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Review

Autophagy: A necessary event during erythropoiesis

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ABSTRACT

Autophagy is a well-known cellular process involved in many physiological and pathological processes. During erythropoiesis, autophagy plays an important role participating in the clearance of unnecessary organelles such as ribosomes and mitochondria (mitophagy) allowing the correct formation of mature red blood cells. The dysfunction of autophagy proteins hamper the correct erythroid maturation, leading to anemia, the release of immature cells from the bone marrow and other hematological abnormalities. Autophagy plays different roles depending on the type of pathology. In leukemia cells, it has been demonstrated that autophagy could be either detrimental, leading to an increase of the apoptosis rate, or protective, acting as a key process that augments proliferation and survival of cancer cells. Thus, understanding the relationship between autophagy and erythropoiesis opens new avenues for the discovery of biochemical and pharmacological targets and for the development of novel therapeutic approaches.

Introduction

Erythropoiesis is a finely regulated process in which red blood cells are generated from immature precursors in the bone marrow. In humans, during the last stages of gestation, hematopoietic stem cells (HSC) migrate from the liver to the bone marrow to initiate hematopoiesis [1,2]. A population of HSC, recognized as CD34 + cells, gives rise to the megakaryocytic-erythroid progenitor, which originates the erythroid progenitor (EP) linage [3–5]. Through the stimulation by high levels of erythropoietin (EPO) and other cytokines (e.g. IL-3, IL-6, IL-1), EP differentiate into the first erythropoietic cells, which are named burst forming units (BFU-E) and colony forming units (CFU-E). Both cell types are involved in the production and accumulation of hemoglobin (Hb) that is necessary to proerythroblast formation. This process is known as the proliferation stage [6,7]. For Hb biosynthesis, cells take up iron from two major sources. One pathway for iron acquisition is the binding of Fe^{3+}-bearing transferrin (Tf) to the specific transferrin receptor (TfR) or CD71, located on the cell surface. Following clathrin-dependent endocytosis of the Tf/TfR complex, the pH within the endosome is lowered through the action of ATP-dependent H^{+} pumps [8], initiating receptor-stimulated iron release from the Tf. Iron is then released from Tf within the endosome into the cytosol through the divalent metal transporter 1 (DMT1), and at this endosomal pH, apoTf remains tightly bound to the TfR with high affinity. The apoTf/TfR complex then returns to the plasma membrane, thus avoiding its degradation within the lysosome. ApoTf is then released from the TfR into the plasma to bind more Fe^{3+} [9,10]. Another iron source is that provided by the degradation of altered RBC by the reticuloepithelial system, in which hemoglobin is released and the prosthetic group heme is metabolized. The latter process constitutes the main source of iron in a ferrous (Fe^{2+}) form. The free heme is highly oxidative and produces reactive oxygen species (ROS), which are toxic for all tissues. Therefore, the heme degradation by the hemo-oxygenase (HO) into carbon dioxide, biliverdin and iron is critical [11–13]. To achieve heme degradation, the heme-hemopexin complex is scavenged by the low density lipoprotein receptor (LDL), the low density lipoprotein-related protein-1 (LRP1) and megalin (LRP2), through endocytosis via clathrin-coated pits. After heme degradation, iron is either stored in the cell as ferritin and hemosiderin or used to form new hemoglobin [13–15].

The heme neosynthesis occurs in the mitochondrial matrix through an enzymatic process in which iron is incorporated into the protoporphyrin IX protein generating new heme. This molecule is then exported into the cytosol and assembled with globin proteins to form hemoglobin [16]. In direct relationship with hemoglobin formation, two isoforms of the Feline Leukemia Virus subgroup C receptor 1 (FLVCR1) have an important function. One of them is the FLVCR1a which is in the plasma membrane and exports heme from the cytosol to the extracellular space, preventing its accumulation and cytotoxic effects. The FLVCR1b isoform is in the mitochondria and exports the synthetized heme into the cytosol. Both isoforms are directly associated with the differentiation of erythroblasts, since the correct amount of

