Altered Calcium and Red-ox homeostasis underline defective haematopoiesis in Fanconi Anemia

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A defective hematopoiesis underlies the severe pathophysiological conditions of Fanconi anemia (FA) patients. A fine tuning of the bone marrow (BM) niche micro environment appear crucial in myeloproliferation and hematopoiesis where molecular events associated with calcium and red-ox gradients dictates the process. Calcium and red-ox signaling are complex regulatory networks which might play both agonistic as well as antagonistic roles. Both modulate structural and functional elements of the process. In FA the peculiar association between a low \([Ca^{2+}]_i\) and an high pro-oxidative metabolism may distinguish its pathological drifting and cancer-proneness. Notably these conditions are associated with specific leukemic contexts typical in FA. Whether the dysregulated calcium metabolism observed in FA be a useful diagnostic tool and/or a predictivity marker of FA associated outcome appear however unlikely given the difficulty to manipulate it.

Introduction

We recently reported how Fanconi anemia (FA) cells (lymphoblasts, lymphocytes and primary fibroblasts) from three different complementation groups (A, C and G) display a low basal intracellular calcium \([Ca^{2+}]_i\) content\(^1\). While this finding may appear peculiar, it might underscore the proneness of FA disease toward a myeloid disorder which might progress to acute or chronic leukemia. Indeed low \([Ca^{2+}]_i\), was shown to be a distinctive feature of chronic myeloid leukemia (CML) and myeloid cells \(^2, 3, 4, 5\); this is interesting in the perspective of the high cancer susceptibility and prevalence of malignancies of myeloid origin in FA \(^6\). A dysregulated calcium homeostasis therefore might be potentially useful in the diagnostic and therapeutic management of the FA patients. Here we try to explore this possibility.

FA is one of the rare inherited bone marrow (BM) failure syndromes which features a very high cancer-predisposition including leukemia. Approximately 20% of the patients have some type of malignancy, 45% of which are leukemias. Eighty-four percent of these leukemias are acute myeloid leukemia (AML). While AML is typical of adults its exceedingly high incidence in FA (AML, 700 folds and myelodysplastic syndromes (MDS) 6000 folds) is probably associated to the very young age of manifestation of the disease with respect to the incidences expected in the general population\(^7\).

In FA patients a progressive failure in BM eventually results in pancytopenia. Hematologic complications arise early with low platelet counts and thrombocytopenia followed by leukopenia which progresses to aplastic anemia. Deficiency in all blood lineages implies hematopoietic stem
and progenitor cell (HSPC) dysfunction \cite{8}. FA patients have few CD34+ cells, the BM fraction enriched for hematopoietic stem cells (HSCs) capable to differentiate in all blood components upon transplantation. Significant depletion of the CD34+ fraction was observed in very young FA patients, well before the onset of pancytopenia suggesting the developmental origin of the FA stem cell defect \cite{9}. Animal model experiments do support this concept \cite{10, 11, 12, 13}. Thus FA phenotype impacts HSC pool expansion and seeding of the fetal BM niche during development. Several findings strongly supports that the defective HSPC process in FA is associated with defective DNA damage, red-ox unbalance and aberrant cytokine expression which jeopardize the maintenance and fate of HSPC \cite{14, 15}. In this context the contribute of a dysregulated calcium signaling is taken into consideration.

A crucial role in myelo-proliferation and development of hematopoietic progenitors appear strictly dependent upon a fine tuning of the micro-environment at the level of BM niche which comprise an highly heterogeneous cell population. The complex signal network generated among these cells and the habitat conditions in which this system is nestled \cite{16, 17} was pre-iconized in the “stem cell niche” hypothesis \cite{18}.

