Editorial Comment

Rethinking the coagulation cascade. By Hoffman M, et al.

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第 27 回学術集会において Duke 大学の Hoffman 先生が行った教育講演の内容をまとめたのが本論文である。主に *in vitro* の現象として解明されて来た血液凝固と、疾病としての血栓症発症あるいは止血血栓の形成の間の関連には改めて問い直さなければならない多くの問題があることを明確に指摘した優れた総説である。

血液凝固のメカニズムとして、内因系凝固カスケード、外因系凝固カスケードは基本事項と理解されている。臨床検査法としても内因系を評価する a-PTT, 外因系を評価する PT, INR が出血性疾患のスクリーニング、抗血栓薬の薬効評価に広く使用されている。

著者が指摘する通り、a-PTT を延長させる第 XII 因子の欠損が出血性合併症を惹起しないことは a-PTT により評価される内因系凝固反応の止血における役割に疑問を持たせる。組織因子を有する細胞、ないし細胞由来の微粒子の役割を重視すべきであるとの主張は説得力がある。Figure 4, Figure 5 で示しているように、組織因子と活性化血小板が生体内での凝固系の活性化とフィブリン形成に必須の役割を果たすという説明、血小板の活性化の過程における第 VIII 因子 / von Willebrand 因子複合体の解離の関与、などに関しては個々の研究者によって議論があろう。

さらに TAFI, 第 XIII 因子の関与まで含めて著者は Figure 7 に生体内の凝固メカニズムのモデルを呈示している。

- 1) 生体内のフィブリン産生メカニズムと試験管内の血液凝固メカニズムは同一でないこと
- 2) 前者には組織因子と活性化血小板が重要な役割を果たすこと

などの著者が本図により propose している主張の根本の部分は完全に同意できる。

プロトロンビン時間 (PT, INR), 活性化部分トロンボプラスチン時間 (a-PTT) などの臨床検査は、先人が出血性疾患のスクリーニング法として確立した貴重な財産である。

一方、財産を当然のこととして引き継いだわれわれは何時の間にかスクリーニング検査と病態を混同してしまうようになった。確立されたスクリーニング検査が優れたものであればあるほど、スクリーニング検査と同じ現象が体内で起こっていると誤解してしまう。スクリーニング検査では強力が抗血栓効果を予想された薬物でも実際の抗血栓効果は全くない場合も、逆にスクリーニング検査では抗血栓効果がなさそうに思える薬物でも生体内の血栓形成を強力に抑制する薬物が有る可能性を考えることが新たな抗血栓薬の開発に役立つと思われる。

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Rethinking the Coagulation Cascade

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Key words: tissue factor, platelet, coagulation cascade, thrombosis

HOW WELL DO WE REALLY UNDERSTAND COAGULATION?

In the 1960s two groups proposed a model of coagulation composed of a sequential series of steps in which activation of one clotting factor led to the activation of another, finally leading to a burst of thrombin generation^{1) 2)}. Each clotting factor was thought to exist as a proenzyme that could be converted to an active enzyme.

The original cascade models were subsequently modified to include the observation that some procoagulants were cofactors and did not possess enzymatic activity. In addition the clotting sequences were divided into so-called extrinsic and intrinsic systems and eventually evolved into the scheme shown in **Figure 1**. As can be seen, the extrinsic system consisted of factor VIIa and tissue factor, the latter being viewed as extrinsic to the circulating blood. The factors in the so-called intrinsic system were all viewed as being intravascular. Both pathways could activate factor X, which, in complex with its cofactor Va, could convert prothrombin to thrombin. A phosphatidylserine-containing phospholipid surface and calcium were considered essential

for the activity of the coagulation complexes. We assess the components of the extrinsic pathway clinically with the prothrombin time (PT) test and the components of the intrinsic pathway as the activated partial thromboplastin time (aPTT) test. While this concept of coagulation was extremely valuable, many people recognized that the intrinsic and extrinsic systems could not operate in vivo as independent and redundant pathways as implied by this “cascade” model. It was clear that even though deficiencies of each of the factors in the intrinsic pathway could have equally long aPTT values, they had dramatically different risks of hemorrhage (**figure 2**). For example, deficiencies of Factor XII were not associated with significant hemorrhage, deficiencies of factor XI might or might not be associated with hemorrhage, but deficiencies of factors VIII and IX were consistently associated with hemorrhage.

