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THROMBOSIS AND HEMOSTASIS

Comment on Ruben et al, page 3463

Shining a light on thrombin activation

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In this issue of *Blood*, Ruben et al[1] determine structures of the prothrombinase complex, both bound to lipid nanodiscs and in a ternary complex with prothrombin. This study illustrates, for the first time, how factors Va (fVa) and Xa (fXa) associate on a membrane surface, as well as how prothrombin binds in a conformation that leads to the generation of meizothrombin.

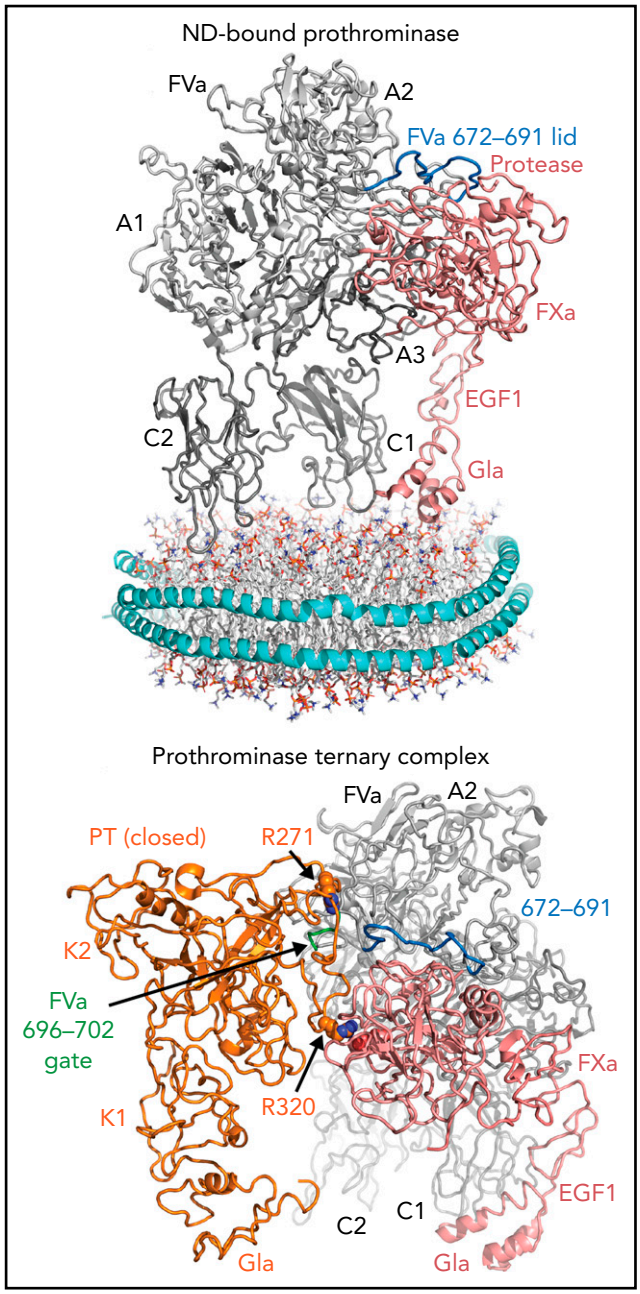
Hemostasis is maintained by a series of proteolytic events that convert inactive zymogens into active serine proteases at the sight of vascular injury, ultimately leading to the formation of an insoluble fibrin mesh.[2] The penultimate step in the blood coagulation cascade is the proteolytic conversion of prothrombin to thrombin by the prothrombinase complex,[3] consisting of fVa and fXa in the presence of calcium and negatively charged phospholipids on the membrane surface of activated platelets or endothelial cells.

Activation of prothrombin is a complex process that has been characterized in detail, yet no direct structural information has been reported.[3] The complete generation of thrombin requires 2 site-specific cleavages of prothrombin, at R271 and R320, which is described via 2 distinct pathways.[4] Recent studies have shown that the prothrombinase complex prefers initial cleavage at R320, generating the catalytically active meizothrombin, followed by cleavage at R271 to release mature thrombin, which serves to cleave fibrinogen to initiate the formation of the insoluble fibrin mesh.[5] The alternative pathway results from initial cleavage at R271, generating the inactive intermediate prothrombin-2, followed by subsequent cleavage at R320 to generate mature thrombin. The structural features of prothrombin have been recalcitrant to structural studies owing to conformational heterogeneity. Recent single-molecule FRET experiments illustrated that prothrombin exists in an equilibrium between an "open" and "closed" state.[6] Using site-directed mutagenesis, constructs of prothrombin have locked both conformations, yielding zymogen states that were amenable to structure determination by X-ray crystallography.[7,8] Further studies have demonstrated that meizothrombin is generated from cleavage in the "closed" conformation, inducing movement to the open conformation for secondary R271 cleavage.[5]