In BM, HSPCs reside in two different niches, the endosteal and the vascular. These contains several BM cells including osteoblasts, osteoclasts, mesenchymal stem cells, sinusoidal endothelium, perivascular stromal and immune cells. These cells play different roles in HSPC regulation \cite{19}. The majority of HSPCs remain quiescent at any given time. In the osteoblastic niche the microenvironment promotes HSC maintenance and quiescence. Here BM is relatively hypoxic and HSPCs are surrounded by an habitat displaying low levels of intracellular ROS \cite{20}. Oxygen gradient increases closer to the vascular niche and around blood vessels, where the microenvironment favours HSPC proliferation and differentiation thereby provides myeloid and lymphoid hematopoietic cells to the peripheral blood circulation \cite{21}. These cell populations are defined by a specific signature of adhesive molecules, cytokines and chemokines which define their “hold” in a certain niche and both oxygen and calcium gradients influence HSPC sub-population migration and differentiation\cite{16}.

Haematopoietic maintenance and differentiation are complex processes as yet not completely elucidated. Different models, vastly superimposable but however still not well known and characterized, have been reported to describe these processes \cite{22}.

The relative roles played by calcium fluxes and homeostasis and oxidative stream in the regulation of the HSC maintenance or differentiation are not well characterized in all these models. Nevertheless we can focus on some of the elements of this interplay that are better characterized.

**The CXCR4/CXCL12 axis**

In the first model mesenchymal stromal cells (MSC) which by themselves can differentiate into the different precursors of bone, fat, and cartilage, provide both the scaffold and the trophic/signaling support for the endosteal and vascular niches. The interactions between MSCs and HSCs are mediated by several structural elements \cite{19, 23}.

The main signaling event in interplay between quiescence and maturation of HSPCs is the interaction between CXCL12, a cytokine secreted by MSC, and CXCR4, a G-protein coupled receptor in the HSPC membranes. The binding between CXCL12 and CXCR4 is essential for maintenance of the HSC in a quiescent state. Secretion of CXCL12 from MSC is an event mediated by cell contact dependent by connexin gap junctions. Inhibition of these junctions impairs the secretion of CXCL12 and the maintenance of HSC in the BM. Calcium fluxes through the gap junctions controls CXCL12 secretion through the cAMP–protein kinase A (PKA) pathway \cite{23}. Also the CXCL12 activity is characterized by the reactivity of 4 conserved cysteine that form 2 disulfide bonds \cite{24}. An altered secretion or activity of CXCL12 causes the CD34+ progenitor cells to be depleted. On the other end, in the endosteal niche, hypoxia appears necessary for the long-term maintenance of HSCs. Indeed these cells do express high level of hypoxia-inducible factor-1 (HIF-1), the metabolic activity of these cells being dependent on glycolysis. Up regulation of CXCR4 expression was reported to be dependent on reducing conditions \cite{25}. The maintenance of the CXCL12/CXCR4 axis appear thus necessary for the support of quiescent HSPCs. Depletion of CXCL12 or CXCR4 results in HSC migration and differentiation \cite{26} but also calcium signaling \cite{23, 27} and red-ox modulation \cite{16, 24, 28} results in the disruption of this condition.

**Gfer and the mitochondrial biogenesis**

According to the second model the evolutionarily conserved growth factor erv1-like (Gfer) is involved in the mechanism of HSC quiescence and maintenance \cite{29}. Gfer, whose action is mediated through a signaling involving Ca\textsuperscript{2+}/calmodulin kinase IV (CaMKIV), acts in the cell nucleus where it inhibits HSC proliferation through the Jab-1 (c-jun activation-domain binding protein-1) and CDK1/p27kip1 pathway \cite{30}. Gfer is also involved in
mitochondrial biogenesis as a red-ox sensor functioning in selection of protein import into the mitochondrial inter-membrane space (IMS). Gfer action in the maintenance of mitochondrial fission–fusion dynamics is mediated through interplay with the GTPase dynamin-related protein 1 (Drp-1). As sulfhydryl oxidase, Gfer catalyzes the formation of disulfide bonds between critical cysteine residues in the GTPase domain of Drp inhibiting excessive fission. Mutations in human GFER are associated with an infantile autosomal recessive myopathy, characterized by loss of mitochondrial function and excessive mitophagy. All these questions are relevant since mitochondrial biogenesis is strictly associated with maturation steps in HSPC. Upregulation of mitochondrial mass in mouse HSC appears closely associated with the appearance of CD34+ expressing cells, a marker related to the loss of long-term repopulation ability, with the passage from a glycolytic to an oxidative phosphorylation-based metabolism which sets the conversion from low to high mitochondrial membrane potential and from quiescent to active HSC.

