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Mouse Models of Hemostasis

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Abstract

Hemostasis is the normal process that produces a blood clot at a site of vascular injury. Mice are widely used to study hemostasis and abnormalities of blood coagulation because their hemostatic system is similar in most respects to that of humans, and their genomes can be easily manipulated to create models of inherited human coagulation disorders. Two of the most widely used techniques for assessing hemostasis in mice are the tail bleeding time (TBT) and saphenous vein bleeding (SVB) models. Here we discuss the use of these methods in the evaluation of hemostasis, and the advantages and limits of using mice as surrogates for studying hemostasis in humans.

Introduction

The term hemostasis is commonly used to indicate the cessation of bleeding at a site of injury by formation of a sealing blood clot [1–3]. More broadly, hemostasis implies that the coagulation response is limited to the site of the injury, allowing blood flow to continue normally in the intact circulation. The hemostatic response is one of several homeostatic mechanisms that prevent acute changes in blood volume and pressure, allowing an organism to maintain normal tissue perfusion.

Formation of a blood clot involves a tightly orchestrated set of interactions between cellular and plasma constituents of the blood, and cellular and extracellular components of the blood vessel (1–3). While *in vitro* and *ex vivo* techniques to study protein-protein, cell-cell, and cell-protein interactions have been instrumental to our understanding of the biochemistry of blood coagulation, the complexity of the system requires animal models to put these findings into proper context.

Here, we discuss the use of mice as surrogates for humans in studying hemostasis, with an emphasis on models that measure duration and extent of bleeding. We focus on the use of these assays to assess inherited or acquired defects in plasma coagulation, but the techniques are applicable to studying platelet contributions as well.

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Advantages of using mice to study hemostasis

Inbred mouse strains have been used for decades to study the physiology and pathology of blood coagulation [4–8]. Mice offer several advantageous features that, combined with their relatively low-cost of upkeep, have established them as important model organisms in hemostasis and thrombosis research. The blood plasmas of mice and humans contain similar complements of coagulation factors and regulatory proteins [5–7]. In general, human plasma coagulation proteins function adequately when infused into mice. With some notable exceptions, hemostatic reactions involving murine blood vessels and platelets are similar to their human counterparts.

Our ability to manipulate the mouse genome to create total or conditional knockouts of a gene of interest has been key to identifying and studying the contributions of various proteins to hemostasis and to a variety of pathologic processes. Even when gene deletion causes embryonic death, strategies to restrict the deficiency state to the adult organism or transgenic approaches to express low levels of protein sufficient to prevent embryonic lethality are available [3,5,9,10]. Knock-in and over-expression strategies facilitate *in vivo* structure-function analyses, disease modeling, and creation of “humanized” mice to study drugs that specifically target human proteins.

Limitations of mice for studying human blood coagulation

There are important physiologic, anatomic, and biochemical differences between mice and humans that must be considered when employing mice as surrogates for humans in hemostasis studies. The three orders of magnitude size difference results in large differences in mechanical forces exerted on mouse and human tissues. The greater propensity for humans with hemophilia (deficiency of factor VIII [fVIII] or factor IX [fIX]) to bleed into joints (hemarthrosis) than do fVIII or fIX deficient mice is at least partly explained by the different magnitudes of force exerted on the tissues of 70 kilogram bipeds and 0.025 kilogram quadrupeds [11–14]. Along similar lines, it is our experience that simple skin incisions are usually well tolerated in hemophiliac mice in the absence of factor replacement, as long as care is taken not to nick underlying structures. However, larger skin injuries, such as punch biopsy wounds are prone to severe delayed bleeding unless small amounts of the missing factor are provided [15,16].

There are notable differences in the coagulation mechanisms of mice and humans that complicate certain analyses. One of the best characterized involves the protease-activated receptors (PARs) expressed on platelet surfaces [17–20]. Human platelets express PAR-1 and PAR-4, and cleavage of either by thrombin promotes platelet activation. Murine platelets express PAR-4 and also PAR-3, which functions as a cofactor for PAR-4, but do not express PAR-1. This situation has created significant difficulties in pre-clinical evaluations of anti-platelet agents that target PAR-1.

