2\), and adrenaline, activates G proteins, namely G\(_i\), G\(_q\), G\(_\alpha_{12}\), and G\(_\alpha_{13}\). A detailed overview of G protein signaling in platelets is provided by Offermanns et al in this review series.\(^74\) Because studies on in vivo thrombosis in mice deficient in G proteins helped to understand platelet physiology significantly, some aspects will be addressed briefly.

Mice lacking the \(G\alpha\) family member \(G\alpha_\alpha\) demonstrated a comparable phenotype as mice lacking the \(\alpha_{2A}\) adrenergic receptor, including resistance to lethal thromboembolism induced by collagen/adrenaline, but not collagen/ADP.\(^75\) No such findings were reported from mice lacking \(G\alpha_\alpha\) family members \(G\alpha_\epsilon\) and \(G\alpha_\beta\), indicating that \(\alpha_{2A}\)-adrenergic recep-
tors are coupled to $G_\alpha$ to mediate inhibition of adenylyl cyclase.75

Mouse platelets that lack $G_\alpha$ do not aggregate and fail to secrete their granule contents in response to thrombin, ADP and TXA$_2$. Accordingly, these mice display massively prolonged bleeding times and are protected from both collagen/adrenaline-induced thromboembolism76 and arterial thrombosis in different models (B.N., unpublished observations), demonstrating a central role for $G_\alpha$ in platelet activation. Interestingly, lack of $G_\alpha$ does not interfere with the ability of TXA$_2$ and thrombin to induce platelet shape change, indicating that this process is mediated by G proteins other than $G_\alpha$, namely $G_{12/13}$, but not $G_\gamma$.77 These findings were further substantiated in $G_\alpha$ and $G_\gamma$ mutant mouse lines. In $G_\alpha$-$G_\gamma$-deficient platelets, the rapid platelet shape change induced by low concentrations of stimuli like TXA$_2$, thrombin, or collagen is completely abolished. Accordingly, mice deficient in $G_{13}$ show a markedly increased bleeding time and fail to form stable occlusive thrombi in a model of carotid artery injury.78 Thus, $G_{13}$ is essentially required for platelet activation, whereas $G_{12}$ appears to be dispensable for this process.

Platelets deficient in both $G_{13}$ and $G_\alpha$ do not respond to TXA$_2$, ADP, or thrombin and do not aggregate in response to collagen. However, primary adhesion to collagen occurs normally in those mice79 suggesting that platelet adhesion can occur largely independent of G protein–mediated signaling, whereas thrombus growth requires activation of at least 2 different G proteins. This is further supported by findings demonstrating that concomitant stimulation of $G_\alpha$ and $G_{12/13}$80,81, or $G_\gamma$ and $G_{12}$82,83 is sufficient to induce platelet aggregation. Thus, full platelet activation through G protein–coupled receptors is a highly integrated process, involving $G_\alpha$, $G_{12/13}$, and G/G$_\gamma$-mediated signaling pathways. It has to be kept in mind that soluble agonists such as ADP, TXA$_2$, adrenaline, or thrombin do not act on their own, but stimulation of platelets with either agonist results in the release of another. This, of course, is also reflected by an interdependency of the subsequently activated signaling pathways. For example, mice deficient in $G_{13}$ (downstream of P2Y$_{12}$) show decreased ADP-induced platelet aggregation, but the responses to thrombin and TXA$_2$ are also impaired although these agonists act primarily through $G_\alpha$ and $G_{13}$.84,85

The final common mechanism of the different signaling pathways initiated via GP Ib-IX, GPVI, and the abovementioned soluble agonists is activation of integrin $\alpha_{IIb}\beta_3$, the principal receptor for adhesion and aggregation. An important process is inhibition or reversal of ligand binding to $\alpha_{IIb}\beta_3$, which is regulated by a number of soluble substances including prostacyclin (PGI$_2$) and nitric oxide (NO).86 Platelet endothelial cell adhesion molecule 1 (PECAM-1), an immunoglobulin superfamily receptor whose immunoreceptor tyrosine-based inhibitory motifs (ITIMs) recruit SHP-1 and SHP-2 tyrosine phosphatases, has been demonstrated to dampen signaling through GPVI and GPlb-IX in vitro.87,88 Recently Falati et al elegantly demonstrated in a laser injury model that the thrombi formed in PECAM-1–deficient mice were larger, formed more rapidly, and were more stable than those in controls.48 In addition, in the FeCl$_3$ model, time to vessel occlusion was significantly decreased in PECAM-1$^{-/-}$ mice. These data demonstrate for the first time in vivo that inhibitory signals are involved in the regulation of the balance between platelet activation and inhibition and possibly contribute to the restriction of a thrombus to the site of injury.

