Comment on Tumburu et al, page 3116

The sickle erythrocyte yields another DAMP

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In this issue of *Blood*, Tumburu et al[1] report abnormal retention of mitochondria in circulating erythrocytes and the presence of mitochondrial DNA (mtDNA) in plasma in sickle cell disease (SCD). Furthermore, they identify cell-free mtDNA (cf-mtDNA) as a danger-associated molecular pattern (DAMP) in SCD. Although mtDNA has been known as a DAMP molecule for some time,[2] this interesting study showed that SCD increases cell-free DNA (cfDNA) and cf-mtDNA at baseline and crisis in humans and Townes sickle mice (see figure).

The evidentiary support for their core observations is considerable. Healthy mature red blood cells do not contain mitochondria, except in rare disease conditions, such as Rett syndrome.[3] The investigators used multiple techniques to demonstrate that SCD increases mitochondrial retention in the sickle erythrocyte. These data provide intriguing support for the idea that the sickle erythrocyte is likely the primary cellular source for circulating cf-mtDNA.

The investigators quantified and sequenced cf-DNA, as well as determined the effects of DNA subtypes on neutrophil extracellular trap (NET) formation, to explore the role of cf-mtDNA in SCD. They demonstrated that SCD increases cf-mtDNA levels approximately five fold to 10-fold at baseline compared with levels in healthy controls. However, the mechanism(s) by which cf-mtDNA causes and quantitatively contributes to inflammation in SCD patients remains unclear.

Although the investigators focused attention on NET formation, additional studies will be required to clarify this issue. For example, we and other investigators have compared the effects of SCD on relative changes in another DAMP: high mobility group box-1 (HMGB1). It was reported that SCD increases HMGB1 by approximately threefold[4] and ~1.8-fold[5] in patients at baseline relative to healthy controls. These data are consistent with the results presented by Tumburu et al. Interestingly, however, cf-mtDNA in SCD patients in crisis does not change relative to cf-mtDNA in SCD patients at baseline. In contrast, HMGB1 in SCD patients in crisis increases by approximately twofold[4] and ~1.7-fold[5] compared with their levels at baseline. If inflammation is proportional to the level of DAMPs released, then the observation that cf-mtDNA did not increase during crisis (see figure) requires further exploration. For example, could cf-mtDNA have increased locally in tissue beds most severely damaged during crisis?

Alternatively, DAMPs come in all shapes and sizes, and the effects of physical and biochemical differences can be exquisitely nuanced. Accordingly, if the cf-mtDNA released during crisis is different from the cf-mtDNA released at baseline, such a biochemical change in the DAMP may alter biological activity. Because DNA methylation plays an important role in epigenetics, the investigators analyzed cf-mtDNA for any detectable differences. They showed that cf-mtDNA released during crisis was more hypomethylated than was cf-mtDNA released by SCD patients at baseline and even more hypomethylated than cf-mtDNA released by healthy controls. This is an intriguing observation that the investigators build on when they compared the effects of genomic DNA or platelet mtDNA in RPMI 1640 media with healthy human plasma and SCD plasma, which contained high concentrations of cf-mtDNA on NET formation. Their data clearly showed that SCD plasma increased NETosis. However, because experimental conditions were not directly comparable, they cautiously speculated that cf-mtDNA in plasma from SCD patients increased NET formation, possibly because it was hypomethylated. Future studies using comparable conditions may help to determine the relative contributions of this growing list of DAMPs on inflammation and NETosis in SCD at baseline and crisis. Indeed,
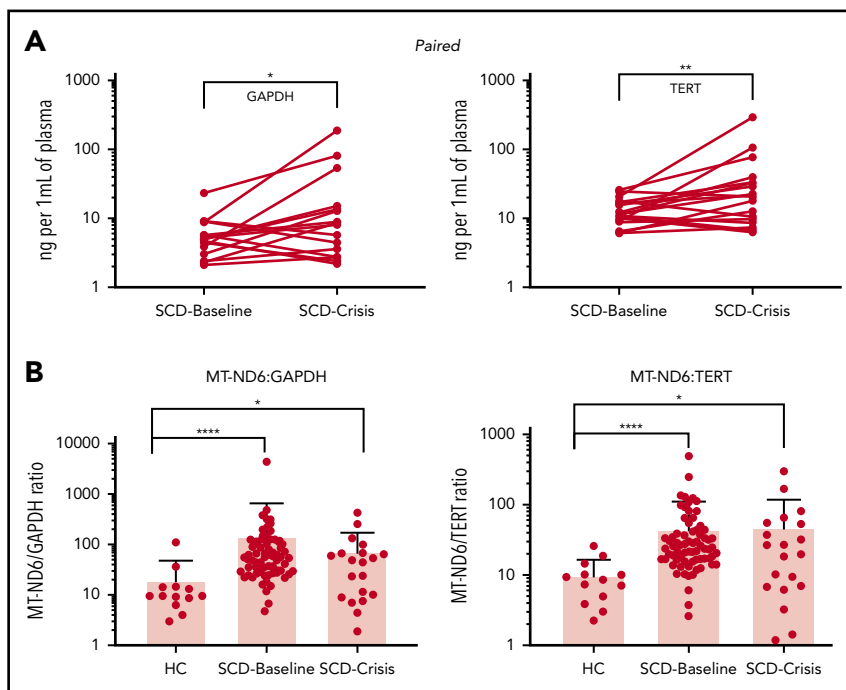
(A) Paired samples: SCD-Baseline and SCD-Crisis pairs (n = 18). Circles represent the mean of each sample. *$P < .05$, **$P < .01$, nonparametric Wilcoxon matched-pairs signed-rank test. (B) Quantitation of cf-mtDNA/cf-nDNA ratio using the following mitochondrial and nuclear targets: *MT-ND1/GAPDH, MTND1/TERT, MT-ND6/GAPDH,* and *MT-ND6/TERT*. Error bars represent the sample mean ± standard deviation. *$P < .05$, ***$P < .0005$, ****$P < .0001$, nonparametric Kruskal-Wallis test with Dunn's multiple-comparison test. cf-nDNA, cell-free nuclear DNA. See the complete Figure 1 in the article by Tumburu et al that begins on page 3116.