The prothrombinase complex has also lacked structural characterization; however, several models have been proposed based on complementary studies. Although there have been several structures determined for thrombin and fXa, which have led to the development of next-generation anticoagulants that are direct inhibitors of these serine proteases, the factor V (fV)/fVa complete structures have only recently been determined with cryogenic electron microscopy (cryo-EM).[9] FVa plays a critical cofactor role in the prothrombinase complex, increasing the catalytic activity of fXa for prothrombin by >1000-fold.

fV circulates as an inactive protein procofactor consisting of an A1-A2-B-A3-C1-C2 domain architecture. Through activation by thrombin, fVa is specifically cleaved at R709, R1018, and R1545. Activation releases the unconserved B domain that keeps fV in an inactive state. The nascently generated C-terminus of the A2 domain (residues 654-709) has been shown to impact prothrombinase assembly and catalytic activity, but this region is largely undefined in the fVa structure. The cofactor function played by fVa is to associate fXa and prothrombin in an optimal arrangement whereby Xa can site-specifically cleave prothrombin, initially at R320, on a negatively charged membrane surface. Membrane association for each component of the prothrombinase ternary complex is achieved either through the C domains (for FVa) or through γ-carboxyglutamic acid (Gla) domains (for prothrombin and FXa). Ruben et al have for the first time determined the structure of prothrombinase bound to lipid nanodiscs as well as in a ternary complex with prothrombin in its "closed" state, in a conformation that illustrates how R320 is oriented toward the fXa active site, leading to the generation of meizothrombin.

The lipid nanodisc-bound structure, which represents a membrane-bound assembly, was determined to lower resolution (5.3 Å). The structure illustrates how fVa associates with membrane surfaces through its C domains and supports previous models for membrane association.[10] The majority of the prothrombinase complex occurs between the fVa A2 domain and the protease domain of fXa. Intrinsically disordered in the previously determined fVa structure, the 672 to 691 segment of fVa A2 domain C-terminus forms a lidlike cover over the protease domain of fXa, representing a conformational shift from the fV structure by >7 Å. Other electrostatic and hydrophobic interactions also occur between the fVa A2 domain and the protease domain of fXa, presumably contributing to the formation of a prothrombinase-active conformational state. Minor contacts are also observed between the A3 and EGF2 domains, as well as the C1 and Gla



Cryo-EM structures of lipid nanodisc-bound prothrombinase (top) and prothrombinase in a ternary complex with prothrombin (bottom). Top, FVa is represented in gray, with the A1, A2, A3, C1, and C2 domains labeled. FXa is shown in pink with the Gla, EGF1, and protease domains labeled, forming connections with the FVa A2, A3, and C1 domains. The protease domain of FXa is held in place with an FVa A2 domain "lid" (residues 672-691, shown in blue). The lipid nanodisc is modeled below to illustrate how these domains bind membrane surfaces. Bottom, prothrombinase (FVa/FXa) bound to prothrombin in its "closed" form. Prothrombin is shown in orange, with the Gla and Kringle (K1 and K2) domains labeled, which makes a small contact with the FVa A2 domain 696 to 702 loop (green). R320 projects toward the specificity site adjacent to the active site of FXa (pink). R271 sits above the 696 to 702 loop. EGF1, epidermal growth factor-like domain 1; ND, lipid nanodisc; PT, prothrombin.

domains, of fVa and fXa, respectively (see upper figure).

Remarkably, the prothrombinase structure is conserved in a ternary complex with prothrombin in the absence of lipid nanodiscs, strongly suggesting this is an authentic, physiologically relevant assembly. The long segment of the A2 domain C-terminus, 680 to 709, was previously implicated in prothrombin binding and prothrombinase activity; however, only a small portion (696-702) serves to separate the 2 sites of cleavage. Here, this is proposed to induce directionality in prothrombin cleavage, sequestering R271 away from the fXa active site while directing R320 immediately for proteolysis. Surprisingly, only modest interactions occur between the protease domain of prothrombin with the A2 domain of fVa. Several previous contacts reported through indirect measurements were not supported by the prothrombinase ternary complex determined in this study, including no direct interactions of the Kringle or Gla domains of prothrombin with either fVa or fXa. The overall structure of the ternary complex is compatible with all membrane association domains being presented along the same plane to bind phospholipid surfaces simultaneously, forming a domelike structure (see lower figure).

The 2 structures reported by Ruben et al represent a landmark advancement in the understanding of how thrombin is activated and provides an optimistic route toward successfully elucidating the structures of more membrane-associated coagulation complexes in the future. Lipid nanodisc technology, single-molecule FRET measurements, and cryo-EM advances can all now be combined to generate great new insights into how blood coagulation occurs rapidly and specifically at the sites of vascular damage and may lead to more innovative clinical interventions to improve treatments for thrombotic and hemophilic diseases.

