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Eryptosis in polycythemia vera and essential thrombocythemia*

Eryptoza w czerwienicy prawdziwej i nadpłytkowości samoistnej

Paweł Kopka[1,B,D,E,F], Katarzyna Bliźniewska[1,B,D,E,F], Paulina Sicińska[2,A,B,C,D], Piotr Duchnowicz[2,B,C,D], Bożena Bukowska[2,A,D], Jacek Treliński[1,A,D], Krzysztof Chojnowski[1,A,D]

[1] Department of Hemostasis Disorders, Medical University of Lodz, Department of Hematology, Copernicus Memorial Hospital, Lodz, Poland
[2] Department of Environmental Pollution Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, Lodz, Poland

Summary

Aim: Polycythemia vera (PV) and essential thrombocythemia (ET) are Philadelphia–negative myeloproliferative neoplasms with documented apoptosis impairment at the level of hematopoietic stem cell. However, so far no study has evaluated apoptosis of circulating blood neoplastic cells, including the suicidal death of erythrocytes – eryptosis.

Material/Methods: Erythrocytes from 61 patients (24 PV and 37 ET) naïve to and treated with hydroxyurea (HU) and 13 healthy individuals were analysed using flow cytometry to quantify phosphatidylserine (PS) externalization from Annexin-V-binding, calpain activity from 7-amino-4-chloromethylcoumarin (CMAC)-fluorescence, cell volume from forward scattered light (FSC) and cell shape from side scattered light (SSC).

Results: Significantly increased levels of calpain activity and PS exposure were observed in both ET and PV naïve patients, indicating enhanced eryptosis. Among HU-treated patients, a significant increase in calpain activity in the ET group and a decrease in the PV group were observed compared to patients without cytoreductive therapy. Among PV patients, FSC was substantially higher in the HU-treated group than in the naïve group, whereas no significant differences were found between HU-treated and HU-naïve groups of ET patients.

Conclusions: The enhanced eryptosis in ET and PV patients may be a form of systemic compensation of the pathological bone marrow overproduction of erythrocytes. HU, the basic cytoreductive drug used in ET and PV, may affect eryptosis in PV and ET in different ways depending on disease. The JAK2V617F mutation was not observed to have any effect on eryptosis in ET.

Keywords: **Essential thrombocythemia • Polycythemia vera • Eryptosis • Hydroxyurea • JAK2V617F mutation**

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Author's address: Paweł Kopka, Department of Hemostasis Disorders, Medical University of Lodz, Department of Hematology, Copernicus Memorial Hospital, Ciolkowskiego 2, 93-510 Lodz, Poland; e-mail: pablozdw@onet.eu

Abbreviations: **AML** – acute myeloid leukemia, **CALR** – calreticulin, **CMAC** – 7-amino-4-chloromethylcoumarin, **ET** – essential thrombocythemia, **FACS** – fluorescence-activated cell sorting, **FITC** – fluorescein isothiocyanate, **FSC** – forward scatter, **HCT** – hematocrit, **HSC** – hematopoietic stem cell, **HU** – hydroxyurea, **JAK2** – Janus Kinase 2, **MF** – myelofibrosis, **MPL** – myeloproliferative leukemia, **MPN** – myeloproliferative neoplasm, **PAF** – platelet activating factor, **Ph** – Philadelphia chromosome, **PLT** – platelets, **PS** – phosphatidylserine, **PV**– polycythemia vera, **RBC** – red blood cell, **SSC** – side scatter, **STAT** – signal transducer and activator of transcription protein family, **WBC** – white blood cell.

INTRODUCTION

Polycythemia vera (PV) and essential thrombocythemia (ET) are examples of Philadelphia chromosome–negative chronic myeloproliferative neoplasms (Ph-negative MPNs) – a group of clonal proliferative bone marrow diseases characterized by excessive growth of multipotent hematopoietic stem cells (HSCs), resulting in the overproduction of mature functional blood cells [33]. PV is characterized by a trilineage proliferation of the erythroid, myeloid and megakaryocytic cell line, usually resulting in increased numbers of erythrocytes, but also leucocytes and blood platelets in many cases. ET is generally a disorder of the megakaryocyte lineage with an excess platelet production. Although ET and PV are defined as separate entities, they show a great deal of overlap in their morphological and clinical characteristics, such as increased risk of bleeding, thrombosis, microcirculatory symptoms, leukocytosis, splenomegaly and the potential for transformation to myelofibrosis or acute myeloid leukemia [29]. ET and PV are also known to share the same molecular mutation, i.e. activating somatic mutation involving the JH2 pseudokinase domain of Janus kinase 2 (JAK2V617F), which is present in more than 95% of patients with PV and approximately 60% of patients with ET [32]. Additionally, certain ET cases with JAK2V617F mutation are associated with erythroid and granulocytic hyperplasia and can progress to a true PV or may remain a *forme fruste* of PV [5]. Permanent activation of JAK2 detected in JAK2V617F--positive HSCs triggers several tyrosine kinase-dependent cellular signaling JAK/STAT (Janus kinase/signal transducers and activators of transcription) pathways involved in cell proliferation and resistance to apoptosis promoting, culminating with the myeloproliferation and accumulation of myeloid cells [32].