Key observations made by several groups have led to a revision of earlier models of coagulation. A major observation was that a complex of factor VIIa/TF activated not only factor X but also factor IX³⁾. Other important observations led to the conclusion that the major initiating event in hemostasis in vivo was the formation of a factor

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Figure 1

Figure 2

VIIa/TF complex at the site of injury^{4) 5)}. However, in spite of these advances, it was not clear why an intact extrinsic pathway could not compensate for the lack of factor IX or VIII in hemophilia.

CAN AN EMPHASIS ON THE ROLE OF CELLS IMPROVE OUR UNDERSTANDING OF COAGULATION?

It was recognized from the earliest studies of coagulation that cells were important participants in the coagulation process. Of course, normal hemostasis is not possible in the absence of platelets. In addition, TF is an integral membrane protein and thus its activity is normally associated with cells. Since different cells express different

levels of pro- and anti-coagulant proteins as well as having different complements of receptors for components of hemostasis, it is logical that simply representing the cells involved in coagulation as phospholipid vesicles may overlook the important contributions of cells in directing hemostasis *in vivo*. Our group hypothesized that including the contributions of living cells in a concept of hemostasis might more closely model the process of hemostasis *in vivo*. Thus, we developed an experimental model of cell-based hemostasis that eventually led us to the conceptual model of hemostasis that is reviewed in the first part of this article.

Initiation and Amplification of Coagulation – The Role of the TF-bearing Cell

The goal of hemostasis is to produce a platelet and fibrin plug to seal a site of injury in the blood vessel wall. This process of hemostasis is initiated when TF-bearing cells are exposed to blood at a site of injury.

TF is a transmembrane protein that acts as a receptor and cofactor for factor VII. Once bound to TF, zymogen factor VII is rapidly converted to factor VIIa through mechanisms not yet completely understood but which may involve factor Xa and autoactivation. The resulting factor VIIa/TF complex catalyzes two important reactions: 1) activation of factor X; and 2) activation of factor IX. The factor Xa and IXa formed on the TF-bearing cells have very distinct and separate functions in initiating blood coagulation⁶⁾. The factor Xa formed on the TF-bearing cell interacts with its cofactor Va to form prothrombinase complexes and generates a small amount of thrombin on the TF cells (Figure 3). By contrast, the FIXa activated by FVIIa/TF does not act on the TF-bearing cell and does not play a significant role in the initiation phase of coagulation. If an injury has occurred and platelets

Figure 3

Figure 4

have adhered near the site of the TF-bearing cells, the FIXa can diffuse to the surface of nearby activated platelets. It can then bind to a specific platelet surface receptor⁷⁾, interact with its cofactor, FVIIIa, and activate FX directly on the platelet surface.

Most of the coagulation factors can leave the vasculature and their activation peptides are found in the lymph⁸⁾. Therefore, it is likely that most (extravascular) TF is bound to FVIIa even in the absence of an injury, and that low levels of FIXa, FXa and thrombin are produced on TF-bearing cells at all times. However, this process is kept separated from key components of hemostasis by an intact vessel wall. The very large components of the coagulation process are platelets and FVIII bound to multimeric vonWillebrand factor (vWF). These components normally only come into contact with the extravascular compartment when an injury ruptures the vessel wall. Platelets and FVIII-vWF then leave the vascular space and adhere to collagen and other matrix components at the site of injury.

Binding of platelets to collagen or via vWF leads to partial platelet activation. However, the coagulation process is most effectively initiated when enough thrombin is generated on or near the TF-bearing cells to trigger full activation

of platelets and activation of cofactors (figure 4). Although this amount of thrombin may not be sufficient to clot fibrinogen, it is sufficient to initiate events that “prime” the clotting system for a subsequent burst of platelet surface thrombin generation. Experiments using a cell-based model have shown that minute amounts of thrombin are formed in the vicinity of TF-bearing cells exposed to plasma concentrations of procoagulants, even in the absence of platelets. The small amounts of factor Va required for prothrombinase assembly on TF-bearing cells are activated by factor Xa⁹⁾, or by non-coagulation proteases produced by the cells¹⁰⁾. The small amounts of thrombin generated on the TF-bearing cells are responsible for:^{11) 12)} 1) activating platelets; 2) activating factor V; 3) activating factor VIII and dissociating it from VWF and 4) activating factor XI. The activity of the factor Xa formed by the factor VIIa/TF complex is restricted to the TF-bearing cell, because and factor Xa that dissociates from the cell surface is rapidly inhibited by TFPI or AT in the fluid phase. In contrast to factor Xa, factor IXa can diffuse to adjacent platelet surfaces because it is not inhibited by TFPI and is inhibited much more slowly by AT than is factor Xa.