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CLINICAL TRIALS AND OBSERVATIONS

Comment on Zhang et al, page 3493

HLH treatment: smarter, not harder

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In this issue of *Blood*, Zhang et al[1] present the largest prospective study reported to date demonstrating the clinical benefit of a first-line targeted therapy for children with hemophagocytic lymphohistiocytosis (HLH). HLH is a rare, life-threatening systemic illness that is characterized by unrestrained T-cell activation and cytokine-mediated hyperinflammation, referred to as a cytokine storm. Clinicians are under enormous pressure to act quickly and decisively to interrupt the cycle of immune activation and tissue destruction when faced with a critically ill child with suspected HLH. Although high-dose glucocorticoids coupled with chemotherapy are commonly used, this approach is associated with significant risks. Ruxolitinib has emerged as a promising treatment option in HLH because of its favorable toxicity profile. The study of Zhang and colleagues provides strong support for ruxolitinib as front-line treatment of HLH and represents a major step forward in updating the approach to this life-threatening disease.

The Histiocyte Society's HLH 1994 and 2004 clinical trials form the basis for treatment of HLH in children. These studies were designed primarily to improve outcome in patients with inherited defects in lymphocyte cytotoxicity, that is, familial HLH (FHL), which was uniformly fatal prior to their development.

Initial therapy consists of etoposide and dexamethasone to suppress inflammation by reducing T-cell activation, followed by allogeneic hematopoietic cell transplant for patients with genetically verified FHL or refractory/recurrent disease. This strategy results in long-term survival in 61% of cases. Unfortunately, the treatment is both profoundly myelosuppressive and broadly immunosuppressive and associated with pretransplant mortality in 20%.[2] Importantly, the need for chemotherapy-based treatments for patients who do not have FHL, that is, those with various forms of secondary HLH, is not established.

In recent years, the development of more-targeted, less-toxic therapy for HLH has emerged as a subject of intense interest and study. Although T-cell activation is important in the pathophysiology of HLH, elevated levels of inflammatory cytokines, such as interleukin-1β (IL-1β), IL-2, IL-6, IL-18, tumor necrosis factor (TNF), and interferon-γ (IFN-γ), are increasingly recognized as major drivers of disease activity. The importance of neutralizing inflammatory cytokines was first noted in macrophage activation syndrome (MAS), a form of secondary HLH, where blockade of IL-1β with anakinra, an IL-1 receptor antagonist, is highly effective.[3] More recently, neutralization of IFN-γ with emapalumab led to its Food and Drug Administration approval for treatment of FHL in patients resistant to or intolerant of standard chemoimmunotherapy.[4]

Ruxolitinib, an inhibitor of Janus kinase 1 (JAK1) and JAK2, blocks the signaling of cytokines through JAK/STAT (signal transducer and activator of transcription) pathways, endowing it with the ability to simultaneously inhibit the action of IFN-γ, IL-2, IL-6, and other proinflammatory cytokines (see figure). Ruxolitinib has demonstrated activity in mouse models of primary and secondary HLH.[5] Case series of adults with refractory HLH provide evidence that ruxolitinib can be effective as salvage therapy. However, there are few reports on the use of this medication as front-line therapy in adults or children with HLH.[6-8]

Zhang et al tested the efficacy of ruxolitinib monotherapy in this single-center, single-arm prospective study of 52 children who fulfilled standard diagnostic criteria for HLH. Cotreatment with glucocorticoids was permitted. Patients were stratified by their initial response to the JAK inhibitor, and those with an unfavorable response received individualized intensification treatment with chemotherapy (mostly etoposide and methylprednisolone ± other agents) in addition to ruxolitinib. The primary endpoint was the overall response rate to ruxolitinib monotherapy; this was achieved in 69% of patients. A complete response to ruxolitinib monotherapy was observed in 42% of patients, all of whom demonstrated response by day 3, completed 28 days of ruxolitinib, and subsequently remained disease free. Of the 58% of patients who required addition of "intensive" chemotherapy, about half had treatment response. Ruxolitinib monotherapy followed by intensification therapy in poor responders resulted in 83% overall survival. Among the 52 patients, HLH was associated with Epstein-Barr virus (EBV) in 34 cases (65%). The patients with EBV-HLH were significantly more likely, whereas those with chronic active EBV-HLH were less likely to survive.

There are several important findings to highlight. The study provides strong evidence that a rapid and durable response to ruxolitinib alone can be achieved in a significant subset of children with HLH, obviating their need for toxic