**Platelet Aggregation and the Gap Between Adjacent Platelets**

Platelet aggregation is essential for the formation of the hemostatic plug at sites of vascular injury. This process is primarily mediated by $\alpha_{IIb}\beta_3$, which on activation immobilizes a number of different adhesive substrates to the membrane of the activated platelet. The immobilization step is a prerequisite for both thrombus growth and stabilization. Accordingly, mice lacking $\beta_3$ integrin resemble the phenotype of Glanzmann thrombasthenia with absent platelet aggregation, reduced clot retraction, and greatly reduced fibrinogen uptake into platelets.10 These mice have markedly prolonged tail-bleeding times and display spontaneous hemorrhage in all developmental stages. In intravital microscopy studies performed in mesenteric arterioles, $\beta_3$-null mice do not form any thrombi.89 However, $\alpha_{IIb}\beta_3$ is not simply an anchorage for adhesive substrates but signals through the membrane once ligands have bound. The contribution of this outside-in signaling process to adhesive strengthening and irreversible platelet aggregation has been demonstrated in vivo: mice expressing a mutated cytoplasmic tyrosine motif in the integrin $\beta_3$ chain and impaired outside-in signaling rebleed from tail wounds.90

Several adhesive substrates are known to bind to $\alpha_{IIb}\beta_3$ to bridge and further activate platelets in a growing thrombus, in particular fibrinogen, vWF, and fibronectin. Fibrinogen is a dual player in the process of hemostasis in that it functions as a ligand of $\alpha_{IIb}\beta_3$ but also as the primary building block for the formation of fibrin following thrombin cleavage. The availability of gene targeted mice either lacking fibrinogen or expressing modified forms of fibrinogen has provided the possibility to explore the roles of fibrinogen in vivo. Fibrinogen-null mice develop overt bleeding after birth and show neither platelet aggregation nor blood clotting in vitro.91 Mice expressing a truncated form of fibrinogen, Fa$\gamma$A5, which is unable to bind to $\alpha_{IIb}\beta_3$, can still be converted into fibrin, display a generally normal hematological profile. However, they have an extended bleeding time following surgical challenge.92 Notably, intravital microscopy studies in FeCl$_3$-injured mesenteric arterioles revealed that both gene targeted mice are still able to form thrombi.89,93 These thrombi were unstable and embolized frequently in both mouse models: in fibrinogen-null mice, by being stripped from the interface of thrombi and the vessel wall; and in Fg$\gamma$A5-mice, by rupture through the central or upper portion of the thrombi. These data impressively support the idea that fibrin formation is essential for anchoring thrombi to the injured vessel wall, whereas fibrinogen itself plays an important role in thrombus stability by bridging $\alpha_{IIb}\beta_3$ between neighboring platelets.

Fibrinogen does not have an exclusive role in platelet aggregation; vWF has been shown to be necessary to establish interplatelet bridges at high shear rates in vitro.94
vital microscopy in vWF-null mice proved this idea: although stable thrombi did still form under conditions of arterial flow in the absence of vWF, an open channel remained within the thrombus in most animals.\textsuperscript{99} vWF is necessary for thrombus growth once local shear rates become high, ie, if the diameter of the vessel lumen is decreased below a critical value. However, the fibrinogen-null mouse has shown that vWF alone is not sufficient to achieve stable platelet aggregation, supporting the hypothesis that concurrent binding of vWF to α\textsubscript{IIb}β\textsubscript{3} and GPIb\textalpha allows initial interplatelet contacts, whereas fibrinogen is necessary for a permanent linkage between activated α\textsubscript{IIb}β\textsubscript{3} on neighboring platelets to finally ensure stable aggregate formation. Paradoxically, double-knockout mice lacking both fibrinogen and vWF can still form thrombi,\textsuperscript{89} but these thrombi are very fragile and frequently release small emboli. Interestingly, the platelet content of fibronectin is increased 3-fold in these mice, making it an attractive alternative candidate for platelet cohesion. A conditional knockout mouse with reduced plasma fibronectin levels (\textless 2\% compared with wild type) displayed a normal tail-bleeding time and in vitro parameters of coagulation.\textsuperscript{95} Intravital microscopy revealed that fibronectin deficiency delays thrombus formation in arterioles by a continuous shedding of small aggregates from stably anchored thrombi.\textsuperscript{96} No vessels occluded at the site of injury, demonstrating that fibronectin is an important and formerly under-recognized mediator of platelet/platelet interactions that fosters the continued growth and stability in a forming thrombus.