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heme in the cytosol stimulates the overexpression of globin genes to form hemoglobin. The silencing or knockout of FLVCR1 in mice or in zebrafish is known to cause abnormalities in the expansion and differentiation of erythroid progenitors, leading to lower BFU-E and CFU-E counts, anemia, and impairment in hemoglobin production [17,18]. Proerythroblasts then undergo four mitosis events in 3–4 days to generate the basophilic, polychromatophilic and orthochromatic erythroblasts. In the latter stage, the expulsion of the nucleus and the elimination of some intracellular organelles (e.g. the Golgi apparatus and the endoplasmic reticulum) occurs to release the nascent reticulocytes into the bloodstream, Reticulocytes will then complete their maturation 1 to 2 days later [6,19,20].

As the erythroid maturation progresses, important cellular changes such as the diminution in the cell size and the condensation of the nucleus occur [20–22]. The remodeling of cytoplasmic and membrane components leads to a correct differentiation of the erythroid cell. During maturation, the plasma membrane amounts of TfR are modified depending on the specific function that each erythroblast cell has, but in the reticulocyte stage, this receptor is downregulated once the cell completes the Hb production [23–25]. This change in TfR levels is regulated through its internalization by endocytosis where TfR is targeted to MVBs and sorted into the internal vesicles. Subsequently, these MVBs fuse with the plasma membrane and the exosome-associated TfRs are released outside the cell [26]. In general, most of the cell surface proteins which function in cell-cell and cell-extracellular matrix interaction or adhesion are highly expressed in the proerythroblast and in the first stages of erythroblasts. These proteins are important for the interaction with macrophages in bone marrow erythroid niches and, as erythropoiesis continues, they have to be eliminated to allow the release of reticulocytes into the bloodstream. CD44, an adhesion surface protein, is used as a maturation marker from basophilic erythroblasts to reticulocytes. Flow cytometry and immunoblotting analyses have shown that the levels of CD44 are 30-fold lower in orthochromatic blasts than in proerythroblasts; thus, the determination of CD44 expression levels allows a better differentiation between the 5 stages of erythroblasts than TfR does [25,27]. Likewise, the α4 chain of the reticulocyte-adhesion molecule α4β1 integrin, which participates in the formation of focal adhesions into the hematopoietic niche, is also eliminated during the maturation process. Interestingly, the α4 subunit downregulation allows the release of the reticulocyte and prevents erythrocyte attachment to the vascular endothelium [28]. Other changes are associated with this differentiation process to generate mature reticulocytes such as the activation of hemoglobin synthesis, the remodeling of the cytoskeleton, the modifications of cell-surface proteins and the loss of remnant internal compartments. As a result of all these processes, the mature blood cells are finally generated [22,25].

Autophagy and red blood cell maturation.

Autophagy is a crucial process that takes place during the final stage of the erythroid differentiation through which cytosolic macromolecules, and even whole organelles are transported to the lysosomes for degradation [29,30]. This process begins with the extension of an specialized membrane originating mainly in the endoplasmic reticulum, the mitochondria, and the Golgi cisternae, known as the phagophore [31,32]. The phagophore surrounds the molecules and organelles to be eliminated, forming a vesicle called the autophagosome when both membrane ends connect [32,33]. This organelle interacts with endosomal structures, generating a prelysosomal hybrid organelle termed the amphisome. In previous studies, we have determined the components of the molecular machinery required for this interaction [34]. Finally, autophagosomes or amphisomes fuse with lysosomes, leading to degradation of the sequestered material by an enzymatic proteolytic process. Studies in both yeasts and mammals have allowed the characterization of at least 40 *Atg* (autophagy-related) genes, which encode proteins that participate in autophagy [35,36].

