Original Article

Bone Marrow Erythroblastic Dysplasia on Morphology Correlates Significantly with Flow Cytometric Apoptosis and Peripheral Blood Eryptosis

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Abstract

Introduction: Erythrocytic damage and death in response to physiochemical, infectious, metabolic, and pharmacological insults have been extensively studied in several diseases. Their relationship with erythroid precursors' apoptosis and morphological dysplasia, however, remains largely unexplored, despite several shared triggers and pathogenetic mechanisms. **Materials and Methods:** We compared peripheral blood phosphatidylserine (PS) exposure and calcium influx in 53 patients with early and late apoptosis of CD71 + ve marrow erythroblasts using flow cytometry. Flow cytometric results were then correlated with dyserythropoiesis in the bone marrow as scored by experienced morphologists. **Results:** Median patient age was 32 years (range: 1–75 years); 38 (72%) had hemoglobin (Hb) ≤11.0 g%. Patients overall had significantly higher Annexin V binding (PS exposure) and Fluo-3AM signal (calcium influx) vis-à-vis 20 healthy controls. Dyserythropoiesis on morphological evaluation correlated significantly with PS exposure ($r = 0.618$, $P = 0.014$) and Fluo-3AM binding ($P = 0.002$). Patients with dyserythropoiesis had significantly higher apoptosis compared to those without dyserythropoiesis ($P = 0.006$). In the peripheral blood, Annexin V binding and Fluo-3AM fluorescence correlated strongly with each other ($r = 0.885$, $P < 0.001$). PS exposure and Ca^{2+} influx were increased in 64% of cases. These patients had significantly lower Hbs and reticulocyte counts and increased red cell distribution widths and circulating nucleated red blood cell numbers. **Conclusions:** This is the first study to compare and demonstrate links between dyserythropoiesis, peripheral blood eryptosis, and erythroblastic apoptosis. Eryptosis and apoptosis' interrelationships in patients with diverse hematological disorders link the marrow environment to peripheral blood.

Keywords: Apoptosis, blood, cell death, eryptosis, flow cytometry

Introduction

Erythrocytes are involved in multiple dynamic physiological and pathological processes across the human body. Their ubiquitous presence and constant motion also render them vulnerable to cell injury, senescence, and death.[1] Removal of aged and/or damaged erythrocytes occurs through phagocytosis by reticuloendothelial cells, hemolysis, or eryptosis.[2] Eryptosis refers to a suicidal or endogenously triggered form of erythrocytic death that resembles apoptosis but is distinguished by the absence of mitochondrial depolarization and nuclear condensation.[3,4] It shares several similarities with apoptosis including cell shrinkage, loss of intracellular potassium, plasma membrane blebbing, and breakdown of phosphatidylserine (PS) asymmetry in the cell membrane.[5] Eryptosis is initiated by a variety of insults to erythrocytes including physical, chemical (including oxidative and drug-related), infectious, metabolic, or osmotic stimuli. It can also be abrogated by several chemical compounds, making it a common and useful component of toxicological studies.[6,7]

Eryptosis is postulated to be a protective mechanism to decrease the lifespan of injured or diseased erythrocytes. Their removal from circulation avoids or limits the detrimental sequelae of

Received: 17-10-2022 **Revised:** 25-12-2022
Accepted: 31-12-2022 **Published:** 07-02-2023

Access this article online

Quick Response Code:

Website: http://www.jmau.org/

DOI: 10.4103/jmau.jmau_97_22

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How to cite this article: Hans C, Sharma P, Saini R, Sachdeva MU, Khadwal AR, Bose PL, *et al*. Bone marrow erythroblastic dysplasia on morphology correlates significantly with flow cytometric apoptosis and peripheral blood eryptosis. J Microsc Ultrastruct 2025;13:1-7.

hemolysis, especially the inflammatory response due to the release of heme and intracellular generation of free radicals.[3,6,8]

Pathologically increased eryptosis is, however, more commonly deleterious. Erythrocytes with exposed PS adhere to endothelial cells through the CXCL16/scavenger receptor, interfere with circulation, bind to platelets, and trigger microvascular thrombosis.[3,9,10] Eryptosis amplifies inflammatory and immune responses as healthy erythrocytes acting as cytokine sinks are removed.[11,12] However, most commonly, it leads to anemia, and often, the same stimuli that cause an increase in eryptosis simultaneously impair erythropoiesis.[6,8]