The role of 2 other soluble adhesive proteins that bind to α\textsubscript{IIb}β\textsubscript{3}, vitronectin and thrombospondin, is not yet well understood. Whereas vitronectin-null mice were initially found to have an enhanced rate of thrombus formation, suggesting an antithrombotic effect of vitronectin,\textsuperscript{97} subsequent experiments demonstrated thrombus instability in those animals.\textsuperscript{98} In addition, the initially reported faster thrombus formation in the absence of vitronectin could not be confirmed in the same model, leaving the question about its relevance open for the moment. Thrombospondin-null mice display reduced platelet recruitment to the site of injury under high shear conditions, which appears to be attributable to a role of thrombospondin-1 in protecting (sub)endothelial vWF from cleavage by ADAMTS13.\textsuperscript{99}

Maximal morphological, secretory, and procoagulant responses do not only require binding of the abovementioned adhesive substrates but also an interaction via the gap between adjacent platelets to enable “contact-dependent signaling.” Occupancy of α\textsubscript{IIb}β\textsubscript{3} by one of its ligands and subsequent outside-in signaling is a well-understood part of this type of platelet–platelet interaction\textsuperscript{100}; only recently, CD40L (CD154), a member of the tumor necrosis factor family, was identified as a new ligand of α\textsubscript{IIb}β\textsubscript{3}. Mice lacking CD40L can still develop large thrombi, but these thrombi frequently rupture and embolize.\textsuperscript{101} Thus, outside-in signaling via CD40L/α\textsubscript{IIb}β\textsubscript{3} interaction is critical for thrombus stabilization under conditions of arterial flow. Other ligand–receptor pairs have also been implicated in contact-dependent signaling, including ephrin/Eph receptor kinases and Gas6/Ax1-Tyro3-Mer receptor kinases.\textsuperscript{103–105} Gas6, a member of the vitamin K–dependent protein family, is stored in α-granules and becomes secreted following platelet activation. It binds to Gas6 receptors on the platelet surface, suggesting an autocrine stimulatory mechanism. Gas6-null mice do not undergo spontaneous bleeding and have normal tail-bleeding times. However, the animals form significantly smaller thrombi under both arterial and venous conditions of flow.\textsuperscript{105} In addition, Gas6-null mice are protected from platelet-dependent thromboembolism. Comparable, but less pronounced, results have been found in mice lacking the Gas6 receptor Mer.\textsuperscript{106} Angelillo-Scherrer et al\textsuperscript{107} demonstrated that each of the Gas6 receptors (Tyro3, Axl, and Mer) is important in transmitting a plug-stabilizing effect of Gas6, probably attributable to Gas6 receptor crosstalk, which affects outside-in signaling via the α\textsubscript{IIb}β\textsubscript{3} integrin: deficiency of any of these receptors protected mice against thrombosis. Gas6/Gas6 receptors represent a mechanism that amplifies the response of other agonists, while not evoking any response by itself. Whether these observations made in mouse models are of relevance in human platelet aggregation awaits clarification.\textsuperscript{108}

Further amplifier systems can be expected to fill the gap between adjacent platelets in the future, one of which are members of the SLAM (signaling lymphocyte activation molecule) family, including CD84 and CD150. These proteins become tyrosine phosphorylated in a platelet aggregation–dependent fashion, making them potent candidates for secondary aggregation within the gap. In the FeCl\textsubscript{3} model of in vivo thrombosis, CD150-null mice display delayed thrombus formation and increased embolization of small thrombi but normal tail-bleeding times.\textsuperscript{109}

