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MYELOID NEOPLASIA

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Mutant calreticulin: when a chaperone becomes intrusive

Mario Cazzola FONDAZIONE ISTITUTO DI RICOVERO E CURA A CARATTERE SCIENTIFICO POLICLINICO SAN MATTEO; UNIVERSITY OF PAVIA

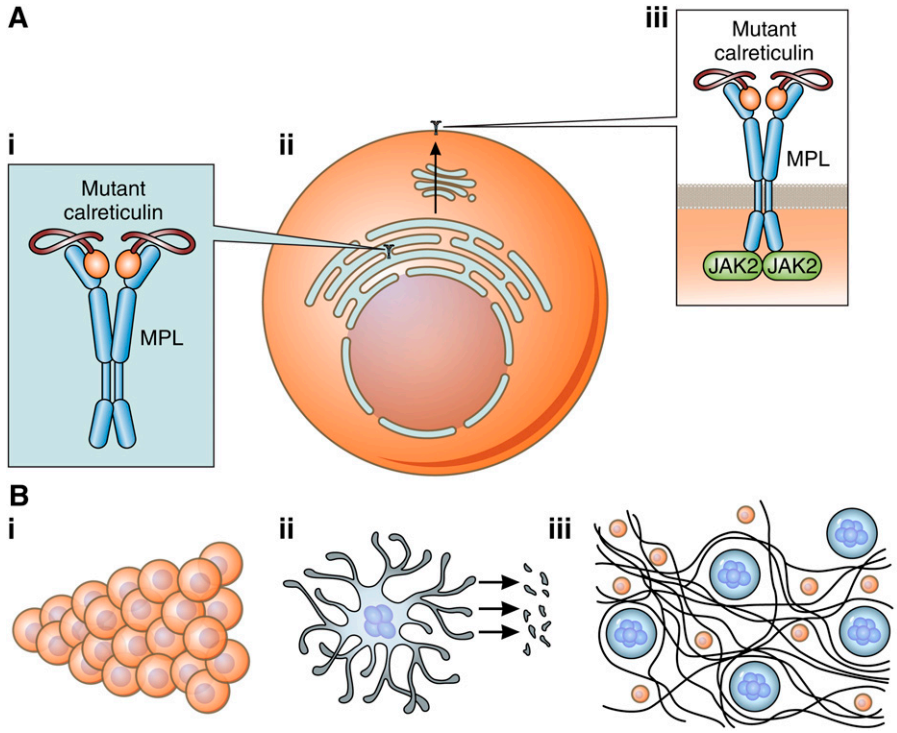
In this issue of *Blood*, Marty et al, Chachoua et al, and Araki et al report results of studies unraveling the molecular pathogenesis of *CALR*-mutant myeloproliferative neoplasms (MPNs). Together, these 3 reports define a novel disease paradigm, whereby a mutant chaperone constitutively activates receptor signaling through an abnormal interaction with the thrombopoietin (TPO) receptor (MPL).[1-3]

When Klampfl et al detected somatic mutations of calreticulin (encoded by the *CALR* gene) in patients with primary myelofibrosis (PMF) with wild-type *JAK2* and *MPL*, it was difficult to understand why mutation in a housekeeping gene, required for the maintenance of basic cellular function, would cause an MPN.[4] In a follow-up study of several types of myeloid neoplasms, we made the illuminating observation that somatic *CALR* mutations occurred almost exclusively in patients with thrombocytosis: essential thrombocythemia (ET), PMF, and the rare myelodysplastic/MPN defined as refractory anemia with ring sideroblasts associated with marked thrombocytosis.[4] This clearly established a strong causative relationship between *CALR* mutation and excessive platelet production, although the underlying molecular mechanism(s) involved were unclear.