In addition to the pool of extravascular, cell anchored TF, a number of reports have now

Figure 5

documented the presence of TF antigen and active TF protein in the circulation. This probably exists largely in association with membrane vesicles that have been shed from the surface of monocytes or endothelial cells^{13) 14)}. Such membrane vesicles can be shed from many cell types, particularly in the setting of inflammation or during apoptosis. Their presence in the blood has been reported in association with a wide range of inflammatory and prothrombotic states, including atherosclerotic vascular disease, severe infections and malignancy.

Propagation of thrombin generation and formation of the fibrin clot – The Role of Platelets

Platelets play a major role in localizing clotting reactions to the site of injury, since they adhere and aggregate at the sites of injury where TF is also exposed. They provide the primary surface for generation of the burst of thrombin needed for effective hemostasis during the propagation phase of coagulation (figure 5). Platelet localization and activation are mediated by vWF, thrombin, platelet receptors and vessel wall components such as collagen¹⁵⁾.

Once platelets are activated, the cofactors Va and VIIIa are rapidly localized on the platelet surface¹⁶⁾. As noted above, the factor IXa formed by the factor VIIa/TF complex can diffuse through

Figure 6

the fluid phase and also bind to the surface of activated platelets. Likewise, factor XI also binds to platelet surfaces and is activated by the “priming” amount of thrombin^{12) 17)}, bypassing the need for factor XIIIa. The platelet-bound factor XIa can activate more factor IX to IXa. The specific receptors on activated platelets that bind factor IXa also promote formation of active factor IXa/VIIIa (tenase) complexes^{18) 19)}. Once the platelet “tenase” complex is assembled, factor X from the plasma is activated to factor Xa on the platelet surface. Factor Xa then associates with factor Va to generate a burst of thrombin sufficient to clot fibrinogen and stabilize the initial platelet plug in a fibrin meshwork (as illustrated in figure 6). Factor XIII, activated by thrombin, crosslinks fibrin and further stabilizes the hemostatic plug. Thrombin also activates TAFI, which helps to prevent lysis of the fibrin clot²⁰⁾. Although the steps of the cell-based model have been depicted as individual reactions, they should be viewed as an overlapping continuum of events (figure 7 from²¹⁾).

Plasma protease inhibitors are critical in keeping the coagulation reactions confined to specific cell surfaces by inhibiting proteases that escape into the fluid phase. They also impose a threshold effect on the coagulation process²²⁾.

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Figure 7

Thus, in the presence of inhibitors, coagulation does not proceed unless procoagulant factors are generated in sufficient amounts to overcome the effects of inhibitors.

The role of factor XI in hemostasis has been a point of some controversy, since even severe factor XI deficiency does not result in a hemorrhagic tendency as severe as that seen in severe factor VIII or IX deficiency. This observation can be explained if factor XI is viewed as an “enhancer” or “booster” of platelet thrombin generation. In factor VIII and IX deficiency, the individual has a markedly decreased ability to generate factor Xa on the platelet surface. Thus, patients with a severe deficiency of either factor VIII or factor IX have little or no platelet surface tenase activity, and hence platelet surface thrombin production is also markedly reduced. In contrast, patients with factor XI deficiency would always possess some platelet surface tenase activity. Such patients only lack the ability to “boost” platelet surface factor X activation by producing extra factor IXa.

Our knowledge of the platelet contribution to thrombin generation continues to evolve. There is evidence that there is more than one

population of activated platelets, one of which has been referred to as COAT (Collagen And Thrombin stimulated) platelets²³⁾. These platelets have enhanced thrombin-generating ability due to enhanced binding of both tenase and prothrombinase components^{24) 25)}. The in vivo relevance of these findings is not yet clear, but it may be that the greatest procoagulant activity is generated on platelets that have bound to collagen matrix and also been exposed to thrombin. Once the exposed collagen is covered by a platelet/fibrin layer, any additional platelets that accumulate are not activated to the “COAT” state – thus tending to damp down the procoagulant signal as the hemostasis proceeds.