Erythropoiesis in the bone marrow is a complex and highly-regulated process of the generation of erythrocytes from multipotent stem cells. Traditional models dichotomize anemia into that due to impaired medullary erythropoiesis, as is seen in inherited and acquired bone marrow failure syndromes, thalassemias, and congenital dyserythropoietic anemia; versus anemia occurring due to peripheral erythrocytic destruction or pooling, as in immune hemolysis and erythrocytic enzymopathies and membranopathies. Emerging opinion, however, suggests that abnormalities of erythropoiesis as well as of circulating erythrocytes contribute to anemia in most if not all conditions, as exemplified by sickle cell disease, anemias associated with inflammation, malignancy, renal failure and other chronic diseases, myelodysplastic syndromes, and infections such as severe malaria and sepsis.[13,14]

Dyserythropoiesis refers to the defective development/maturation of erythrocytes. It may be congenital or later-onset and acquired or inherited. Typically, a large number of dyserythropoietic erythrocytes are destroyed within the marrow during their abnormal maturation process in an apoptosis-based process called ineffective erythropoiesis. In some conditions with less severe dysmaturational changes, erythrocytes with morphological and functional abnormalities such as anisopoikilocytosis and fragmentation may enter the circulation.[13,15]

Apoptosis of erythroid progenitors in the marrow is triggered by the activation of intrinsic and extrinsic pathways. The same mechanisms are also frequently exacerbated in pathologic conditions characterized by anemia (i.e., hemoglobinopathies, hematological malignancies, myelodysplasia, and aplastic anemia). Oxidative stress, a major mediator of eryptosis, is also a key mechanism of ineffective erythropoiesis.[13-15]

The overlap between etiologies and pathogenetic mechanisms of red cell eryptosis and erythroblastic apoptosis suggests that both processes possibly act synergistically. To explore this possibility, we examined paired peripheral blood and bone marrow samples to detect correlations, if any, between them and morphological dyserythropoiesis.

Materials and Methods

This prospective study was conducted between July 2019 and September 2020 in the hematology department of a 2000 + bedded, tertiary care, state-funded teaching hospital, and research institute. Ethylenediaminetetraacetic acid (EDTA)-anticoagulated peripheral blood and bone marrow specimens from 53 patients undergoing bone marrow examination for on a definitive clinical indication were tested. Bone marrow aspirates that were inadequate for any reason, including hemodilution, were excluded. Patients with marked erythroblastopenia (<0.5% erythroblasts in the aspirate) were also excluded. Since standardized reference ranges for peripheral blood eryptosis were unavailable in the literature, we enrolled 20 healthy individuals (14 males and 6 females) aged between 23 and 40 years who were nonsmokers, nondiabetic, normotensive, and not on any medications. The control subjects had normal range blood counts, erythrocytic indices, and reticulocyte counts but were not matched for age/sex with the patients. They were also tested at the same time as the patients for various eryptosis tests on circulating erythrocytes. The study was approved by the Institutional Ethics Committee (Intra-Mural/Thesis) P.G.I.M.E.R. in June 2019. It adhered to the tenets of the Declaration of Helsinki. All participants or their legal guardians provided informed consent.

Two milliliter peripheral venous blood sample in EDTA was obtained from each patient as well as the control. In addition, from the patients, a minimum of 0.5 mL of EDTA anticoagulated bone marrow specimen was also obtained. Peripheral blood testing included a complete hemogram on D×H800 automated hematology analyzer, Beckman Coulter Inc., Florida, USA, with reticulocyte count and manual differential count, and Leishman-stained blood film examination. Eryptosis was assessed flow cytometrically by staining erythrocytes for PS exposure by Annexin V binding and assessment of calcium influx using Fluo-3AM dye.

Bone marrow flow cytometry was done for early and late apoptotic erythroblasts using the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA). This kit contains propidium iodide (PI)-based DNA staining solution (code 51-66211E) which was substituted by methyl green (MP Biomedicals, Santa Ana, CA; CI. 42585) as a brighter, safer and experimentally equivalent PI-alternative.[16-18]

Morphological examination of all bone marrow aspirate smears was done by an expert hematopathologist. The final diagnostic impression, the status of erythropoiesis (including the presence of dyserythropoiesis in a 1000-erythroblast count), and results of special studies (cytochemical stains, flow cytometry, and cytogenetic and molecular genetic analyses, as required) were recorded from the laboratory information system.