Calreticulin resides in the lumen of the endoplasmic reticulum (ER), where it functions as a molecular chaperone for many glycoproteins, assisting their regular folding.[5] In addition, the C-terminal domain of calreticulin is responsible for calcium-buffering activity, which controls calcium homeostasis and, in turn, signaling processes. Somatic *CALR* mutations result in insertions and deletions generating a frameshift, and cluster in the last exon (exon 9) of the gene.[4] More than 50 different types of insertions/deletions in *CALR* have been detected, but a 52-bp deletion (type 1) and a 5-bp insertion (type 2 mutation) are the most frequent types, overall found in >80% of all patients with a *CALR*-mutant MPN.[6,7] The type 2 mutation is predominantly associated with an ET phenotype, whereas the type 1 mutation is mainly associated with a myelofibrosis phenotype and a significantly higher risk of myelofibrotic transformation in ET.[6]

To study the contribution of *CALR* mutants to the pathogenesis of MPNs, Marty et al engrafted lethally irradiated recipient mice with bone marrow cells transduced with retroviruses expressing the mutants.[1] CALRdel52 (type 1 mutation)-expressing mice rapidly developed marked thrombocytosis and then progressed to a condition similar to human myelofibrosis. By contrast, CALRins5 (type 2 mutation)-expressing mice had a mild ET phenotype with low propensity to disease progression. Thus, both mutants specifically amplified the megakaryocyte lineage and increased platelet production. It should be noted that Pietra et al[8] have recently reported differential clinical effects of different mutation subtypes in *CALR*-mutant MPNs, and the clinical effects are very similar to those observed by Marty et al[1] in their murine models. Using a cell line model, Marty et al then demonstrate that *CALR* mutants specifically activate MPL to induce constitutive activation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 5/3/1 (STAT5/3/1).[1] In addition, they show that CALRdel52 cannot induce thrombocytosis in *Mpl* knockout mice. Altogether, these observations indicate that *CALR* mutants are sufficient to induce thrombocytosis through MPL activation.

Using cell line–based transcriptional and proliferation assays, Chachoua et al assessed the ability of *CALR* mutants to induce activation of a series of cytokine receptors that signal via the JAK/STAT pathway.[2] They found that pathogenic *CALR* mutants specifically activate the TPO receptor by a mechanism dependent on the presence of the extracellular N-glycosylation residues of MPL and the glycan-binding site at the new C-terminal tail of the mutant calreticulin. Expression of a soluble extracellular MPL domain blocks pathological signaling of CALR mutants via the TPO receptor, provided it is N-glycosylated. Signaling induced by *CALR* mutants via MPL directly leads to dimerization and activation of JAK2, and downstream STAT5/3/1, mitogen-activated protein kinase, and phosphatidylinositol-3 kinase signaling. Chachoua et al also studied patient cells using specific short hairpin RNAs, which allowed them to confirm the crucial role of MPL and JAK2 in *CALR* mutant-induced spontaneous growth of megakaryocytic progenitors.[2]



Schematic representation of the molecular pathogenesis of *CALR*-mutant MPNs based on the findings of the 3 articles of this issue.[1-3] (A) TPO-independent activation of the TPO receptor (MPL) by mutant calreticulin in an individual hematopoietic cell. (i) Abnormal physical interaction between mutant calreticulin and TPO receptor within the ER. (ii) MPL coupled with mutant calreticulin is exported on the cell surface through the Golgi apparatus and/or an alternative pathway. (iii) TPO-independent activation of MPL, and in turn dimerization and activation of JAK2. As a chaperone, calreticulin normally assists proper folding of the TPO receptor (MPL) inside the ER. Mutant calreticulin preferentially interacts with MPL, and the mutant-specific domain of the protein is required for this interaction that leads to stable binding. Here, it is assumed that the MPL dimer is already assembled in the ER, but another possibility is that dimerization occurs on the cell surface. It is also uncertain whether both MPL subunits bind mutant calreticulin, or just one of them does; again, both events might take place. The interaction of mutant calreticulin with MPL activates MPL signaling through JAK2. (B) Biological and clinical consequences. (i) Activated MPL-driven clonal expansion of HSCs. (ii) Excessive platelet production by abnormal megakaryocytes: ET. (iii) Bone marrow reticulin formation: progression from ET to myelofibrosis. Most patients with a *CALR*-mutant MPN have fully clonal hematopoiesis, indicating that the founding mutation drives clonal expansion at the HSC level. Under normal conditions, adult HSCs are TPO-dependent for their survival and maintenance in the osteoblastic niche[10,11]; thus, mutant calreticulin-induced abnormal MPL signaling is likely responsible for clonal expansion of HSCs carrying a *CALR* mutation. At the hematopoietic precursor level, the only cells in which MPL signaling is effective are megakaryocytes, and therefore MPL-signaling activation leads to overproduction of platelets. Mutant calreticulin-expressing megakaryocytes also contribute to bone marrow fibrosis, and in the long-term this process leads to a transition from ET to myelofibrosis. Professional illustration by Patrick Lane, ScEYEnce Studios.