The canonical autophagy pathway involves the inactivation of mammalian target of rapamycin complex 1 (mTORC1) when nutrients are scarce. This leads to the activation, through phosphorylation, of the Unc-51-like kinase complex (Ulk1/Ulk2), a serine-threonine kinase, and the subsequent cascade of the other ULK complex members such as FIP200 and Atg13 [37]. The second complex that is activated is the Beclin1, in which one the members, named Vps34, is translocated into the ER membranes and produces high levels of phosphatidylinositol 3-phosphate, being this molecule necessary for the recruitment of other effectors such as WIPI2b [38–40]. This effector interacts and recruits Atg16L, which binds Atg5 conjugated with Atg12. The formation of Atg5-Atg12-Atg16L complex is the preceding step to LC3 lipidation, whose function is to determine the site where LC3 will be conjugated and activated to LC3-II [41,42]. Atg3, an E2-like protein, is associated to LC3-I and binds to the complex through Atg12, allowing the conjugation of LC3-I with phosphatidylethanolamine to generate LC3-II [43]. LC3-II, which is associated with the inner and outer membranes of autophagosomal structures, is required for phagophore extension, engulfment of cargo and vesicle closure to form the autophagosome [44–46]. The elements destined to be eliminated by the autophagic pathway bind receptor/adaptor molecules like p62, NDP52 that contain a LC3 interacting region (LIR). This domain allows the recognition of elements to be engulfed and eliminated by the phagophore [47,48].

Mitochondria participate in essential cellular process such as ATP production, apoptosis regulation and hemoglobin synthesis, among others. The metabolites produced during oxidative stress or reactive oxygen species (ROS) generated in the respiratory chain, cause mitochondrion aging and membrane damage, leading to the release of *cythocrome c* and pro-apoptotic factors [49–51]. Therefore, the control and clearance of altered mitochondria is detected and marked by the autophagy machinery. When mitochondria membranes suffer depolarization or are damaged, different membrane proteins containing LIR, as BNIP3, NIX and FUNDC1, are exposed (see fig. 1). In this process, mitochondria are engulfed and targeted to the lysosome for its degradation in a process known as mitophagy [35,52,53]. In the last step of reticulocyte maturation, when hemoglobin has been completely synthesized, mitochondria must be eliminated for the correct functioning of mature red blood cells. Normally, mitophagy occurs through the canonical autophagy pathway through the activation of the conventional autophagy proteins in an Atg5-Atg7-dependent manner, allowing the lipidation of LC3 and formation of the autophagosome. Interestingly, it has been demonstrated that Atg7 is also responsible for the clearance of mitochondria in reticulocytes during erythropoiesis. Atg7 knockout mice develop anemia, lymphopenia and reticulocytosis. Interestingly, the knock-out of this gene produces a delayed depolarization and impaired clearance of mitochondria, showing a partially Atg7-dependent autophagy [49,50]. Moreover, Ulk1/Atg1 expression levels correlate directly with the removal of ribosomes and mitochondria by autophagy during reticulocyte maturation. Ulk1 knock-out mice present alterations in blood cells count, and a delayed clearance in both ribosomes and mitochondria, indicating a deficiency in the maturation of red blood cells. Likewise, a population of CD71 negative red blood cells bearing mitochondria has been observed in Ulk1 −/− animals, indicating the existence of an increase in immature cells release into the bloodstream [54].

Notwithstanding, when Atg7, Atg5 and Ulk1 are knocked out, the mitochondrial clearance is not completely hampered, suggesting that this is not the only pathway involved in this process [49,55]. Furthermore, studies have demonstrated that non-canonical autophagy, where unusual cytosolic proteins such as Rab9a participate, could be activated by microorganisms or pathological processes. Commonly, Rab9a is involved in the trafficking between late endosomes and lysosomes. However, in an erythroid leukemic cell line, it has recently been demonstrated that this protein is responsible for mitophagy activation when canonical autophagy is blocked [55,56]. The knocking down of either Atg7 or Ulk1 leads to mitochondrial clearance through a non-canonical Rab9a-dependent autophagy, a process in which this