The most important feature of the cell-based model of coagulation is that it shows us that the “extrinsic” and “intrinsic” pathways are not redundant. Let us consider the “extrinsic” pathway to consist of the FVIIa/TF complex working with the FxVa/Va complex to produce thrombin; and the “intrinsic” pathway to consist of factor XIa working with the complexes of factors VIIIa/IXa and factors Xa/Va to produce thrombin. Using these revised definitions, we can see that the “extrinsic” pathway operates on the TF-bearing cell to initiate and amplify coagulation. By contrast, the “intrinsic” pathway operates on the activated platelet surface to produce the burst of thrombin that causes formation and stabilization of the fibrin clot. Both “Intrinsic” and “Extrinsic” pathways (as defined above) are necessary for hemostasis. Activation of factor X by the FVIIa/TF complex can not make up for a lack of factor X activation by the FIXa/VIIIa complex (as occurs in hemophilia), because the two complexes operate on different cell surfaces. Thus, hemophilia is specifically a failure of platelet surface thrombin generation due to a failure of factor X activation on the platelet surface.

LIMITING PROPAGATION OF THE COAGULATION REACTION – THE ROLE OF ENDOTHELIAL CELLS

Once a clot covers an area of vascular injury, the clotting process must be limited to avoid thrombotic occlusion in adjacent normal areas of the vasculature. If the coagulation mechanism were not controlled, clotting could propagate throughout the entire vascular tree after even a modest procoagulant stimulus.

Endothelial cells play a major role in confining the coagulation reactions to a site of injury and preventing clot extension to areas where an intact endothelium is present. Endothelial cells have two major anti-coagulant/anti-thrombotic activities. The protein C/S/thrombomodulin (TM) system is activated in response to thrombin generation²⁶⁾. Some of the thrombin formed during the coagulation process can diffuse away or be swept downstream from a site of injury. When thrombin reaches an intact endothelial cell, it binds to TM on the endothelial surface. The thrombin/TM complex then activates protein C, which binds to its cofactor protein S and inactivates any factor Va or VIIIa on the adjacent endothelial cell membrane. This prevents generation of additional thrombin in the vasculature. The endothelial cell also possesses other anticoagulant features. The protease inhibitors AT and TFPI are present bound to heparan sulfates on the endothelial surface where they can inactivate proteases that reach the endothelial cell²⁷⁾.

REMOVAL OF THE HEMOSTATIC CLOT – FIBRINOLYSIS

Even as the fibrin clot is being formed in the body, the fibrinolytic system is being initiated to disrupt it. The final effector of the fibrinolytic

system is plasmin, which cleaves fibrin into soluble degradation products. Plasmin is produced from the inactive precursor plasminogen by the action of two plasminogen activators: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). The PAs are in turn regulated by plasminogen activator inhibitors (PAIs). Plasminogen is found at a much higher plasma concentration than the PAs. Therefore, the availability of the two PAs in the plasma generally determines the extent of plasmin formation. tPA release from endothelial cells is provoked by thrombin as well as venous occlusion²⁸⁾. tPA and plasminogen both bind to the evolving fibrin polymer. Once plasminogen is activated to plasmin it cleaves fibrin at specific lysine and arginine residues, resulting in dissolution of the fibrin clot.

Fibrinolysis is essential for removal of clots during the process of wound healing as well as for removing intravascular clots that might otherwise be manifest as thrombosis. Intravascular deposition of fibrin is also associated with the development of atherosclerosis. Therefore, an effective fibrinolytic system tends to protect against the chronic process of atherosclerotic vascular disease as well as the acute process of thrombosis. Conversely, defects of fibrinolysis increase the risk of atherothrombotic disease. For example, elevated levels of plasminogen activator inhibitor-1, an inhibitor of fibrinolysis, are associated with an increased risk of atherosclerosis and thrombosis²⁹⁾ as are decreased levels of plasminogen³⁰⁾. The effectiveness of hemostasis *in vivo* depends not only on the procoagulant reactions, but also on the fibrinolytic process.

bleeding

WHAT DOES THE CONCEPT OF CELL-MEDIATED HEMOSTASIS MEAN FOR CLINICAL LABORATORY TESTING?

