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The regulation of proplatelet production

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Hematologists have long been fascinated by thrombopoiesis. While it has long been accepted that platelets are derived from megakaryocytes,[1] the process by which this occurs and its regulation are still incompletely understood. As they mature, megakaryocytes acquire competency for platelet formation through up-modulation of a vast array of cytoskeletal, membrane and granule regulatory proteins,[2,3] differentiating into cells that are large, polyploid, and have a cytoplasm filled with a complex system of interconnected cytoplasmic membranes (dermarcation membrane system or DMS), as well as stores of ribosomes, alpha granules and dense granules.[4,5] Although the growth and differentiation of megakaryocytes requires thrombopoietin, the role of this cytokine in the subsequent steps of platelet formation is less well established.[6]

Models of proplatelet formation

At least two models have been put forward to explain platelet formation. Based on examination of microscopic images, James Homer Wright proposed that platelets are released from pseudopodial processes (later termed proplatelets) that extend from megakaryocytes into blood vessels.[1] Alternatively, Sharnoff and colleagues suggested that megakaryocytes travel through the circulation to the lungs where they are physically fragmented into platelets within pulmonary capillaries.[7] In the former model, it is hypothesized that the role of the DMS is as a store of membrane to support proplatelet formation, whereas in the latter the DMS defines pre-formed platelet territories.

Although still controversial, recent data favor the proplatelet model of thrombopoiesis. Using live cell microscopy, Italiano and colleagues captured cultured megakaryocytes elaborating branching proplatelet processes.[8] Microtubule bundles within the processes were seen to form discrete loops at the end of the processes, correlating to the microtubule marginal band that defines the outline of the discoid platelet (Figure 1). Importantly, the fragmentation model does not account for this essential platelet structure. Subsequent work by Junt and colleagues confirmed that proplatelet formation was not only an *in vitro* phenomenon, but that YFP-labeled megakaryocytes could be seen to release proplatelet-like bodies into the marrow sinusoids in living mice.[9] These studies also suggested that shear forces in the marrow sinusoid may play a role in stimulating platelet release. Despite these remarkable insights from live cell imaging, a number of questions remain regarding the factors that initiate and regulate proplatelet formation.

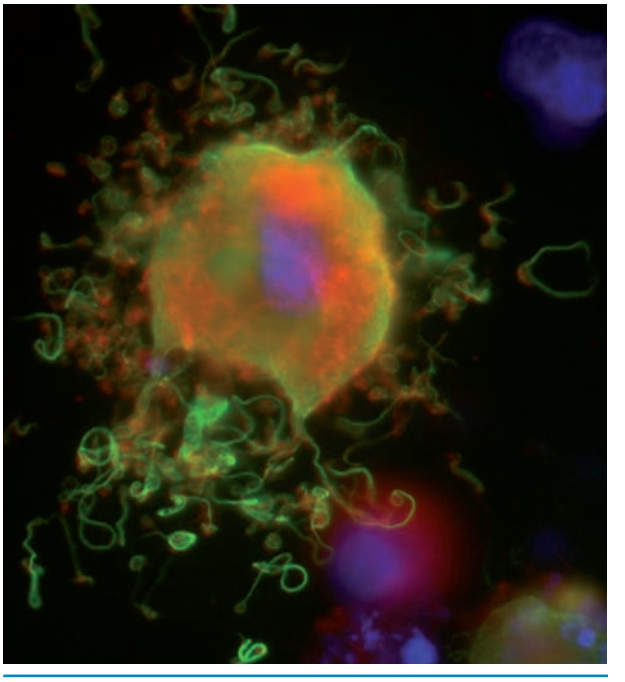

Figure 1. Murine megakaryocyte demonstrating *in vitro* proplatelet formation. Green: tubulin, Red: actin, Blue: DNA. Note formation of microtubule loops at the end of the proplatelet processes.

The role of apoptosis

Proplatelet formation is a terminal process, and once it has been completed the residual megakaryocyte nucleus is engulfed by macrophages within the bone marrow.[8] However, recent studies indicate that localized apoptosis actively contributes to proplatelet formation by an as yet poorly understood mechanism.[10,11] For example, using a mouse model in which megakaryocytes over-express Bcl-x$_L$, Kaluhnzy and colleagues found that inhibition of apoptosis results in abnormal DMS formation, impaired *in vitro* proplatelet formation and a blunted recovery from thrombocytopenia.[12] In addition, Morison and colleagues characterized a large family with autosomal dominant thrombocytopenia due to a novel mutation at a conserved residue cytochrome C that led to enhanced apoptosis.[13] Surprisingly, platelet lifespan was normal in affected family members; however, the observation of proplatelets forming within the marrow space suggested that premature and ineffective proplatelet formation is the mechanism of peripheral thrombocytopenia in this disorder. Further study of the function of the apoptosis machinery in megakaryocytes is needed to understand its contribution to thrombopoiesis.