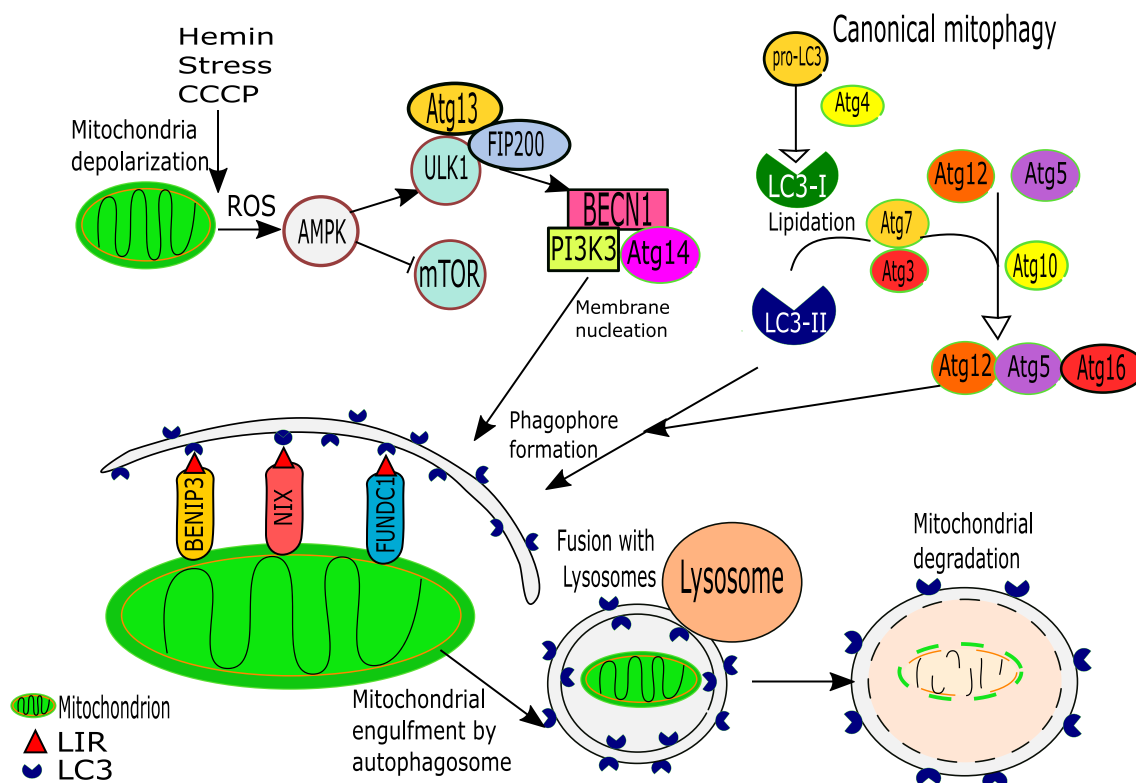

Fig. 1. *Canonical mitophagy*: Exogenous or endogenous stimulation can produce mitochondrial depolarization with the concomitant liberation of reactive oxygen species (ROS). This process activates signaling transduction through AMP-activated protein kinase (AMPK), which inactivates the mammalian target of rapamycin (mTOR). On the other hand, this process activates canonical autophagy proteins such as ULK1 and Beclin-1, leading to membrane nucleation and phagophore formation. In turn, in an enzymatic process which involves Atg7 and Atg5, the activation of LC3 is achieved by conformational changes of the LC3 protein by lipidation with phosphatidylethanolamine forming LC3-II. LC3-II anchors to the phagophore membrane. Depolarized or damaged mitochondria expose proteins on its surface which have a LC3 interacting region (LIR). This allows the interaction with LC3 and the phagophore leading to the surrounding of the organelle and total engulfing when the autophagosome is completely formed. Autophagosomes with the cargo continue a maturation process to finally fuse with the lysosomes for content degradation.

protein is overexpressed, leading to the formation of autophagosomes and elimination of mitochondria. However, this pathway is not yet completely understood, and the recruitment of LC3 has not been explained [56].