Apoptosis, defined as the suicidal death of nucleated cells, can also affect erythrocytes, which lack nuclei and mitochondria, by a process called eryptosis [4, 7, 18]. Despite numerous publications that have appeared on the role of impaired apoptosis in bone marrow and/or peripheral hematopoietic progenitor cells in Ph-negative MPNs, including PV and ET [9, 19, 20, 25, 31], no study has yet examined eryptosis in mature functional red blood cells (RBCs) drawn from PV or other MPN Ph-negative patients. There are two major mechanisms of RBCs clearance from circulation: 1) senescence – the physiological removal of aged erythrocytes [3] and 2) eryptosis – prior to senescence suicidal death of defective erythrocytes injured by various types of cellular stress (oxidative stress, mechanical injury, energy depletion, hyperosmolarity, hyperthermia, xenobiotics or endogenous substances) [10].

Only four studies have examined phosphatidylserine (PS) exposure, one of the hallmarks of eryptosis in patients with Ph-negativeMPNs: three in PV patients [11, 12, 26] and one in ET patients [30]. However, these studies examined the onlyhypercoagulable status in these diseases and they did not evaluate eryptosis per se. Hence, the aim of the present study is to determine the possible role of eryptosis in the pathogenesis of PV and ET. The study also attempts to determine correlations between the studied eryptotic markers and JAK2V617F mutational status, as well as the influence of cytoreductive therapy with hydroxyurea (HU) on eryptosis in PV and ET patients.

MATERIALS AND METHODS

Patients

The study was approved by the Bioethics Committee of the Medical University of Lodz (No. RNN/84/16/KE). All subjects provided written informed consent before participating in this study. ET and PV were diagnosed according to the World Health Organization 2008 criteria [33].

Twenty-one patients with ET and ten patients with PV were previously untreated cytoreductive (naïve patients), whereas the rest of the studied patients were on HU. The clinical data included type of treatment (cytoreduction or not), presence of JAK2V617F mutation and complete blood count findings. The control group consisted of thirteen healthy subjects of similar age and gender.

Erythrocytes isolation

Peripheral blood was collected into tubes with anticoagulant (23 mM citric acid, 45.1 mM sodium citrate, 45 mM glucose), then centrifuged for 10 min at 600 xg at 4°C. After removal of plasma and leukocyte layers, samples were washed three times with 0.9% sodium chloride solution after each washing, and spinning in the same conditions. The erythrocytes were suspended in 0.9% sodium chloride solution to obtain a final hematocrit (HCT) of 50%.

FITC-conjugated Annexin-V staining

PS externalization on erythrocyte membrane is a marker of eryptosis. It can be measured using Annexin-V binding. Annexin-V (Annexin-V-FITC) is a fluorescein (fluorescein isothiocyanate, FITC) labeled, Ca^{2+}-dependent phospholipid binding protein. This protein has the highest binding affinity for PS. The externalization of PS was measured according to the procedure given by the manufacturer for Annexin-V-FITC apoptosis detection kit. The cells were centrifuged at 300 x g for 5 min at 4^0C and diluted with Ringer buffer (density $1x10^6$ cells/mL). The cells were stained with Annexin-V-FITC (1mikroM) in Annexin-V-binding buffer for 15 min at room temperature in total darkness. Flow cytometric analysis (LSR II, Becton Dickinson) was performed at excitation/emission of 488nm/525nm to visualize FITC fluorescence. FMC gate on the erythrocytes has been established for data acquisition and the data were recorded for a total of 10.000 events per sample. Annexin-V fluorescence intensity was expressed as arbitrary units of fluorescence (a.u.)

Calpain activity

Calpain I or μ-calpain (hereinafter called *calpain*) is a protease degrading cytoskeletal and erythrocyte plasma membrane proteins. Activation of calpain is a marker of eryptosis. Calpain activity was analyzed using flow cytometry (LSR II, Becton Dickinson). The analysis was conducted according to the manufacturer's protocol. Red blood cells were centrifuged at 300 xg for 5 min at 4^0C and diluted with Ringer buffer (density $1x10^6$ cells/mL). The cells were treated with cell permeable calpain substrate (t-butoxycarbonyl-Leu-Met) conjugated with 7-amino--4-chloromethylcoumarin (CMAC) at 10 mikroM and incubated for 60 min at 37^0C in total darkness. The cells were centrifuged and washed with Ringer buffer. The analysis for 10,000 cells was performed at excitation/emission wavelengths of 355 nm and 450 nm, respectively. Activity was reported in arbitrary units of fluorescence (a.u.)