Results

The patients' age ranged from 1 to 75 years (median: 32 years); 36 (72%) were males. Clinical indications for bone marrow examination and diagnoses in the 53 cases are listed in Table 1. Overall, 21 cases (40%) showed infiltration by an active malignancy, 15 cases (28%) had been tested to assess response

to antineoplastic therapy, and 4 patients (8%) had been tested as a part of a disease-staging protocol.

Peripheral blood findings

Overall, 38/53 cases (72%) had hemoglobin (Hb) ≤11.0 g%. Only one patient had elevated Hb, a 43-year-old male with polycythemia vera (Hb 18.5 g%).

Table 2 shows the overall data for the tests for eryptosis in the patients and controls. Significant differences were noted between the values from patients-versus-controls for Annexin V and Fluo-3AM binding. There was a strong correlation between Annexin V binding and Fluo-3AM fluorescence (correlation coefficient $r = 0.885$, $P < 0.001$).

Using the control group ranges as reference intervals for eryptosis, cutoffs of >41 and >1270, respectively, were taken for defining increased Annexin V and Fluo-3AM staining. With these limits, increased Annexin V binding and increased Fluo-3AM staining were found in 32/53 (60%) patients. These patients showed significantly greater degrees of anemia, red cell distribution width-coefficient of variation (RDW-CV), and circulating nucleated erythrocytes and significantly lower reticulocyte counts as compared to the others. The mean platelet count in them was also lower, although the difference was statistically not significant ($P = 0.052$). These two groups are compared in Table 3.

The hospital information system was mined on the survival of our patients at 3 months after the bone marrow procedure. Three of the patients had expired (a 32-year-old woman with monocytic acute myeloid leukemia, a 9-year-old boy with relapsed B-ALL, and a 25-year-old man with COVID-19-associated hemophagocytic lymphohistiocytosis [HLH]). All three were among the cases with increased eryptosis.

Bone marrow findings

Erythropoiesis was normoblastic in 32 (60%), megaloblastic in 21 (40%) cases, and dyserythropoiesis ≥10% was seen in 7 (13%) cases. The median erythroid cell percentage was 28% (range: 1%–65%).

Early and late apoptotic erythroblast numbers were compared with the severity of anemia. Patients with moderate-to-severe anemia (Hb <8.0 g%) had significantly higher levels of late apoptotic erythroid precursors than those with higher

Table 1: Clinical indications for bone marrow examination and diagnoses in 53 cases

Diagnosis	Indication for marrow examination	*n*
B-lineage acute lymphoblastic leukemia on therapy	Marrow for remission status and MRD analysis	8
Acute myeloid leukemia on therapy	Marrow for remission status and MRD analysis	6
Acute myeloid leukemia, newly diagnosed	Diagnostic work-up	6*
Chronic myeloid leukemia, *bcr-abl1* positive, in chronic phase, newly diagnosed	Cytogenetic analysis, baseline trephine biopsy evaluation	5
Acute lymphoblastic leukemia, newly diagnosed/relapsed	Diagnostic workup	3
Aplastic anemia	Diagnostic workup	3
Suspected hemophagocytic lymphohistiocytosis	To document marrow hemophagocytosis; exclude alternative causes	3
bcr-abl1-negative myeloproliferative neoplasms	Diagnostic workup	2[$]
Myelodysplastic syndromes with excess blasts-2	Diagnostic workup	2
Chronic myeloid leukemia, *bcr-abl1* positive, on therapy with unexplained cytopenias	Investigation of the cause of cytopenias	2
Hypereosinophilic syndrome	Diagnostic workup	2
Plasma cell myeloma on therapy	Assessment of response to therapy and MRD estimation	2[#]
NHL without infiltration of bone marrow	Staging of NHL	2
NHL with infiltration of bone marrow	Staging of NHL	1
Plasma cell myeloma, newly diagnosed	Diagnostic workup	1
Immune thrombocytopenia	Exclusion of alternative causes of thrombocytopenia	1
Alport syndrome-associated renal osteodystrophy	Investigation of cause of cytopenias	1
Megaloblastic anemia refractory to hematinics	Investigation of cause of refractoriness	1
Neuroblastoma	Staging of neuroblastoma	1
Langerhans cell histiocytosis	Diagnostic and staging workup	1

*One case of acute promyelocytic leukemia, [$]One case each of polycythemia vera and essential thrombocythemia, [#]One patient who was post-CAR T-cell therapy, all hematopoietic and lymphoid neoplasms were classified as per WHO 2016 scheme. MRD: Measurable residual disease, NHL: Non-Hodgkin lymphoma, WHO: World Health Organization, CAR: Chimeric antigen receptor