Some mitochondria-membrane receptor proteins participate in the control of mitochondrial integrity and function as adaptors or markers for autophagosomes, leading to mitochondrial clearance. NIX (a BH3-only member of the Bcl-2 family) has been described as the major mitochondrial protein involved in the regulation of the clearance of this organelle [57,58]. Studies have shown that this protein is upregulated during erythroid cell differentiation and that it is necessary for correct mitochondrial membrane depolarization [57,59]. Likewise, when the erythroleukemia cell line K562 is stimulated with hemin, a heme homologous, erythroid differentiation stimuli such as hemoglobin production and mitophagy induction are triggered [59,60]. *Nix* gene knockdown mice have an impaired erythroid maturation, anemia and erythroblasts hyperplasia [57,61]. *Nix* −/− mice consequently have an impairment in mitochondrial clearance, reticulocytosis and abnormal reticulocyte maturation, which leads to a decrease in the RBC counts [57,58].

More recent studies have demonstrated that during starvation and mitochondrial-membrane depolarization, the autophagy gen that encodes for Beclin 1 protein (BECN1) undergoes an alternative splicing forming BECN1s, which is directly associated with selective mitophagy [62]. In addition, PINK1 (PTEN-induced putative kinase 1), participates in mitochondrial-integrity control, and is also involved in mitophagy of damaged mitochondria [63]. But this selective mitophagy also occurs in some diseases like Fanconi anemia, where FANCC, a protein involved in macroautophagy as virophagy, interacts with Parkin in the membrane of damaged mitochondria which is then directly bound to autophagosomal vesicles [64].

Advanced knowledge about the complexity of the autophagy mechanism have allowed us to understand the importance of some proteins involved in erythropoiesis and the pathophysiology of hematological diseases. This knowledge contributes to the generation of new strategies for preventing and treating leukemia and anemia, among other pathological processes.

Autophagy and hematological diseases.

As mentioned, autophagy has historically been considered a mechanism that is induced under different conditions such as cellular stress and organelle turnover, being responsible for the maintenance of the cellular homeostasis, the energetic balance and development. However, several other functions of autophagy have been demonstrated in numerous pathological processes such as infectious diseases, cardiomyopathy, neurodegenerative diseases, diabetes, diseases associated to aging and cancer. Regarding the latter, autophagy has a dual function, acting as a cell survival mechanism (favoring the growth of established tumors) and as a tumor suppressor (preventing the accumulation of damaged proteins and organelles) [65]. Moreover, several studies have shown in cancer cell lines, that autophagy plays an important role as a cellular mechanism mediating sensitization to cancer therapy, being a useful strategy for the treatment of drug resistant tumors [65–70].

Chronic myeloid leukemia (CML) is a myeloproliferative disorder featured by a disproportionate accumulation of myeloid cells, which is molecularly characterized by the presence of the Philadelphia (pH) chromosome. This disease is due to a reciprocal translocation of the ABL1 gene to the BCR gene resulting in the expression of the oncogenic BCR-ABL1 fusion protein, which is known to be the starting point of this kind of leukemia. The BCR-ABL1 fusion protein has a constitutively

active tyrosine kinase activity that is able to mimic the growth factor stimulation, generating an increased cellular proliferation and decreased apoptosis [71–73]. The K562 human CML cell line has been frequently used to study the erythropoiesis and the red blood cell differentiation process [74–76]. In K562 cells, Chiariello et al. have demonstrated that BCR-ABL is able to modulate autophagy via MAPK15, through its ability to interact with LC3-family proteins and in a LIR-dependent manner [77]. Moreover, the artificial depletion or the pharmacological inhibition of endogenous MAPK15 has proved to inhibit the BCR-ABL1-dependent autophagy, suggesting the ability of this kinase to control autophagy and cell proliferation. Interestingly, some studies have shown that active BCR-ABL is engulfed into the autophagosomes, indicating that cancer cells are able to use autophagy to regulate the levels of this oncogenic protein [78,79]. These results suggest the potential role of MAPK15 as a feasible therapeutic target in hyperproliferative human diseases such as CML [77].