findings from such studies may explain why crisis worsens, even though cf-mtDNA did not increase beyond baseline.

Findings from this report will likely spur the further studies needed to unravel the complex processes by which sickle erythrocytes increase inflammation beyond the damage caused to the vessel wall by sickled erythrocytes. If cf-mtDNA can be specifically targeted and inflammation is reduced in SCD mice, then cf-mtDNA may be an important mediator of inflammation that increases vaso-occlusion by what the investigators suggest is neutrophil activation and NETosis. Direct comparisons of DAMPs in SCD will reveal whether cf-mtDNA is an important mediator of inflammation and vasocongestion or just another biomarker of inflammation.

Conflict-of-interest disclosure: K.A.P. is a founder and member of ReNeuroGen LLC, an early-stage virtual pharmaceutical company developing therapies for the treatment of neuroinflammation in multiple sclerosis, bronchopulmonary dysplasia, vasculopathy in sickle cell disease, ischemic stroke, *silent cerebral infarct, and traumatic brain injury. K.R.R. declares no competing financial interests.* ■

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THROMBOSIS AND HEMOSTASIS

FV/FVa revealed

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In this issue of *Blood*, Ruben et al present detailed structures of coagulation factor V (FV) and FVa, generated through cryogenic electron microscopy (cryo-EM). This study provides, for the first time, structural detail on conformational changes in FV upon its activation, which control not only its ability to enhance coagulation but also how it is regulated and degraded.[1]

Coagulation is initiated upon vascular injury as an innate response to prevent blood loss. FV is central to this process by acting as a cofactor for FXa within the prothrombinase complex.[2] In the absence of vascular injury, FV circulates as a procofactor comprising an A1-A2-B-A3-C1-C2 domain structure. As a procofactor, FV has no procoagulant functions but instead serves as an anticoagulant regulator by enhancing 2 anticoagulant pathways, the tissue factor pathway inhibitor (TFPI) and the activated protein C (APC) pathways.[3] Only once coagulation has been initiated is FV activated to FVa by limited proteolysis at 3 sites, Arg709, Arg1018, and Arg1545, resulting in complete removal of the B domain.[2] The procoagulant functions of FVa are in turn regulated by APC-mediated proteolysis at Arg306 and Arg506.[2,3] Although we have a general understanding of the molecular mechanisms behind the pro- and anticoagulant functions of FV/FVa, structural information has been lacking. Previous studies have provided some structural insight into FVa or its inactive derivatives.[2,4-7] However, these have not included an experimentally determined structure of the human FV A2 domain. This is of particular importance, because this domain is key to both the procoagulant cofactor function of FVa as well as its inactivation by APC.[2] Ruben et al have for the first time determined the structures of both FV and FVa, including their A2 domains (see figure).

The structures illustrate how the 2 FV C domains provide a platform supporting the A domains, similar to the previously published structure of bovine FVai.[6] The A1 and A3 domains sit on top of the C domains, with the A2 domain resting between the A1 and A3 domains and having no contact with the C domains (see figure). In contrast to the A and C domains, the B domain is overall disordered, looping around the protein in a dynamic conformation. Unfortunately, this means that there is no structural insight into the FV B-domain acidic and basic regions, which are essential in maintaining FV in a procofactor state.[2] However, the most N- and C-terminal segments of the B domain, directly connected with the A2 and A3 domains, were more stable, crucially allowing the resolution of the functionally important Arg709 and Arg1545 thrombin cleavage sites.

Through comparing the FV and FVa structures (see figure), the authors show how removal of the B domain from FV resulted in increased disorder in the A domains, mostly in the C-terminus of the A2 domain, as well as (somewhat surprisingly) in the C2 domain (FV/FVa structures are shown from additional angles in Figure 2 of the article by Ruben et al). These changes in conformation and subsequent functional epitope exposure in the A2 domain are most likely necessary for FVa to assemble with FXa in the prothrombinase complex. Furthermore, although the APC Arg306 and Arg506 cleavage sites are largely buried in FV, they become more exposed after thrombin-mediated activation of FV, essentially priming FVa for APC-mediated inactivation. The relatively inefficient cleavage by APC of the Arg506 cleavage site in FV, compared with that in FVa, is therefore explained.[8] The large distance between the Arg306 and Arg506 APC cleavage sites supports independent cleavage. Indeed, biochemical studies have shown a significant difference in cleavage kinetics between the 2 sites. Furthermore, their differing dependence for cleavage on protein S, the cofactor of APC, may be partially explained by this spatial separation.[3,9]