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**Effectors of calcium signaling**

Calcium action is mediated through several actors inside cells. Among these various signal transducer, we will just focus upon three families, the protein kinase C family (PKCs), the Calcium-calmodulin activated kinase family (CaMKs) and the calpains, which are those principally involved in HSPC signaling and might have, for different reasons, a value in the FA HSPC deficiency.

1 - PKCs

PKC is a family of serine and threonine protein kinases activated downstream triggering of several surface cell receptors and Ca2+ influx. Different PKC family members display very different requirements in terms of calcium concentration for activation. PKC activity and Ca2+ homeostasis modulation are implied in the pharmacologic treatment of the CML where typically a low [Ca2+]i is described. CML cells summarize the characteristics of a population of cells that retains the characteristic of undifferentiated cells in the mesenchymal stroma. This might also be the case for those MDS patients which will progress to AML, as for FA patients.

Imatinib (IM) was reported to correct the low [Ca2+]i in K562 and CML patient-derived mononuclear cells acting at InsP3-receptor in ER. IM and IM-related drugs are first line treatments for the CML-positive Philadelphia chromosome both in adults and children. The drug is approved for treatment of stem cell transplanted patients and in blast crisis. IM acts inhibiting the binding between ATP and the BCR-ABL tyrosine kinase. This action results in a complete regression in a large percentage of CML-treated patients while it is ineffective in treating patients with mutations in the kinase domains. IM is also able to significantly down-regulate the expression of several cytokines (IL-6 and IL-8) and transcription factors (NF-kB and AP-1), known to be implied in the pathological manifestations of CML. IM is effective in killing CML cells defective in the calcium signal transduction that is expected in cells featuring low [Ca2+]i. However actions mediated by IM as well by desatinib, another TK inhibitor employed in CML therapy, activate PKC-δ, a PKC isoforms which does not require Ca2+ for activation. The same holds true also in CML killing mediated by IFN-α which results in enhanced PKC-δ activation. However PKC triggers the action of TPA and TPA-like tumor promoters and exacerbates the apoptotic response to a variety of DNA damaging agents and is also able to activate Akt-related pro-survival signals. In conclusion modulation of the calcium homeostasis and its downstream signals does not appear to be the primary target of the drugs currently employed for the treatment of CML.

2 - Ca2+/CaMKs

The same conclusion can be drawn concerning the complex system of the Ca2+/CaM Kinases. While these are a highly complex and heterogeneous class of Ca2+-dependent kinases they do act as signal transducers to connect pro-oxidant conditions into specific downstream responses. A balance favouring CaMKII over CaMKIV was recently reported as crucial in maintenance of HSPC quiescence and CaMKIV was also involved in the Gfer mechanism. Since increasing calcium fluxes would favour the activity of the proliferation-associated CaMKII likely a low [Ca2+]i should limit this condition. However several papers have reported that appropriate oxidative stress activate CaMKII even in absence of intracellular free [Ca2+]i.

3 - u-Calpain

An increased activity of μ-calpain, a Ca-dependent cysteine protease, was reported in cells from several FA complementation groups by the group of Lambert. The increased activity of μ-Calpain was associated to a critical deficiency of the structural protein αII Spectrin which associates the chromosomal instability (CI) and defective
DNA interstrand cross-link repair typical of the FA cells. \( \mu \)-Calpain is mainly involved in the shaping and modification of several structural elements inside cells (cytoskeletal elements, actin, mitofilin, microtubules, Golgi and ER) and outside cells (cell adhesion and migration and matrix elements, laminin) \cite{49, 50} and its activity was chiefly associated with immature and quiescent HSPC\cite{51}. The high activity of \( \mu \)-calpain in FA cells might be counterintuitive in the context of a low \( [Ca^{2+}]_i \) in FA however it was reported that the catalytic site of \( \mu \)-Calpain, which differs from m-Calpain for its lower calcium requirements, is more flexible. This may imply that \( \mu \)-Calpain is active in very low \( [Ca^{2+}]_i \)\cite{52}.