Araki et al used the TPO-dependent megakaryocytic cell line UT-7/TPO previously generated by their laboratory.[3] Using this cell line, they demonstrate that mutant, but not wild-type, *CALR* activates MPL and downstream signaling molecules including JAK2, STAT5, and extracellular signal-regulated kinase 1/2, and subsequently promotes the TPO-independent growth of UT-7/TPO cells. Araki et al also provide evidence that mutant calreticulin preferentially binds to MPL, and that the mutant-specific domain of calreticulin is required for this interaction.[3] Additionally, they show the cell surface localization of mutant calreticulin with no paracrine activation capacity. Finally, they demonstrate that MPL is required for TPO-independent megakaryocytopoiesis in induced pluripotent stem cell–derived hematopoietic stem cells (HSCs) harboring a *CALR* mutation.

A late-breaking abstract at the 2015 American Society of Hematology meeting reported findings indicating that physical interaction between mutant calreticulin and the TPO receptor is required for hematopoietic transformation and development of MPNs.[9]

The figure summarizes the currently available evidence concerning the molecular pathogenesis of *CALR*-mutant MPNs. The interaction between mutant calreticulin and the TPO receptor likely occurs within the ER, where chaperoning of proteins takes place.[5] MPL coupled with mutant calreticulin is then exported to the cell surface, and this liaison results in constitutive activation of mutant megakaryocytes. Many patients with a *CALR*-mutant MPN have a granulocyte mutant allele burden of ~40% to 50%, indicating that they have fully clonal hematopoiesis. This suggests that activation of MPL must provide an advantage at the stem cell level, consistent with the crucial role of TPO and MPL in maintaining adult quiescent HSCs.[10,11] At the hematopoietic precursor level, the only cells that are activated by MPL signaling are megakaryocytes, and therefore *CALR*-mutant clonal hematopoiesis results in selective overproduction of platelets. Thrombocytosis is the initial phenotype of all *CALR*-mutant MPNs, but a portion of patients develop bone marrow fibrosis progressing to myelofibrosis with myeloid metaplasia.[6] Type 1 *CALR* mutation involves a high risk of myelofibrotic transformation, likely because it specifically impairs the calcium-buffering activity of calreticulin in activated megakaryocytes.[8]

Will our better understanding of the pathogenesis of *CALR*-mutant MPNs translate into better management of patients? Unfortunately, we cannot rely upon specific MPL inhibitors at present. However, a study recently published in this journal has shown that interferon α can inhibit platelet overproduction and also clonal hematopoiesis in *CALR*-mutant ET.[12] In addition, an interesting study has recently shown that megakaryocytes drive fibrosis in animal models of MPNs, and that aurora kinase A is required for myelofibrosis development.[13] This study also shows that targeting megakaryocytes with aurora kinase A inhibitors has the potential to provide therapeutic benefit. Ad hoc clinical trials are needed to evaluate whether this inhibition will be safe and effective in treating patients with myelofibrosis.