It should be clear from the preceding discussion that our commonly used clinical coagulation tests do not really reflect the complexity of hemostasis *in vivo*. That does not mean that the PT and aPTT are useless. We just need to understand what they can and cannot tell us. These “screening” coagulation tests are abnormal when there is a deficiency of one or more of the soluble coagulation factors. They do not tell us what the risk of clinical bleeding will be. As noted at the beginning of this article, two patients with identical aPTT values can have drastically different risks of hemorrhage. All of our common coagulation tests including the PT, aPTT, thrombin clotting time, fibrinogen levels, and coagulation factor levels tell us something about the plasma level of soluble factors required for hemostasis. Their clinical implications must be evaluated by the ordering physician. Thus, just because the PT and aPTT are within the normal range it does not follow that the patient is at no risk for bleeding. Conversely, a mild elevation in clotting times does not mean that the patient is at significant risk for bleeding.

Many whole blood coagulation tests are jockeying for position as a means of evaluating overall hemostatic status in selected clinical settings. While whole blood tests have the advantage that they may reflect the contributions of platelets to the hemostatic process, they still do not reflect the contributions of the TF-bearing cells and local tissue conditions. Thus any laboratory test requires skilled interpretation and clinical correlation in evaluating the true risk of

WHAT CAUSES BLEEDING IN PREVIOUSLY NORMAL PATIENTS?

Many patients that experience significant hemorrhage do not have a congenital underlying bleeding tendency that can be identified prior to a bleeding episode. Bleeding following surgical or accidental trauma, or during a medical illness is often associated with the development of an acquired coagulopathy. The hallmark of coagulopathy is microvascular bleeding. This means oozing from cut surfaces and minor sites of trauma, such as needle sticks. Microvascular bleeding can lead to massive blood loss.

Causes of coagulopathic bleeding include consumption of coagulation factors and platelets, excessive fibrinolysis, hypothermia and acidosis.

Consumption of Coagulation Components

We normally think of disseminated intravascular coagulation (DIC) when we talk of consumption. However, clotting factors and platelets can also be consumed during appropriate physiological attempts at hemostasis. In this case it is appropriate to replace the depleted factors with transfusion therapy.

DIC can be much more complicated to manage³¹. The mainstay of treatment is to treat the underlying disorder, such as sepsis. In early or mild/compensated DIC administration of low dose heparin may be considered to control the procoagulant response to inflammation, infection or malignancy. However, in more severe or advanced DIC replacement therapy may be necessary to treat the bleeding tendency associated with depletion of coagulation factors and platelets.

Excessive fibrinolysis

The process of fibrinolysis is initiated whenever

coagulation is initiated. When attempts at hemostasis are unsuccessful, a significant amount of fibrinolytic activity may still be generated and thwart subsequent efforts at hemostasis. Thus, fibrinolytic inhibitors have proven to be useful in some cases in hemophilic bleeding. However, in most cases of coagulopathic bleeding there is little evidence that inhibitors of fibrinolysis are useful.

Hypothermia

Many patients become hypothermic during medical illness or following surgical or accidental trauma³²⁾. Hypothermia can directly interfere with the hemostatic process by slowing the activity of coagulation enzymes. Less well recognized is the finding that platelet adhesion and aggregation is impaired even in mild hypothermia³³⁾. Thus, in hypothermic coagulopathic patients, raising the core temperature can have a beneficial effect on bleeding by improving both platelet function and coagulation enzyme activity.

Acidosis

Acidosis can have as profound an effect on procoagulant function as hypothermia, though the two metabolic abnormalities often coexist. A drop in the pH from 7.4 to 7.2 reduces the activity of each of the coagulation proteases by about half³⁴⁾. Thus, acidosis should be considered as a potential contributor to coagulopathic bleeding in medical and surgical patients.

WHAT HAPPENS AFTER THE BLEEDING STOPS?