The cytoskeleton in proplatelet formation

Reorganization of the megakaryocyte cytoskeleton, including tubulin, actin and myosin, precedes and promotes thrombopoiesis. Dynamic reorganization of tubulin is essential for proplatelet formation, as shown using chemical inhibitors of tubulin polymerization and depolymerization.[8] Subsequent work demonstrated that proplatelet elongation requires the sliding of microtubules past one another and can be inhibited by blocking the function of the microtubule-associated motor protein dynein.[14] The predominant β-tubulin isoform in platelets is β1-tubulin, which is unique to the megakaryocyte lineage. Mice lacking β1-tubulin are thrombocytopenic and exhibit impaired proplatelet formation compared to controls, although they are still capable of generating platelets.[15] Due to disruption of the marginal band, *β1-tubulin*-null platelets are spherical rather than discoid in appearance.[15] Recently, the importance of tubulin in platelet production has been correlated clinically, as familial mutations of β1- tubulin have been discovered in humans and in dogs in association with autosomal dominant macrothrombocytopenia.[16,17]

Actin and myosin also play important roles in proplatelet formation, likely promoting the branching and amplification of proplatelet tips.[8] Additionally, myosin IIA and its upstream regulator RhoA restrain the onset of platelet formation. Consistent with this model, mutations that reduce myosin II activity, such as those occurring in MYH9-related disease, are associated with the premature initiation of proplatelets within the marrow space.[18,19] Mutations in the actin-regulating Wiskott Aldrich syndrome protein (WASP) are also associated with ineffective thrombopoiesis,[20] which may combine with peripheral platelet destruction to cause thrombocytopenia in patients with Wiskott Aldrich syndrome.

Integrin receptors provide a potential mechanism to link these cytoskeletal changes in the megakaryocyte to the extracellular marrow environment. For example, type I, but not type III or IV, collagen inhibits proplatelet formation,[21] whereas fibrinogen promotes it.[22] Correspondingly, the integrin α2bβ3 is essential for proplatelet formation in response to fibrinogen[22] and mutations resulting in constitutive activation of α2bβ3 further increase thrombopoiesis.[23] One interpretation of this constellation of findings is that the differential response to extracellular factors provides a mechanism to prevent platelet release until the megakaryocyte reaches the vascular niche.[24]

The von Willebrand factor receptor GPIb is another important regulator of proplatelet formation. Antibodies to GPIb have been found experimentally to inhibit megakaryopoiesis and platelet production *in vitro*,[25,26] a finding which may have clinical relevance to immune thrombocytopenia syndromes in which antibodies to GPIb are common. In addition, autosomal recessive inheritance of mutations involving GPIb causes macrothrombocytopenia in humans (Bernard-Soulier syndrome, BSS). Hypothetically, the interaction between GPIb and von Willebrand factor would be one way for the megakaryocyte to sense shear in the sinusoid. However, Kanaji and colleagues expressed a hybrid protein consisting of the intracellular domain of GPIbα and the extracellular domain of the interleukin-4 receptor in *GPIbα*-null mice and found that platelet production was partially rescued, suggesting that in the absence of interaction with von Willebrand factor the association of GPIbα with cytoskeletal components such as filamin and 14-3-3ζ is still able to contribute to thrombopoiesis.[27]

Questions of platelet size

Any model of proplatelet formation must account for the variation in platelet size that occurs under conditions of increased platelet destruction and in certain heritable diseases. A population of large platelets is characteristically seen during recovery from acute thrombocytopenia such as occurs in immune-mediated platelet destruction. This has been attributed to the premature release of young platelets and precedes measurable changes in megakaryocyte ploidy.[28,29] Curiously, large platelets are not prominent in reactive thrombocytosis and, therefore, the kinetics of platelet production is probably an important factor in this phenomenon. Abnormally large platelets are also characteristic of several of the inherited platelet disorders including the above mentioned BSS and MYH9-related disease, as well as gray platelet syndrome and thrombocytopenia due to mutations involving GATA1.

The marked macrothrombocytopenia of BSS has drawn platelet biologists to study this disease in order to better understand the role of GPIb in thrombopoiesis. Paulus and colleagues observed that the platelet territories defined by the DMS in megakaryocytes from patients with BSS are larger than in those from controls.[30] More recently, Balduini and colleagues studied patients with BSS due to a heterozygous mutation of GPIbα and found that their megakaryocytes produce 50% fewer proplatelets than control megakaryocytes, and those proplatelets have abnormally large tips.[31] Because further clarification of the mechanisms leading to macrothrom-

bocytopenia is technically difficult in patients, Strassel *et al.*, in a study published in this issue of Haematologica,[32] turned to a mouse model to address these questions.