While mice and humans have similar complements of plasma coagulation factors, some proteins may serve different functions in the two species. For example, in humans factor XI (fXI) deficiency causes a variable tendency to bleed excessively with trauma or surgery

[21,22]. A similar phenotype has been noted in fXI deficient cattle, dogs and cats [23–25]. FXI deficient mice, on the other hand, have not demonstrated a consistent propensity to bleed abnormally with any hemostatic challenge [26–28, and unpublished observations]. In humans, most fXI circulates in plasma in complex with high molecular weight kininogen (HK) [29]. In mice, most fXI is bound to glycosaminoglycans on blood vessels [30]. These findings raise the possibility that the primary function of fXI in mice is not hemostasis, and brings into question the suitability of the mouse as a model for human fXI deficiency.

Mouse Models of Hemostasis

Several approaches have been used to study hemostasis in mice, but most are not well standardized. Here we concentrate on two of the most widely used and best characterized models, the tail bleeding time (TBT) and saphenous vein bleeding (SVB) models. We will briefly discuss models based on injury to the liver or cuticle. Differences in mouse strain and methods of anesthesia may influence bleeding in the TBT and SVB models [31–33]. When we describe our experiences with a model, they are based on work with C57Bl/6 mice anesthetized with pentobarbital or ketamine.

Tail Bleeding Time (TBT) Model

TBT assays are the most widely used approach for assessing hemostasis in mice [8,34–41]. The assays are relatively simple to perform, with a small impact on the health of the animal. In the basic model, a razor or scalpel is used to transect the tail at a predetermined distance from the tip (1 – 5 mm), or at a point with a specific circumference [38–40]. The bleeding tail stump is usually immersed in normal saline warmed to 37 °C, and time to cessation of bleeding is determined. Blood loss is assessed by measuring hemoglobin in the saline. Alternatively, the transected tail may be left exposed to air, with frequent wicking of blood using filter paper. The hemoglobin on the filter paper can then be quantified.

In our hands, wild type (WT) mice usually bleed for 150 to 300 seconds after tail transection (Figure 1A) [27]. However, there can be significant variability, and regular users of the TBT are familiar with the occasional control animal with prolonged bleeding. Tail transection causes injury to multiple types of tissues (skin, bone, connective tissue) including three large blood vessels (a central artery and two lateral veins) each of which may respond differently to injury. Furthermore, the anatomy of the tail varies substantially between individual animals. These factors likely contribute to variation in injury severity and subsequent bleeding that is not amenable to technique standardization.

We found that transecting the tail at a location where it is 2 mm wide (as determined by a template) is associated with less variability than transecting at a specific distance from the tail tip. While a recent study found that the type of anesthetic used (pentobarbital, ketamine or isoflurane) did not affect the TBT in wild type mice [new 42], we and others have noted that injury-induced thrombosis varies considerably depending on anesthetic type [27, new 42]. This may reflect differences in drug effects on vascular tone, blood velocity through certain vascular beds, platelet reactivity, and perhaps plasma enzymatic reactions. Furthermore, while bleeding in wild type mice may not be affected appreciably by anesthetic

types, this may not hold for mice with hemostatic defects. For this reason, we use a single anesthetic (pentobarbital) for our work with the TBT.

Bleeding in the TBT assay is not continuous. That is, it consists of bleeding punctuated by periods where hemostasis appears to have been achieved. Broze and colleagues proposed that this is due to cycles of vasoconstriction and vasodilatation [31]. When evaluating bleeding after tail transection, then, one should record both the time to initial cessation of bleeding and the time at which bleeding stops without rebleeding. A modification of the basic TBT model that replaces tail transection with an incision along one of the lateral tail veins has been adopted by several groups [31,43]. This technique causes less severe bleeding that is more reproducible than with standard tail transection.

The TBT is sensitive to deficiencies of plasma coagulation factors associated with severe bleeding phenotypes in humans. Mice lacking fVIII or fIX (models of human hemophilia A and hemophilia B, respectively) usually exsanguinate after tail transection unless the tail tip is cauterized (Figure 1A) [14,27,44,45]. Bleeding usually stops shortly after tail transection in hemophiliac animals in a manner that appears similar to WT mice. However, subsequent rebleeding in the hemophiliac mice is severe and usually fatal without intervention. We observed a similar bleed-stop-rebleed pattern in fIX deficient mice after puncturing the carotid artery with a 25-gauge needle (unpublished observation). In the TBT, bleeding in fVIII [31,42–44,46–48] and fIX deficient [31,49,50] mice responds to factor replacement and other therapeutic interventions used to treat humans with hemophilia.