Table 2: Results of various tests for eryptosis in peripheral blood

Test number	Test name	Patients (*n*=53)	Controls (*n*=20)	*P**
1	PS exposure by annexin V binding	45.4±15.1 (20-107)	31.1±8.3 (12-41)	***<0.001***
2	Ca^{2+} influx by fluo-3AM indicator	855.6±334.9 (77-1607)	668.2±320.5 (53-1270)	***0.017***

*Two-tailed *t*-test. Significant values are shown in bold italics. All values are given as mean±SD (range) of the mean fluorescent intensities for AV and Fluo-3AM. SD: Standard deviation, PS: Phosphatidylserine, AV: Annexin V

Hb ($P < 0.001$). Although there was a trend for increasing early apoptosis with declining Hb, this did not reach statistical significance [$P = 0.846$].

Bone marrow erythroblastic apoptosis showed significant correlations with peripheral blood PS exposure ($P = 0.001$), Fluo-3AM binding ($P = 0.006$), and Hb ($P = 0.009$). It, however, did not correlate with the RDW-CV, reticulocyte count, or nucleated erythrocyte number [Table 3].

Eryptosis and apoptosis versus morphological dyserythropoiesis

All patients with ≥10% dyserythropoiesis in the bone marrow aspirate on morphological evaluation also showed increased eryptosis. The percentage dyserythropoiesis in the bone marrows (of all 53 patients) correlated significantly with peripheral blood PS exposure ($r = 0.618$, $P = 0.014$) and Fluo-3AM binding ($P = 0.262$). Patients with dyserythropoiesis had significantly higher erythroblastic PS exposure in the bone marrow (i.e., early apoptosis) compared to those without dyserythropoiesis [$P = 0.006$]. Figure 1 shows 3 case vignettes representing varying levels of bone marrow early apoptotic and late apoptotic/necrotic cell death of erythroblasts.

Discussion

The current study is, to the best of our knowledge, the first in world literature to directly compare eryptosis with erythroblastic apoptosis. Eryptosis is increasingly described in numerous pathological settings associated with anemia.[1,4,8,19-24] There is increasing clinical interest in its modulation.[4,13,15,25] Several shared triggers of apoptosis and eryptosis are well-established: both are induced by energy depletion, osmotic shock, hyper/hypothermia, and reactive oxygen species. All of these result in the activation of Ca^{2+} permeable cation channels, rapidly increasing cytoplasmic calcium concentrations.[6,9,26] PS

Table 3: Comparison of peripheral blood and bone marrow findings in 15 patients with increased Annexin V as well as Fluo-3AM signal (used as a definition of unequivocally increased eryptosis) versus all the others

Parameter	Patients with unequivocally increased eryptosis ($n=15$)	Patients without unequivocally increased eryptosis ($n=38$)	*P*
Peripheral blood findings	-	-	-
Hb (g%)	7.4±3.1	10.6±4.2	***0.010***
Total WBC count (×10⁹/L)	27.6±19.3	33.7±25.8	***0.409***
Platelet count (×10⁹/L)	57.4±77.0	125.1±122.3	***0.052***
RDW-CV (%)	26.2±8.2	18.5±6.9	***0.001***
Corrected reticulocyte count (%)	1.5±0.5	2.3±0.8	***<0.001***
Nucleated erythrocytes/100 WBCs	2.9±4.5	0.8±2.7	***0.042***
Bone marrow findings	-	-	-
Erythroid percentage out of all acquired events	1.5±0.8	1.3±1.1	***0.525***
Early apoptotic erythroblast percentage in marrow*	19.7±12.3	13.4±14.6	***0.146***
Late apoptotic erythroblast percentage in marrow#	18.8±13.7	6.9±8.9	***0.001***

*Annexin V positive, methyl green negative, #Annexin V and methyl green dual-positive. Significant *P* values shown in bold italics. WBC: White blood cell, Hb: Hemoglobin, RDW-CV: Red cell distribution width-coefficient of variation