Thrombopoiesis in mouse models of Bernard-Soulier syndrome

Murine models of BSS, including mice with targeted disruption of either *GPIbα*[33] or *GPIbβ*[34] recapitulate the phenotype of the human disease with macrothrombocytopenia and platelet dysfunction. Megakaryocytes from *GPIbα*-null mice have a poorly developed DMS and a reduced internal membrane pool.[35] In this issue of Haematologica Strassel *et al.* characterize megakaryopoiesis and proplatelet formation in the *GPIbβ*-null mouse in an attempt to further elucidate the mechanisms of macrothrombocytopenia in BSS.[32] The strengths of their elegant work are the similarities between the phenotype of megakaryocytes from the *GPIbβ*-null mice and those from patients with BSS, the stepwise evaluation of thrombopoiesis in the mice from megakaryocyte commitment to platelet survival, and quantitative analysis of their observations. The results provide clear evidence that macrothrombocytopenia in *GPIbβ*-null mice is not due to decreased platelet survival or impaired megakaryocyte maturation, but to abnormal DMS formation and impaired proplatelet formation. Curiously, the microtubule bundles at the core of the proplatelet and within the marginal band are measurably thicker than those in wild type platelets, suggesting that GPIbβ regulates microtubule organization in an as yet undefined way. Nevertheless, in this model the *GPIbβ*-null platelets retain their discoid shape, a finding that is in contrast to prior observations that platelets from BSS are spherical.[36] Taken together, this work and others demonstrate that there is a growing interest in and understanding of the mechanisms of proplatelet formation, which will provide insight into both acquired and inherited thrombocytopenia syndromes.

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Definition, diagnosis and treatment of immune thrombocytopenic purpura

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In this issue, Arnold and colleagues document by a systematic review of controlled studies that eradication of *H. pylori* infection increases the platelet count in patients with immune thrombocytopenic purpura (ITP).[1] This observation, together with data from a previous systematic review,[2] requires that we address two questions. 1) What is the role of *H. pylori* infection in the pathogenesis of ITP? 2) What is the role of eradication of *H. pylori* infection in the management of ITP? These questions require a discussion of the definition, diagnosis, and current treatment of ITP. This discussion focuses on adults, as *H. pylori* infection is rare in children and two current systematic reviews have only identified studies of ITP in adults.[1,2]

Definition and diagnosis of immune thrombocytopenic purpura

ITP is defined as isolated thrombocytopenia with no clinically apparent associated conditions or other causes of thrombocytopenia.[3] This definition provides the basis for the initial patient evaluation. No specific criteria establish the diagnosis of ITP; the diagnosis relies on the exclusion of alternative disorders, such as the examples listed in Table 1. Exclusion of recognized alternative etiologies of thrombocytopenia was the basis for the original name for ITP, *idiopathic thrombocytopenic purpura*.[3] A current perspective has proposed the name *primary immune thrombocytopenia* for ITP, to distinguish ITP from identifiable alternative *secondary* etiologies.[4]

Should *H. pylori* infection be considered an alternative disorder, the same as HIV and hepatitis C infections? Does the diagnosis of *H. pylori* infection exclude the diagnosis of ITP? The current status of *H. pylori* infection, as indicated in Table 1, is uncertain. *H. pylori* infection may be an alternative, additional, or incidental unrelated disorder; all three possibilities are suggested by different studies. It is the inconsistency among current studies which has prevented broad acceptance of the association of *H. pylori* infection with ITP.

There are striking geographical disparities of both the frequency of *H. pylori* infection among patients with ITP and the frequency of platelet count responses following eradication of *H. pylori* infection, and these two frequencies correlate with each other.[1,2] In countries with a high prevalence of *H. pylori* infection and high platelet count response rates following eradication, such as Japan (where most studies of *H. pylori* eradication in ITP have been performed) and Italy, testing for *H. pylori* infection has been recommended as a standard diagnostic procedure in adults with suspected ITP and eradication therapy is recommended as the initial treatment in *H. pylori*-infected patients.[5,6] These studies suggest that thrombocytopenia associated with *H. pylori* infection may be an alternative disorder, similar to the thrombocytopenia associated with HIV and hepatitis C infections. In contrast, only three studies have been reported from the United States and Canada and they have all reported lower frequencies of both *H. pylori* infection and platelet count response rates following eradication treatment.[7-9] These data suggest that *H. pylori* infection may be merely an incidental observation, not excluding the diagnosis of ITP and not clinically important for management. In between these alternative interpretations are studies reporting partial platelet count responses, suggesting that *H. pylori* infection may contribute to the thrombocytopenia in ITP but is not the sole cause.[2] An explanation for the different platelet count response rates following *H. pylori* eradication may be the presence of different genotypes of *H. pylori* in different geographical regions.[6] For example, most *H. pylori* strains in Japan express the product of the cytotoxin-associated gene A (CagA); the frequency of CagA-positive strains of *H. pylori* in Western countries is lower.[2]