While fXI deficient humans may experience excessive trauma-induced bleeding, fXI deficient mice are indistinguishable from WT mice in the TBT model (Figure 1A) [27,31,51,52]. The plasmas of mice lacking individual components of the plasma contact activation system (factor XII [fXII], prekallikrein and HK) display significant defects in surface initiated clotting assays *in vitro*; however, their TBTs are comparable to those of WT mice [53–56]. This is consistent with the absence of a bleeding diathesis in humans lacking fXII, prekallikrein or HK.

Prolonged TBTs have been observed in WT mice treated with anticoagulants including heparin [27,31], warfarin [41], and small molecule inhibitors of thrombin [57–59] and factor Xa [60]; the anti-platelet agents aspirin [27,31] and clopidogrel [39]; and with over-expression of the coagulation regulator protein C [61].

Mice with platelet abnormalities equivalent to the human conditions Glanzmann thrombasthenia (caused by mutations affecting either subunit of the GP IIb/IIIa complex) and Bernard-Soulier syndrome (caused by mutations of one of the three subunits that make up the GP Ib-IX-V complex) demonstrate severe hemostatic defects in the TBT [31,62–64]. The effects of disrupting other platelets receptors/proteins affect the TBT to varying degrees and have been reviewed elsewhere [65].

Saphenous Vein Bleeding (SVB) Model

The SVB model was developed by Whinna with the goal of establishing an assay to study hemostasis in hemophiliac mice that has better sensitivity and lower variability than the TBT

[66–68]. Bleeding in the SVB model can be adjusted to show a graded response to plasma factor concentrations between 1 and 20% of normal by changing the degree of injury. This allows the assay to more closely mirror the varying degrees of bleeding observed in patients with severe (<1%), moderate (1 to 5%) or mild (>5%) hemophilia. Monroe adapted the model for the study of anticoagulation therapy [69].

Mice under general anesthesia are placed in a supine position with the hind limbs gently restrained to a procedure mat warmed to 37 °C. The ventral surface of the hind limb is shaved, and a longitudinal skin incision is made along the length of the limb exposing the saphenous vein. The vein is punctured once with a 23-gauge needle to create an entry hole. After initial bleed stops (1–2 minutes), a blade of a Student Vannas spring scissor is inserted through the entry hole into the distal part of the vessel and a longitudinal incision is made. We make an ~1 mm incision, but the length can be varied to change the bleeding severity. With an ~1 mm incision, bleeding in fIX deficient mice correlates with plasma fIX levels between 1% and 20% of normal. Blood is wicked away from the injury site using gauze until bleeding stops. The clot is then gently disrupted to restart bleeding. The goal is to remove the clot with minimal manipulation of the blood vessel. We disrupt clots by gently running gauze along the clot surface in the direction of blood flow. Others use the tip of a 30-gauge needle to dislodge the clot. Each time bleeding stops, the clot is disrupted to restart blood flow. The vessel is observed for 30 minutes, and normal saline is applied periodically to prevent the surgical field from drying. The number of clots formed over the course of 30 minutes (Figure 1B) and the duration of each bleed (Figure 1C) are recorded. The total amount of blood lost may also be determined.

While we find the SVB model is subject to less intra-operator variability than the TBT model, it is more technically demanding. We observed considerable inter-operator variability, likely reflecting subtle differences in technique. Differences in the size of the vessel opening alters the sensitivity of the assay to plasma coagulation factor levels, and is likely one source of variability. The manner in which clots are disrupted also alters the hemostatic response [28,70].