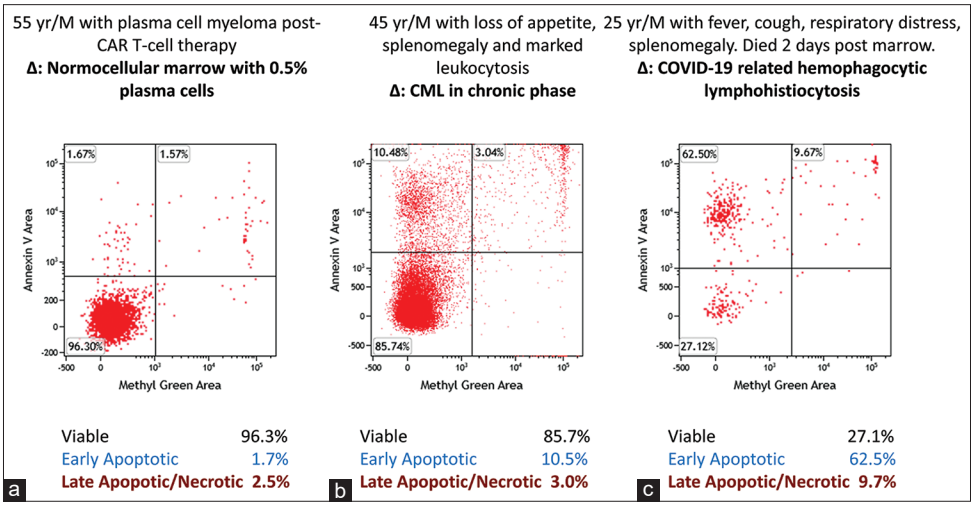

Figure 1: Three representative cases displaying varying levels of viable as well as early and late apoptotic erythroblasts in the bone marrow: (a) A 55-year-old male with myeloma on therapy shows very low numbers of annexin V and methyl green positive cells; (b) A 45-year-old male with chronic myeloid leukemia shows intermediate levels, while (c) A 25-year-old male with severe viral infection shows markedly increased number of apoptotic cells

exposure occurs in both processes. PS-exposed cells adhere to endothelial cells and platelets, triggering microvessel thrombosis.[6,9,27] Apoptosis is a key player in many canonical biological pathways including embryonic development, tissue homeostasis, several immunological reactions, and in the abrogation of the inflammatory response.[28] Similar roles for eryptosis are likely as contributions of erythrocytes in hemostasis, immune function, pathogen removal, and maintenance of acid–base and redox equilibria are increasingly well-defined.[11,29]

Being an exploratory study, we chose to enroll consecutive patients undergoing bone marrow examination (excluding them only if they had extremely low numbers of erythroblasts in the bone marrow). This particular paired-sample study design had some limitations. Prior studies on eryptosis have tried to enroll "clean" patients to avoid confounding factors. For instance, studies have excluded patients who were administered systemic antibiotics in the past 2 months, those on immunosuppressive therapies for up to the last 3 months, or those who had been transfused in the preceding 4 months. Some others excluded patients with systemic autoimmune and connective tissue disorders, hemoglobinopathies, those with hematological malignancies, or recipients of organ transplants.[11,21,30-32] In contrast, we enrolled a broad group, unified only by the fact that they required a bone marrow examination, since it offered us an opportunity to synchronously study in a specific hematopoietic precursor and its corresponding mature progeny (erythroblasts and erythrocytes), processes that were highly likely to be correlated with each other (apoptosis and eryptosis). We were limited (due to ethical reasons) to studying only those conditions where the patient has to anyway undergo a marrow procedure for a clinical indication, and not for this research. We could not age-and sex-match the control and patient groups, due to financial reasons that were also not studied for this reason. In addition, the heterogeneity of diagnoses in our cohort meant that this study design would not reveal the effects of variables such as therapy administered as well as the multiple underlying etiologies that will also influence eryptosis. This cellular-level study did not analyze molecular signaling pathways involved in eryptosis, for example., the p38 mitogen-activated protein kinase, caspases, casein kinase 1, Janus kinase 3, and protein kinase C.

In our main results, Annexin V binding and Fluo-3AM staining were the most reliable tests for eryptosis in our experience that correlated highly with each other. These are also the most commonly utilized assays in publications worldwide due to their sensitivity as well as technical convenience.[4] Reference ranges for these assays are difficult to come across, due to interlaboratory differences, and using our 20 healthy controls' data as the baseline, we found 64% and 36% of patients, respectively, to have elevations of Annexin V and Fluo-3AM binding. To maintain high specificity for eryptosis, we only considered those 15 patients (28% of the total) in whom both measures were elevated as showing unequivocally increased eryptosis. This approach was validated when these 15 cases showed significantly worse anemia, RDW-CV, reticulocyte counts, and nucleated erythrocytes numbers vis-à-vis the others. Since very low Hb, thrombocytopenia, high RDW, and circulating nucleated erythrocytes are known to predict increased mortality in various health-care settings,[33-35] it was unsurprising that all three patients who died showed increased eryptosis. This raises the intriguing possibility of whether elevated eryptosis in an intensive care, infectious disease, or oncological settings could be a potential predictor of future increased mortality, a theme not yet explored in literature.

