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and expansion of the mutation known to be resistant to the selected TKI, as predicted.

This comprehensive prospective study provides an alternative method for sensitive KD mutation testing, one that is perhaps easier to adopt given the number of institutions that have established NGS testing for other purposes. Earlier detection of low-level KD mutations will inform clinicians of the need to alter the current therapy before the disease progresses to blast crisis CML, which is a real possibility as shown in this study. The dismal outcomes associated with blast crisis CML[5] may be circumvented by an earlier switch to an optimal TKI, which would reduce transformation rates and improve survival.

However, several limitations associated with NGS make routine application of this technology for the proposed purpose challenging. Although Soverini et al were able to provide results within an average of 11 days (range, 7-24 days), many laboratories would struggle to achieve this turnaround time,[6] which would thwart the feasibility of obtaining an actionable result before overt treatment failure or disease progression. Moreover, the costs associated with NGS can be prohibitive in a diagnostic setting; thus, batch testing would be required to reduce the expense associated with testing each sample separately, adding to the delay in obtaining results. In comparison, SS is fast and inexpensive, although it has a mutation detection limit of 10% to 20%,[7,8] which negates its applicability in low-level mutation testing. Before NGS-based assays can be adopted into mainstream diagnostics, these issues will need to be addressed by each institution that intends to implement them for the purpose of KD mutation testing.

An issue that now needs to be addressed is the actual consensus definition of low-level mutation. The NGS-based assay used by Soverini et al in their study, classified low-level KD mutations as being undetectable by SS, which correlates to a variant allele frequency of 3% to 20%. However, as technology improves, it is likely that the sensitivity of NGS will increase, with the potential to detect mutations that have an allele frequency considerably less than 3%. What should the actionable threshold for recommending a switch in TKIs? Furthermore, should we be screening patients deemed to be at high risk for treatment failure at earlier time points, even before a warning response is recognized? This could conceivably include screening selected patients at diagnosis to detect low-level actionable KD mutations that would optimize TKI selection and prevent the development of TKI resistance. Although these issues will need to be considered in the near future, the findings from Soverini et al make a strong argument to support the use of NGS in patients who do not achieve optimal molecular responses; facilitating rational selection of TKI and ideally prevent overt drug resistance and disease progression.

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RED CELLS, IRON, AND ERYTHROPOIESIS

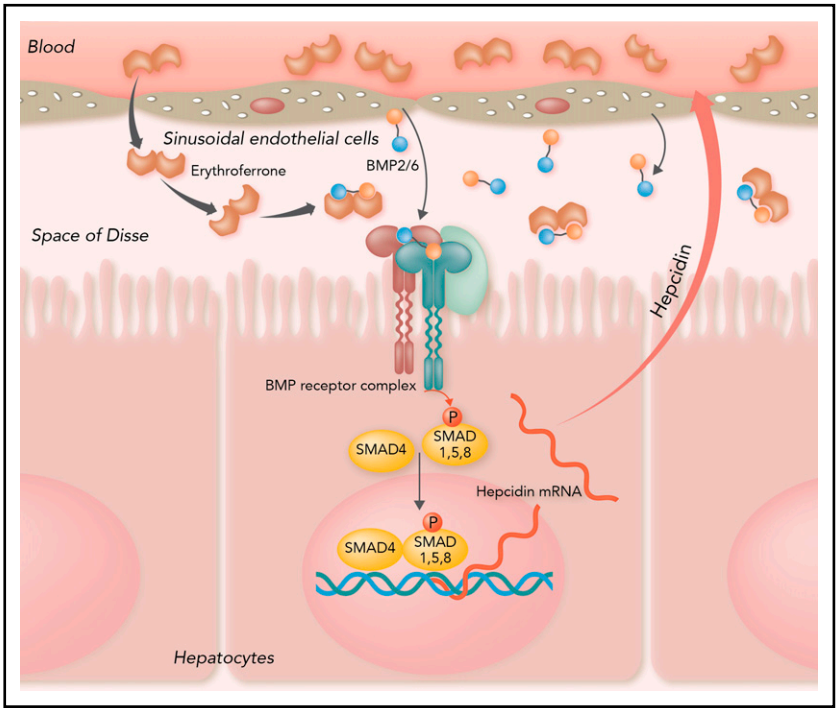
Comment on Arezes et al, page 547

Drugging erythroferrone to treat anemias

Tomas Ganz | University of California, Los Angeles

In this issue of *Blood*, Arezes et al report on the development of a monoclonal antibody that neutralizes the activity of the iron-regulatory erythrokine erythroferrone (ERFE). In a mouse model of β-thalassemia intermedia, the monoclonal antibody increased hemoglobin levels and decreased serum iron concentration and systemic iron overload.[1]