The SVB model was designed to be sensitive to fVIII or fIX deficiencies and bleeding is reduced by factor replacement and other treatment modalities used to treat hemophiliacs [67,71–74]. Untreated fIX-deficient mice have relatively few clotting events (Figure 1B, 1 to 3 over 30 minutes, compared to 25 to 30 for WT mice), and each bleeding event is substantially longer than in WT mice (Figure 1C). While the SVB model, like the TBT, does not detect a difference between fXI deficient and WT mice in our hands [28], administering a large amount of human fXI to mice lacking fIX (hemophilia B) increases the number of clots and reduces the duration of bleeding (Figure 1B and 1C, data highlighted in gray) [28]. This is likely due to fXIa activation of factors V and X that partially bypass the need for fIX. [75]. This supports the impression that the SVB model is useful for studying novel therapeutic strategies in hemophilia other than factor replacement. The SVB model has also been quite useful for assessing the effects of anticoagulation therapy on hemostasis [69,76,77].

Recently, a variation on the SVB model has been described that incorporates intravital fluorescence microscopy to study clot formation in real time [78]. Labeled antibodies to fibrin and platelets are infused before saphenous vein injury is inflicted with a laser. The laser punches a hole in the vessel wall without transecting it. There is no need for a longitudinal incision in the vessel, as the purpose of the incision in the surgical model is to prevent vasoconstriction, which is not an issue with the laser. The effects of repeated injury to the same site may be assessed. Alternatively, separate injuries can be created along the length of the vessel. In addition to the standard parameters, platelet fibrin formation and adhesion/aggregation are observed in real time. The surgical SVB model is sensitive to changes in both coagulation factors and platelet adhesion/aggregation [79]. In contrast, the laser-based model detects subtle differences in platelet function and von Willebrand factor activity, but is relatively insensitive to plasma factor deficiencies but does [78,80]. The reasons for the differences in the two versions of the SVB model have not been established. In some respects, the laser-based model behaves like the template bleeding times used for many years in clinical practice, with sensitivity to platelet defects and relative (but not absolute) insensitivity to defects in thrombin generation. Perhaps surgical injury causes a greater degree of tissue factor exposure to blood than does laser injury.

Liver Laceration Model

This model has been used in a variety of species, most often to assess the anti-hemostatic effects of novel therapeutic agents, and to test strategies for anticoagulant reversal [81]. We used it to investigate hemostasis in fXI-deficient mice and in WT mice treated with antibodies to fXI or fXII (unpublished data). In our version of the model, mice are placed on a warming pad in a supine position. The abdomen is shaved, and the liver is exposed via an anterior right subcostal incision. The left lobe of the liver is externalized by applying gentle pressure posteriorly, and a 5-mm transverse incision is made with a #10 scalpel blade starting one cm from the lobe's inferior edge, and parallel to the inferior edge. Blood is collected into a small tray and the total blood loss determined [81–83]. Heparin and other anticoagulants increase bleeding in this model. Standardizing the degree of injury (particularly the depth of the wound) is a challenge, as is collecting all blood issuing from the wound.

Cuticle Bleeding Model

The nails of many mammals contain a vascular central region called the quick. Cutting into the quick produces bleeding that can be measured. The original model was developed to study therapeutic agents in hemophiliac dogs, and showed a correlation between intensity of therapy and reduced bleeding [84,85]. It has been adapted for other species, most notably rabbits and, less frequently, mice. In mice, the entire cuticle of the middle digit is removed with sharp scissors, and blood is collected with filter paper [86,87]. It is difficult to determine if a consistent injury is being inflicted in mice because of the small size of the digits. There is probably a component of crush injury in addition to the laceration. Furthermore, it is not clear that this model is more reproducible than other models, and some of the groups that reported its initial use have transitioned to other models for subsequent studies.

Conclusions

Mice are invaluable tools for assessing the contributions of plasma, platelet, and blood vessel constituents to normal and pathologic coagulation. Murine bleeding models are commonly used to assess prospective anticoagulants, and to determine the ability of pro-hemostatic therapies to correct bleeding defects. Because of its relative ease of use, the TBT is widely used to evaluate hemostasis in mice. We feel the SVB model is an improvement on the TBT that is particularly useful for studying hemophilia and anticoagulation therapy (and its reversal). While these assays are used routinely in pre-clinical studies, they have limitations as models of human bleeding disorders.