**Thrombus Formation and Coagulation**

Thrombus formation is inseparably connected to the initiation of the coagulation cascade to finally produce fibrin. Plasma protease factor VIIa in complex with the membrane protein tissue factor is of central importance in this process. This was elegantly demonstrated in mice expressing low levels of tissue factor, which form only very small thrombi that do not incorporate fibrin.\textsuperscript{110} Subjecting bone marrow chimera to a model of laser injury in cremaster muscle arterioles, these authors provided evidence that blood-borne tissue factor contributes to the formation of fibrin during thrombus formation, whereas vessel wall tissue factor alone was unable to sustain thrombin generation and fibrin formation throughout the thrombus. There is additional in vivo evidence that blood-borne tissue factor comes from leukocyte-derived microparticles.\textsuperscript{111} These microparticles do express P-selectin GP ligand (PSGL)-1 and are recruited into the thrombus via PSGL-1/platelet P-selectin interaction: neither P-selectin–nor PSGL-1–null mice are able to incorporate sufficient amounts of tissue factor into the thrombus. These results are in line with observations showing that elevation of procoagulant microparticles (a subset of which contained tissue factor) restored hemostasis in hemophilic mice in a P-selectin–/PSGL-1–dependent manner.\textsuperscript{112} However, contradictory results were obtained using models of carotid artery ligation and vena cava ligation.\textsuperscript{113} Analyzing bone marrow chimera, low blood-borne tissue factor did not limit thrombus formation in a wild-type mouse, and high blood-borne tissue factor
did not augment thrombus formation in a low tissue factor animal. These authors conclude that vessel wall-derived tissue factor plays a pivotal role in thrombosis. This discrepancy might be explained by the different nature and severity of the vessel wall injury as well as the different vascular beds used in the models. It must be kept in mind that the existence of active tissue factor in blood is still a matter of debate, and further studies will be required to evaluate the relative contribution of blood-borne versus vessel wall tissue factor in thrombosis.

Other mechanisms contributing to fibrin formation in vivo are also possible, one of which is the so-called “intrinsic pathway,” induced by coagulation factor XII (FXII) activation. The longstanding concept that this pathway is irrelevant for clotting in vivo has recently been changed: Renne et al demonstrated that mice lacking FXII are protected against collagen- and epinephrine-induced thromboembolism, although they do not experience spontaneous or excessive injury-related bleeding (Figure 2). Intravital fluorescence microscopy and blood flow measurements in 3 distinct arterial beds revealed a severe defect in thrombus formation and stabilization. Exogenous human FXII restored arterial thrombus formation completely. In contrast to tissue factor, which is a transmembrane protein that needs to be transported to the developing thrombus (eg, as a constituent of microparticles), FXII activation is likely to appear on the negatively charged surface of activated platelets. The most important aspect of the in vivo findings is the possibility that FXII might be involved in (pathologic) thrombus formation, but not in (physiologic) hemostasis, as bleeding times in FXII-/- mice are normal. Importantly, this assumption was further corroborated in an intraluminal filament model of murine stroke. In this model, FXII-null mice were protected from pathological thrombosis without an increase in infarct-associated hemorrhage. Comparative results were obtained in wild-type mice after injection of a peptide-based FXII inhibitor; again, impaired pathological fibrin formation resulted in neuroprotection without increased bleeding. Mice deficient in the FXII substrate FXI were similarly protected from vessel-occluding fibrin formation, suggesting that FXII indeed contributes to pathological clotting through the “intrinsic pathway.” In accordance with this finding, absence of FXI was previously demonstrated to protect mice from occlusion of the carotid artery in a FeCl₃ model without increasing the tail-bleeding time. In addition, treatment of rabbits with antibodies against FXI remarkably reduced thrombus growth in a model of balloon injury of the iliac artery. The latter findings are an important example for the strength of studying thrombus formation in vivo, as these data could point to a presumably “ideal” target for antithrombotic therapy after 50 years of in vitro research.

**Conclusions**

Experimental animal models have substantially contributed to a better understanding of the mechanisms underlying thrombus formation. Despite the unquestionable contribution of the mouse system and the knockout technology, creative, and novel approaches in imaging and image analysis were equally important to meet the challenges of analyzing the complex mechanisms underlying platelet-dependent thrombotic processes in vivo. Both approaches together were not only helpful in finally proving longstanding hypotheses but have rather been used to discover new and previously unexpected mechanisms and causal relationships. However, the results obtained in our experimental models have raised new questions. For example, the observation that deficiencies in certain molecules, including FXII, plasma fibronectin, or Gas6 result in profound inhibition of “experimental occlusive thrombus formation” without detectable effects on “experimental hemostasis” raises the interesting possibility that hemostasis and thrombosis might be 2 mechanistically different processes. To date, no evidence in support of this hypothesis has been provided in humans.

Further work in this field will mainly have to focus on (1) dissecting what kind of stimulus or injury triggers effects that best reflect pathophysiology of acute ischemic events in humans and (2) identifying mechanisms that trigger thrombus formation but are dispensable to arrest bleeding. These directions will help us to develop better strategies to control acute ischemic cardiovascular and cerebrovascular syndromes. In any case, appropriate animal models will be instrumental in this way.

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