Once hemostasis is established the process of wound healing can begin. The hemostatic plug must be stable enough to maintain hemostasis, yet be removed as the tissue defect is permanently closed. Fibrinolysis is accomplished by the action of plasmin, probably in concert with other leukocyte proteases. The neutrophils that initially

accumulate at a site of injury are replaced over the course of a few days with macrophages that engulf and degrade cellular debris and components of the fibrin clot. The macrophages also secrete cytokines and growth factors that facilitate the migration of fibroblasts and endothelial cells into the wound site. In the case of a skin wound, the dermis is replaced by highly cellular and vascular granulation tissue, while the surface epithelium proliferates and migrates from the margins to cover the surface of the wound. Many of the activities involved in wound healing are influenced by thrombin. Thrombin plays a major role in platelet activation and degranulation. Several key cytokines modulating wound healing are released from activated platelets, including transforming growth factor beta (TGF β 1), and platelet-derived growth factor (PDGF). Of course, the amount and rate of thrombin generated during hemostasis influences the initial structure of the fibrin clot – the framework on which cell migration takes place. In addition, thrombin has chemotactic and mitogenic activities for macrophages, fibroblasts, smooth muscle cells and endothelial cells. Thus, we hypothesized that the rate of thrombin formation would influence the rate and/or effectiveness of wound healing. This hypothesis is provisionally supported by the sentiment held by many hematologists that patients with hemophilia heal more slowly than hemostatically intact patients. However, there is no published literature documenting any such difference in wound healing.

DOES HEMOSTASIS INFLUENCE SUBSEQUENT WOUND HEALING?

We selected a skin punch biopsy model of wound healing³⁵⁾ to test our hypothesis in hemophilia B mice³⁶⁾. We reasoned that excessive

bleeding could be easily noted in this model and controlled by either direct pressure or coagulation factor replacement. We placed 3 mm punch biopsies on the backs of normal or hemophilia B mice under sterile conditions. The mice were monitored for bleeding over the next eight hours. Mice with excessive bleeding were given one dose of (human) FIX into the peritoneum. The mice were allowed to rest undisturbed the night following placement of the biopsy wounds, then checked again twice daily for evidence of bleeding over the next two days. The gross appearance and size of the biopsy wounds were monitored once daily. After eight days the mice were killed, the skin of the back was removed, and pinned flat on cork during fixation in 10% buffered formalin for six hours. The site of each wound was then cut out of the skin block, processed routinely and embedded in paraffin. Sections through the center of the wound sites were cut and stained with hematoxylin and eosin for histologic review.

We found that the wild type mice, consistent with many previous reports in the literature, had complete closure (re-epithelialization) of their skin wounds by 7 days after wound placement (**figure 8**). Somewhat to our surprise, most of the factor IX-deficient (KO) mice did not have excessive bleeding at the time of injury. Only two of 13 KO mice had bleeding at the time of injury. However, five additional mice developed bleeding over the next 8 hours. This is consistent with the picture in human patients with hemophilia, who often experience delayed bleeding. Those KO mice that experienced excessive bleeding following wounding were “rescued” with a single dose of human FIX to prevent exsanguination. No wild type mice had delayed bleeding and there was no mortality to the 16 wild type mice. However, three of 13 KO mice died in these initial experiments in spite of the rescue protocol.

Figure 8

The wounds in the KO mice that were not treated with FIX required about 10 days for healing (**figure 8** – compared to an average of 8 days in the wild type mice). The mice that had received FIX as treatment for excessive bleeding exhibited more rapid healing of their wounds than did the untreated KO mice – suggesting that temporary reconstitution of the hemostatic system improved wound healing. However, all of the KO mice developed subcutaneous hematomas that were barely beginning to organize at the time that the skin wounds had completely closed and re-epithelialized. We speculate that the very high level of TF that is present in the epidermis might promote initial clot formation at the surface of the wound, thus accounting for the significant number of KO mice that had no external evidence of excessive bleeding. However, TF is much less abundant in deeper tissues, such as subcutaneous fat and muscle. Later bleeding might then occur below the dermis and result in hematoma formation between tissue planes. These persistent hematomas found in the KO mice, even those treated with FIX, might be a model for hemophilic pseudotumor formation seen in human patients.

Thus, our preliminary data indicate that wound healing is impaired in a mouse model of hemophilia, although we do not yet have

any data on the mechanism. We hope that our experiments will lead to a better understanding of the mechanisms of wound healing and allow us to design treatment regimens that not only provide hemostasis in hemophiliac patients, but also minimize complications and normalize wound healing.

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