First, the requirement for certain hemostatic factors varies depending on the types of tissue injured, as clearly demonstrated by the propensity of hemophiliacs to bleed preferentially into certain areas of the body. When considering the utility of the TBT and SVB models then, the suitability of mouse tail and saphenous vein tissue as models for bleeding in other areas must be considered. Taking an example we are particularly familiar with, humans lacking fXI tend to bleed most severely after trauma to the nasopharynx, mouth and genitourinary tract [21,22]. These tissues have high intrinsic levels of fibrinolytic activity that prematurely degrade clots, and it is hypothesized that fXI-supported thrombin generation stabilizes clots in this setting [22]. Such conditions may not be reproduced with the TBT or SVB models, perhaps partly explaining why fXI and WT mice respond similarly in these assays.

Second, some proteins identified as coagulation factors in humans may serve a different role in mice. The core of the vertebrate thrombin generation mechanism is comprised of a group of vitamin K-dependent proteases and their cofactors [1–3]. It appears that all vertebrates require these proteins for normal hemostasis [88]. However, the roles of ancillary proteins may differ. Again, taking the example of fXI, we identified important roles for this protein in occlusive thrombus formation [27,89] and inflammation [90] in mice, but have not identified a condition under which it is required for hemostasis [27,28]. The fact that most mouse fXI is bound to blood vessels and is not in plasma suggests adaptation for novel functions not relevant to human physiology [30]. Such issues will become more important as antithrombotic strategies targeting fXI move into clinical trials [89,91]. The take home lesson is that absence of bleeding in a laboratory animal model may not accurately reflect the responses of human patients to various drugs and conditions.

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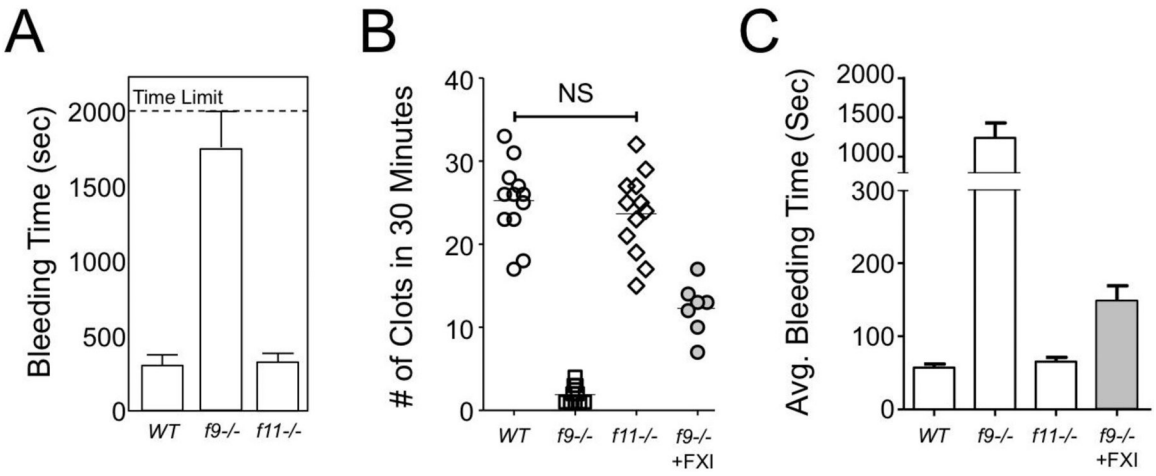

Figure 1. Effects of factor IX or factor XI deficiency on the tail bleeding time and saphenous vein bleeding models.

(A) Groups of ten C57Bl/6 wild type (WT), fIX deficient (f9−/−) or fXI deficient (f11−/−) mice were tested with a standard tail bleeding time assay. Mice were not allowed to bleed for more than 2000 seconds. Data are shown as means of times to final termination of bleeding +/− 1 SD. **(B and C)** Groups of twelve WT (○), fIX−/− (□) and f11−/− (◇) mice were tested in the saphenous vein bleeding model. Data for number of clots in 30 minutes is shown in panel B, and the average duration of each bleeding episode (+/− 1 SD) is shown in panel C. Note that there is no significant difference between WT and fXI−/− mice. The gray circles in panel B and gray bar in panel C show the effect of an infusion of human fXI into f9−/− mice calculated to double the plasma fXI concentration.