In general, chemotherapy or radiotherapy, mediate their effects by stimulating a programmed cell death. Moreover, it has been described that cancer cells have the ability to develop resistance to primary cancer chemotherapy, generating drug resilience with poor clinical prognosis. CML had been considered a fatal disease until de introduction of first and/or second generation tyrosine kinase inhibitors (TKI), which block the enzymatic activity of the BCR-ABL1. These drugs are imatinib, dasatinib, and nilotinib [80]. Although these drugs have changed the therapy against CML, other alternative pharmacological approaches are still necessary to achieve a successful treatment. Several studies have shown that autophagy is essential for the development of the BCR-ABL-triggered leukemogenesis [73,81,82], and also to protect cancer cells from apoptosis induced by the TKI [48,83–87]. In addition, it has been proposed that the treatment of K562 cells or primary CML stem cells with imatinib induces autophagy, favoring the cancer cell survival [83]. On the other hand, it has been largely argued that the heme oxygenase-1 (HO-1), the main enzyme responsible for heme catabolism, plays an important role in the development of resistance to imatinib by chronic myeloid leukemia (CML) cells. A recent work has demonstrated that the overexpression of HO-1 induces autophagy in CML cells, while the inhibition of this pathway sensitizes the cells to imatinib. Likewise, imatinib-resistant CML patients became significantly sensitive to this drug when HO-1 expression was inhibited [17]. In this context, the molecular or pharmacological autophagy inhibition, by either Atg5 or Atg7 knock down or hydroxychloroquine treatment has been used to enhance the TKI-induced apoptosis in CML [73,83]. In line, recent reports have demonstrated that celecoxib, a cyclooxygenase-2 (COX-2) inhibitor, is able to induce necrosis and apoptosis by inhibiting autophagy in CML and acute leukemia cell lines [88,89]. This autophagy inhibitory effect of celecoxib is due to the impairment of the lysosome function, which hampers the autophagic flux. Interestingly, imatinib was tested in combination with celecoxib, showing that the COX-2 inhibitor could reinforce the cytotoxicity of imatinib in imatinib-resistant CML cells [89]. In contrast to the cancer cell survival effect of autophagy induction by some chemotherapy drugs, it has been demonstrated that desatinib (a second-generation tyrosine kinase inhibitor) induces autophagy in mice with Bcr-Abl-positive leukemia, being one of the mechanisms underlying cell death in the leukemic cells that infiltrate the central nervous system (CNS) [90].

As mentioned above, mitophagy degrades damaged mitochondria under diverse stress conditions such as hypoxia, caloric restriction, or during certain developmental processes. Mitophagy is one of the mitochondrial quality control and surveillance mechanisms that is impaired in some pathologies leading to mitochondrial dysfunction. Likewise, several pathologies such as cancer development or progression are closely linked to abnormal mitophagy, being this intracellular mechanism a promising target for anticancer treatment [91,92]. Interestingly, our research group has demonstrated that hemin (a natural regulator of erythropoiesis) is able to induce mitophagy in K562 cells in a NIX-dependent manner. These results suggest that hemin favors erythroid maturation, inducing mitochondrial clearance [59]. On the other hand, it has been demonstrated that hematopoietic stem cell (HSC)-derived early progenitors from mice with decreased autophagy develop many symptoms of human myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). In addition, these autophagy-deficient HSCs showed a disrupted mitophagy, increased mitochondrial mass and higher proliferation and apoptosis levels [93]. As with CML, similar results have been obtained in autophagy impaired AML cells, where the knockdown of Atg7 leads to a marked increase of apoptosis and DNA damage during the treatment with cytarabine and idarubicin. These results suggest that autophagy and its microenvironment play an important role in AML chemoresistance, being the inhibition of Atg7 a possible strategy to enhance chemosensitivity and to improve outcomes in AML therapy [94,95]. Other authors have determined in adult patients with AML and acute lymphoblastic leukemia (ALL) that Beclin-1 and MAB1LC3B expressions were significantly down-regulated whereas the hypoxia-inducible factor-1α (HIF-1α) was upregulated. These changes in the Beclin-1, LC3 and HIF-1 α proteins levels have been associated with poor survival, indicating the essential role of these proteins in the development and progression of acute leukemia [96].