The correlation between morphological dyserythropoiesis and PS exposure, although not unexpected,[30] has not been explored previously. The small number of patients with dyserythropoiesis (n = 7), however, makes reconfirmation in future larger cohorts essential. These seven patients also showed significantly increased PS exposure in their marrows. Raducka-Jaszul *et al*. recently reviewed erythroblastic maturation, apoptosis, and eryptosis in β-thalassemia patients, and highlighted that increased caspase levels and activation occur in both erythroblasts and erythrocytes in this condition.[36] This link is reflected in our results too, although the conditions studied were different. In thalassemic, efforts are underway to use apply novel therapeutic approaches such as transforming growth factor-beta (TGF-β) superfamily ligand traps to improve erythropoiesis by decreasing the proliferation of early erythroid progenitors and enhancing the differentiation of late-stage precursors.[37] Since eryptosis likely exacerbates anemia, hypoxia, and iron absorption in these patients,[38] it is possible that TGF-β superfamily inhibitors such as luspatercept and sotatercept act at least partially by inhibiting eryptosis as well.[38,39] Currently used clinically in thalassemia and myelodysplastic syndromes, evidence from studies such as ours may potentially extend their applications to conditions with anemia due to increased eryptosis, including renal and hepatic failure, malignancies, metabolic syndrome, and myelofibrosis.[1,9,37,40]

Although several of the diagnoses of our patients have previously been shown to result in increased eryptosis,[1,4,8,19-24] notable exceptions are HLH (which has only been described to display eryptosis in a single patient receiving cyclosporin A),[41] newly-diagnosed acute and chronic leukemias (previous reports are of patients receiving therapy),[42] and aplastic anemia. Since the above patients are often secondarily infected and/or are on multiple medications including antibiotics even at the time of referral to a tertiary care center such as ours, it is difficult to ascribe the enhanced eryptosis to the primary disease alone. In patients with polycythemia vera and essential thrombocythemia, enhanced eryptosis has been suggested to constitute a compensation mechanism against the myeloproliferative activity.[43] However, neither of the patients with polycythemia vera and essential thrombocythemia in our cohort had increased eryptosis.

In light of the above previously undescribed associations, caution is warranted as new insights in the field of cell death

become available at a rapid pace. Even though PS exposure on the cell membrane has long been considered a distinctive and specific feature of apoptotic cells that enables their recognition and phagocytosis by reticuloendothelial cells (efferocytosis), it has now also been reported in nonapoptotic types of programmed inflammatory cell death such as necroptosis and ferroptosis and in oncosis.[5,25,44] The ability of Annexin V staining alone to distinguish necrosis from apoptosis has also been challenged,[45] although till date, it remains the most commonly used test for this purpose.[3,4,6,9] Ultimately, these advances indicate that complete knowledge of how the processes of regulated cell death interact with the immune and inflammation pathways is yet not fully elucidated. The increased PS exposure in erythroblasts that we attribute to early apoptosis may be shown in the future to represent only partially related or even entirely different processes. There may even be a physiological role of erythroblast apoptosis in cellular maturation, similar to localized caspase activation in megakaryocytes to enhance proplatelet formation and budding.[46]

Conclusions

The current study's findings elevate eryptosis beyond just a mechanism for the removal of stressed and/or damaged erythrocytes. Demonstration of eryptosis' links to late apoptosis of erythroblasts as well as dyserythropoiesis in the bone marrows of patients with diverse disorders is significant as it throws up numerous intriguing possibilities of connecting the marrow environment's milieu to that of peripheral blood. Discovery of these "missing links" are likely to open up new possibilities of interventions that may ultimately benefit patients with chronic anemias, malignancies, metabolic derangements, and inflammatory and immunological conditions.

Acknowledgments

The authors thank Prof. N. Varma and Mrs. M. Gupta for expert guidance and technical assistance, respectively.

Financial support and sponsorship

Funding was provided by the Special Research Grant of the Medical Education and Research Cell, Postgraduate Institute of Medical Education and Research, Chandigarh, India (C. Hans).

Conflicts of interest

There are no conflicts of interest.

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