During maximally stimulated erythropoiesis (eg, after hemorrhage), hemoglobin synthesis increases up to 10-fold and must be supported by a comparable increase in iron delivery from stores and diet. This response starts within hours after the precipitating event and develops without an appreciable change in serum iron concentration. Five years ago, ERFE[2] was discovered as a new hormone secreted by erythropoietin-stimulated erythroblasts, and it acts on the liver to suppress the production of the iron-regulatory hormone hepcidin. Circulating ERFE levels increase after hemorrhage or in response to stimulation with erythropoietin, and they help maintain stable plasma iron concentrations by mobilizing iron to meet the requirements of erythropoiesis.[2]



Shown is a current model of the mechanism of action of ERFE. ERFE binds and sequesters heterodimeric BMP2/6 secreted by sinusoidal endothelial cells into the space of Disse and thereby prevents BMP2/6 from binding to the hepatocyte BMP receptor. Diminished BMP receptor signaling then decreases hepcidin gene transcription and protein secretion.

Remarkably, plasma ERFE concentrations are greatly increased in anemias with ineffective erythropoiesis,[3,4] probably because of ERFE secretion by a greatly expanded population of mostly dead-end erythroblasts in both the marrow and at extramedullary sites. Plasma ERFE concentrations seem to be inversely related to those of hepcidin, consistent with the proposed suppressive effect of ERFE on hepcidin. In a mouse model of β-thalassemia intermedia, genetic ablation of ERFE reversed the suppression of hepcidin and partly prevented iron accumulation.[4]

The mechanism of action of ERFE has been a mystery, and multiple searches for ERFE receptors turned up no information. ERFE belongs to the C1q-TNF-α–related protein family, characterized by an N-terminal extended region containing a variably long collagen-like multimerization motif and a C-terminal globular head that structurally resembles that of TNF-α or the C1q complement protein. The article by Arezes et al provides 2 important insights. First, they documented that the bioactive segment of ERFE is in the N-terminal extended region, an observation also made independently by another group.[5] Importantly, they also found that ERFE seems to act directly on bone morphogenetic protein (BMP) signaling,[6] the main pathway by which the transcription of the hepcidin gene is regulated. Unexpectedly, the N terminus of ERFE binds the BMP receptor ligands BMP2 and BMP6, which had been implicated as being essential for hepcidin regulation. Here, the relevant physiological ligand may be the BMP2/6 heterodimer, which could be the species entrapped by ERFE (see figure), as suggested by others.[7] The avidity of the interaction between ERFE and these BMPs may be further strengthened by the multimeric structure of ERFE (in this regard, behaving similarly to adiponectin[8]), with multimerization promoted by the interactions between the globular C-terminal regions. Although the existence of a receptor for ERFE is not excluded by these observations, the proposed mechanism of action explains all relevant findings to date.

Unlike its downstream target hepcidin, which circulates at nanomolar concentrations, ERFE is active at picomolar concentrations, making it an ideal target for monoclonal antibody therapy. Arezes et al developed such antibodies, appropriately targeting the bioactive N terminus of ERFE, and demonstrated that these antibodies neutralized the hepcidin-suppressive activity of ERFE in a hepatocyte cell line as well as in mice. Importantly, these antibodies exerted a beneficial effect in the murine β-thalassemia intermedia model, in which they ameliorated anemia, reversed the suppression of hepcidin, decreased serum iron concentrations, and diminished hepatic iron overload. Supporting the feasibility of this approach, largely similar results were also obtained by another group with a different set of antibodies.[9,10] A puzzling effect of anti-ERFE antibodies is that they improve anemia in thalassemic mice, an effect not seen when the ERFE-encoding *Erfe* gene was ablated in these mice.[4] It is possible that the complete loss of ERFE during development elicits some form of compensation not seen when ERFE is neutralized by antibodies in adult mice.

In view of the broad importance of BMP signaling in many organs and tissues, systemic inhibition of BMP signaling by ERFE in iron-loading anemias may contribute to the many nonhematologic manifestations of these diseases, raising hope that some of these may also respond to anti-ERFE therapy. Anti-ERFE antibodies are expected to be well tolerated in humans because ERFE, at least in mice, is a stress hormone that is not essential for health or survival. The addition of yet another preclinical candidate to the rapidly widening pipeline of treatment options for thalassemias and other anemias with ineffective erythropoiesis is a cause for optimism.