**Implication of an altered Calcium homeostasis in FA**

Concerning FA we recently contribute to describe several defects associated with structural elements (lamin and mitofilin), metallo-proteinases, morphological and functional elements (mitochondria and nuclei) \cite{53, 54, 55, 56}. These defects were essentially viewed in the single perspective of a red-ox unbalance, as treatment with selected antioxidants generally restored physiological conditions. However, the finding of an altered \( Ca^{2+} \) homeostasis in FA \cite{1} requires a deeper look to the FA defects with a broader perspective, considering the whole universe of the relationships that the calcium-associated activities and concerns and the relevant overlap between \( Ca^{2+} \) homeostasis and red-ox balance. This is especially true in the choice for an appropriate diagnostic and therapeutic approach. It may be intriguing to note that no significant information links AML to a defined calcium dis-metabolism even if we should remind the sheer differences in the cytogenetic abnormalities observed in FA-AML patients, mostly pediatric patients, in comparison to the adult AML patients \cite{57, 58, 59}. Furthermore, we should note also that the low \( [Ca^{2+}]_i \) in FA is expressed as a physiological stretch before a clearly established baseline condition of dysplasia.

\( Ca^{2+} \) and ROS signaling in physiological conditions are associated and directly proportional so that an increase in calcium flow from the extra- to the intra-cellular environment and the metabolic pathways associated with this signaling are congruent to the signaling induced by the increase in the oxidative stress gradient. By contrast in FA these two processes are inversely proportional or asymmetric. We argued the meaning of this asymmetry as strategic, in FA, to counteract the high oxy-stress associated with the FA phenotype \cite{1}. Indeed, Revankar\cite{3}, in studying the effects of the calcium fluxes associated with the binding of different chemo-attractants to PMNL cells of a population of CML patients, reported that these defects were associated with both a low \( [Ca^{2+}]_i \) and low PKC-\( \alpha \) expression. Are there other perspectives from which to regard this?

Still concerning asymmetry, from a different point of view: the words of Mantel\cite{33} “The quintessential property of HSCs is their ability to undergo an asymmetric, self-renewing cell division’ highlight how asymmetry is a crucial process to maintain long-term hematopoiesis. This HSPC renewing process might be aberrant in FA, causing depletion of HSPCs population. Different factors would contribute to this depletion. An abnormal oxidative stress in the hematopoietic FA niche would prevent the execution of the asymmetric self-renewing cell division and result in the collapse of the haematopoietic system, thus justifying the BM failure observed in Fanconi anaemia patients \cite{60}. This condition has been described several times in serial transplantation experiments of both human and animal HSCs into FA deficient mice \cite{11, 15, 61, 62, 63}. Defective homing, limited self-renewal and decreased Rho GTPase Cdc42 activity were reported as contextual and associated with FA mutation itself \cite{64}. This deficiency might be associated with an intrinsic functional defect in FA mitochondria. Interestingly enough, mitochondria appear to play a role as cell-fate determinants in the asymmetry HSC self-renewal process\cite{65} and we contributed to thoroughly describe the mitochondrial functional defect in FA \cite{53, 54, 55, 56}. On the other hand, it was shown that intra-mitochondrial \( Ca^{2+} \) can control oxidative phosphorylation, i.e., indirectly, the ROS production. Therefore, in low \( [Ca^{2+}]_i \), mitochondria would be expected to be less active in ATP production, but at the same time less prone to collapse, which would prolong the life of FA cells \cite{67}. Concerning these same mechanisms Szabadkai \cite{68} recently reported how the mitochondrial reticulum (MR) appear involved in the control of the apoptotic response through a close inter-active modulation with ER. Oxidative stress-induced release of \( Ca^{2+} \) from ER to MR results in a consequent calcium-dependent wave of mitochondrial membrane depolarization and apoptosis. MR fragmentation reduce the interaction from the two reticula lowering \( Ca^{2+} \) passage and inhibiting membrane depolarization that results in resistance to apoptosis. In FA cells treated with hydrogen peroxide we observed \cite{1, 53} MR fragmentation and low membrane depolarization suggesting an adaptive cell organization \cite{68, 69}.

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