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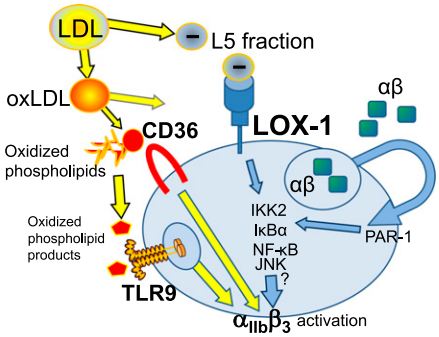

Prothrombotic and proatherogenic LDL fractions and their products operate via platelet pattern recognition receptors, including LOX-1, leading to $\alpha_{IIb}\beta_3$ activation and platelet aggregation. JNK, c-Jun N-terminal kinase; oxLDL, oxidized LDL; PAR-1, protease-activated receptor 1; TLR9, Toll-like receptor 9.

● ● ● PLATELETS AND THROMBOPOIESIS

Comment on Shen et al, page 1336

Prothrombotic lipoprotein patterns in stroke

Eugene A. Podrez and Tatiana V. Byzova CLEVELAND CLINIC

The importance of research focused on the final events of atherothrombosis cannot be overestimated. Platelet hyperreactivity leading to thrombosis is the main reason for mortality and morbidity in patients with cardiovascular disease and stroke, which together remain a leading cause of death in developed countries. In this issue of *Blood*, Shen et al[1] establish another functional link between proatherogenic lipoproteins and platelet-mediated thrombus formation with a specific focus on stroke. In their model, the initiating component is L5, the electronegative subfraction of low-density lipoproteins (LDLs), which was shown to be substantially elevated in patients with ischemic stroke. L5 was shown to activate platelets via its receptor, lectin-like oxidized LDL receptor-1 (LOX-1), and αβ amyloid peptide, which together contribute to platelet hyperreactivity and stroke complications.

It is surprising that thrombosis, being the most critical pathological event that underlies the majority of disabilities in United States,[2] remains relatively poorly understood. Compared with many other diseases, stroke in general seems to attract less attention from researchers as well as funding agencies.[2] While there is a substantial body of literature focused on the role of platelets in cardiovascular complications, the number of articles in PubMed addressing the specifics of platelet activation in stroke is several times lower. The main contributing factor to this phenomenon is, of course, the complexity and multifactorial nature of platelet hyperreactivity.[3] Several previous studies have established not only a correlative but also a mechanistic link between hyperlipidemia and thrombosis. Among those are identifications of new interactions between platelet scavenger receptors and well-characterized products of phospholipid oxidation, which lead to platelet aggregation in hyperlipidemia.[4] However, the heterogeneity of plasma lipids combined with products of their oxidation is extremely high, therefore suggesting the existence of multiple receptors and mechanisms contributing to platelet activation.

The key role of the electronegative subfraction of LDL is not particularly surprising since high levels of L5 have also been shown to associate with metabolic syndrome and high risk of cardiovascular complications in several pathologies.[5] The very same group had previously shown that an L5 subfraction isolated from human patients triggers platelet activation and aggregation via LOX-1 on platelets.[6] Highly electronegative LDL from patients with ST-elevation myocardial infarction was shown to trigger platelet activation and aggregation.

In the present study, Shen et al analyzed lipids from ischemic stroke patients to show that, although total cholesterol, LDL, and high-density lipoproteins (HDLs) are similar between the groups, the concentration of L5 is significantly higher than in controls. In agreement, expression levels of the L5 receptor LOX-1 were elevated in platelets from patients with stroke. Then, L5 isolated from these patients was shown to worsen complications in a ligation-induced cerebral ischemia model. Likewise, the blockade of LOX-1 decreased the infarct volume while improving sensorimotor performance in mice. In another model, where thrombosis was induced in the middle cerebral artery, L5 was again thrombogenic, whereas LOX-1 deficiency had an inhibitory effect, thereby solidifying the role of the L5–LOX-1 axis in the pathology of stroke and its complications. Administration of L5 shortened the bleeding times, and LOX-1 blockade restored the tail-bleeding times back to control levels. LOX-1 deficiency only mildly attenuated hemostasis in the absence of L5, thereby demonstrating that normal levels of platelet aggregation remain