Conflict-of-interest disclosure: T.G. is a scientific founder and shareholder of Silarus Therapeutics and Intrinsic LifeSciences and serves as a consultant to Global Blood Therapeutics, Sierra Oncology, Ionis, American Regent, Akebia, and Regeneron. T.G. has received grant funding from Akebia/Keryx and Sierra Oncology. ■

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THROMBOSIS AND HEMOSTASIS

Comment on Ivanov et al, page 558

Plasma kallikrein's low gear

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In this issue of *Blood*, Ivanov et al[1] demonstrate that the apple domain–containing proenzyme prekallikrein (PK) has single-chain proteolytic activity capable of initiating reciprocal activation with factor XII (FXII).

Most cars have a low-gear mode that enables rapid acceleration from a standing start. Ivanov et al describe a low-gear mode for plasma protease PK in the form of weak single-chain enzyme activity. This finding builds on work from the same group that demonstrated single-chain activity for coagulation FXII. Thus, both enzymes have background proteolytic activity inherent to the intact polypeptide that was traditionally described as an inactive zymogen. This explains why the 2 enzymes appear to consume each other constitutively, and a depletion of PK gives rise to an increased plasma concentration of FXII.[2] High-gear activation of the contact system likely involves additional factors, such as polyphosphate coupled with relocation to the appropriate cell membrane. Once PK, FXII are converted by cleavage of the activation loop to generate 2 chain PKa and FXIIa, the high gear required for bradykinin production and initiation of the intrinsic pathway is engaged.

PK is an apple domain–containing protease closely related to factor XI (FXI) and thus has a different structure than FXII, which most closely resembles hepatocyte growth factor (HGFA). FXII also clusters with plasminogen activators tPa and uPa in terms of overall sequence identity. The first example of single-chain proteolytic activity was characterized for tPa. The remarkable crystal structure for tPa revealed the basis of the single-chain activity. A positive charge from the amine group of tPa residue Lys156 salt bridges the carboxylate of Asp194 to stabilize the active/open conformation of the oxyanion hole and S1 pocket (see figure panel A).[3] The new data for PK provide a compelling argument that single-chain activity can be generalized more widely to serine proteinases in the trypsin and chymotrypsin family. How does the low-gear mechanism work for FXII and PK? These enzymes do not have Lys156, but Gln appears in an equivalent position for FXII, PK as well as chymotrypsin, thrombin, and FXI. A model of the PK structure based on tPA reveals that Gln156 can coordinate the Asp194 carboxylate and stabilizes the open conformation of the enzyme through hydrogen bonding (see figure panel B).

Proteases factor VII (FVII) and FVII activating protease have Met156 and Leu156, respectively, and these hydrophobic residues would not be expected to provide a similar stabilization of Asp194 as Lys156/Gln156, which is consistent with the observed higher zymogenicity for these enzymes. Mutagenesis studies that replaced FVII Met156 with Gln resulted in an increase in proteolytic activity.[4] Proteases thus have a variety of ways to modulate their single-chain activity to a minimum. Factor X has Lys156, and HGFA has Arg156; these proteases have not been characterized as having high levels of single-chain activity like tPa. In this respect, it is interesting that Ivanov et al also demonstrated that FXI does not have single-chain activity toward its substrate factor IX (FIX) despite having Gln156; so how is this achieved? One mechanism is to directly influence the local environment of Asp194 and "pull" it toward the zymogen state by addition of a positive charge to counter the influence of Lys156/Gln156. It was previously demonstrated for tPa that substitution of residue Phe40 for His can reduce single-chain activity. The His40 side chain directly stabilized Asp194, forming an Asp194-His40-Ser32 network termed the zymogen triad, which was originally observed in the chymotrypsinogen structure. The FXII sequence has Phe40, and thus, no zymogen triad exists; however, a crystal structure revealed direct contact between residues Asp194-Arg73 occurs and could be an alternative to stabilize a zymogen-like conformation.[5] FXI has an Asp194-His40-Thr32 zymogen triad but so does PK, so this is not the complete explanation for the lack of single-chain activity in FXI. A second possibility is that the burial of FXI substrate-binding exosites on the apple domains can modulate the single-chain activity.[6] Thus, in the zymogen, FXI cannot recruit substrate FIX without cleavage of the activation loop to release the exosite in the apple 3 domain. In this case, an extra layer of conformational regulation smothers the FXI single-chain activity to prevent inappropriate cleavage.

Single-chain activity may have an evolutionary origin in the precursors of modern regulatory proteases, which were simpler in design, lacking an activation loop and N-terminal ancillary domains. The functional significance of the single-chain activity is evident for PK and FXII because this can drive autoactivation and stimulate a cascade without the requirement of an exogenous protease to cleave the activation loop. Thus, PK can be activated by a nonproteolytic mechanism via prolyl-carboxypeptidase[7] or heat shock protein 90[8] without the need for FXII. Perturbation of the PK protein structure is sufficient to release the clutch and engage the single-chain low-gear

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