Polycythemia vera (PV) is one of the Philadelphia chromosome–-negative myeloproliferative neoplasms, characterized by an overactive Janus kinase (JAK)-(STAT) pathway. PV is estimated to transform into acute leukemia in 5–15% of cases over the course of 10 years. In general, this disease is featured by the development of erythrocytosis and presents significantly elevated levels of the transcription factor nuclear factor-erythroid 2 (NF-E2) [97,98]. This transcription factor plays an essential role in erythroid maturation and is a critical regulator of globin gene expression [98]. Moreover, it has been demonstrated that NF-E2 has an important role in the regulation of mitophagy and ribosome clearance during erythropoiesis. Likewise, it has been reported that the expression of the autophagy proteins NIX and Ulk1 are upregulated in transgenic mice and in granulocytes from PV patients. Furthermore, it has been demonstrated that elevated NF-E2 levels retards mitochondrial depolarization and delays mitochondrial elimination, thus altering erythrocyte maturation [99]. These results provide a crucial role for NF-E2 as a mitophagy regulator in the erythropoiesis.

Acquired aplastic anemia (AA) is a hematologic syndrome featured by pancytopenia and bone marrow hypoplasia in which a profound reduction in hematopoietic stem and progenitor cells occurs. Some studies have demonstrated that autophagy is active in murine CD34 + hematopoietic progenitor cells (HPCs) [100–102]. In contrast, a considerably decreased level of autophagy in CD34 + cells from patients with AA was observed. Likewise, inhibition of autophagy in CD34 + HPCs leads to a decreased proliferation and survival, sensitizing the cells to death and apoptosis [103]. These evidences support the role of autophagy in the hematopoiesis.

Conclusions

The studies discussed in this review support the fairly established role of autophagy in erythropoiesis as well as the role of this pathway in leukemia cells survival and protection against chemotherapy. During red blood cell maturation, cellular remodeling in the reticulocyte occurs due to two main processes, which are overlapped at cellular and molecular levels: vesicular trafficking and autophagy. It has been demonstrated that autophagy inhibition during erythroid differentiation leads to deficient erythroid maturation, demonstrating that this intracellular pathway is an essential process required for erythroid differentiation. For these reasons, understanding the action of autophagy modulators during erythropoiesis could prevent hematopoietic disorders. During the last decades, increasing efforts have been made to develop new strategies for the treatment of leukemia, such as the TKIs

in CML. It has been widely argued that autophagy has an important role in the treatment resistance in leukemia, being considered a cytoprotective mechanism in these tumor cells. However, inhibition of chemotherapy-induced autophagy sensitizes leukemia cells to chemotherapy, leading to programmed cell death. Molecular or pharmacological inhibition of autophagy might serve as a useful strategy for the treatment of drug and radiation resistant leukemia. Therefore, autophagy has been an important target for future treatment of hematologic pathologies, being its inhibition a possible therapeutic strategy, which could improve the efficiency of currently approved therapies.

Practice Points:

- Autophagy is a key catabolic pathway of blood cells involved in cell differentiation.
- Erythroid maturation is deficient when autophagy is impaired.
- Autophagy has an important role as a cell survival mechanism (favoring the growth of established tumors) and as a tumor suppressor (preventing the accumulation of damaged proteins and organelles).
- Modulation of autophagy in normal and pathologic erythropoiesis may facilitate

prevention and treatment of red blood cell-related disorders.

- Autophagy plays an important role as a cellular mechanism mediating sensitization to cancer therapy.

Research Agenda:

- Autophagy in erythroid maturation physiology.
- Molecular components of autophagy for prognosis and diagnosis of leukemia.
- Relationship between autophagy, erythropoiesis and hematological disorders.
- Development of autophagic targets as coadjuvant therapies.
- Genic studies that contribute to the diagnosis of cancer and other blood cell malignances.

Conflict of interest

The authors declare that there are no conflicts of interest.

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