Quantification of red cell size and shape

Cell shrinkage and cell membrane blebbing are the morphological characteristics of eryptosis. The size and shape of PV and ET erythrocytes were analyzed with a flow cytometer (LSR II, Becton Dickinson) using simultaneous separate detection of low angle (FSC) and right angle (SSC) light scattering. The intensity of forward scattered light (FSC) is expected to be proportional to the size (volume) of the cell, whereas side scattered light (SSC) is proportional to the cell shape, which depends on membrane roughness and/or inner complexity of the cell: the polymorphism of nucleus, cell density or granularity. FSC was used as a measure of erythrocyte size and SSC as an indicator of cell surface unevenness because erythrocytes are enucleated cells. For each population of RBCs, FSC and SSC signals were expressed in arbitrary units (a.u.)

Statistical analysis

Data are expressed as medians and interquartile ranges for continuous variables and as count and percentage for categorical variables. Statistical comparisons between two independent groups were made by the Mann-Whitney U test. The multiple intergroup comparisons were performed using the Kruskal-Wallis test followed by Dunn's post hoc test. The differences were recognized to be statistically significant when the p-value was lower than 0.05. Statistical analyses were conducted using STATISTICA software (StatSoft, Inc., Tulusa, USA).

RESULTS

Demographic and hematological characteristics

The demographic, clinical and laboratory data of the groups are summarized in Table 1. PV patients had significantly higher HCT, total platelet (PLT) and white blood cell (WBC) counts compared to controls. Additionally, PV patients had significantly higher HCT, red blood cell (RBC) and WBC counts compared to ET patients. ET group then had significantly higher PLT count compared to controls.

Eryptosis in PV and ET naïve patients versus control

The present study assessed the eryptotic potential separately in ET and PV naïve populations, using two recognized hallmarks of eryptosis: calpain activity (Fig. 1) and PS externalization (Fig. 2). Statistical analysis of ET individuals without HU-treatment (ET HU (-) patients) versus the control and PV individuals without HU-treatment (PV HU (-) patients) versus the control showed significant increase in calpain activity and Annexin--V binding in both ET and PV HU (-) patients compared to controls, with no statistically significant differences between these two groups of patients.

The morphological characteristics of PV and ET RBCs assessed by light scatter properties (FSC and SSC) in flow

Table 1. Characteristics of control and patient groups

Parameters	Control	ET	PV
Number	13	37	24
Sex M/F	5/8	7/30	8/16
Age (years)	56 (46–64)	64 (28–86)	63 (45–85)
Mutant JAK2V61F n (%)	–	18 (49)	24 (100)
HU – treated n (%)	–	16 (43)	14 (58)
HCT (%)	42.2 (33.0–46.8)	40.2 (25.0–47.0)	46.5*,## (40.6–54.3)
Hemoglobin (g/dL)	13.2 (12.3–15.7)	13.4 (7.7–16.2)	15.7 (13.8–18.0)
RBC count ($\times 10^9$)	4.67 (3.61–5.58)	4.43 (2.72–5.43)	4.84# (3.64–7.14)
PLT count ($\times 10^9$)	179 (155–286)	641** (335–1745)	484** (219–1082)
WBC count ($\times 10^9$)	6.95 (4.35–9.99)	7.52 (4.25–17.36)	7.50**,## (4.54–17.36)

Data are reported as number or median (range). ET = Essential thrombocythemia, PV = Polycythemia vera; *p <0.05 vs. controls, **p <0.001 vs. controls, #p <0.05 vs. ET, ##p <0.001 vs. ET

cytometry are presented in Table 2. Significantly higher median values of SSC were noted in both ET and PV HU (-) patients in comparison to controls; however, no statistically significant differences were found between the ET and PV HU (-) groups.

HU and eryptosis in PV and ET

In order to test whether eryptosis in PV and ET could be modified by a cytoreductive drug (HU), the eryptotic and cell morphology parameters of patients with (HU +) and without HU treatment (HU -) were compared in the PV and ET groups separately. The findings are presented in Fig. 1 (for calpain activity), Fig. 2 (for PS externalization) and Table 2 (for cell morphology parameters: FSC and SSC). In both the PV and ET HU-treated groups, calpain activity was higher than control values. Moreover, the activity of calpain in the HU (+) and HU (-) patients varied depending on the type of disease: in ET, calpain activity was significantly higher among the HU (+) patients than HU (-) ones, while in PV, calpain activity was significantly lower in the HU (+) patients than HU (-). Consequen-

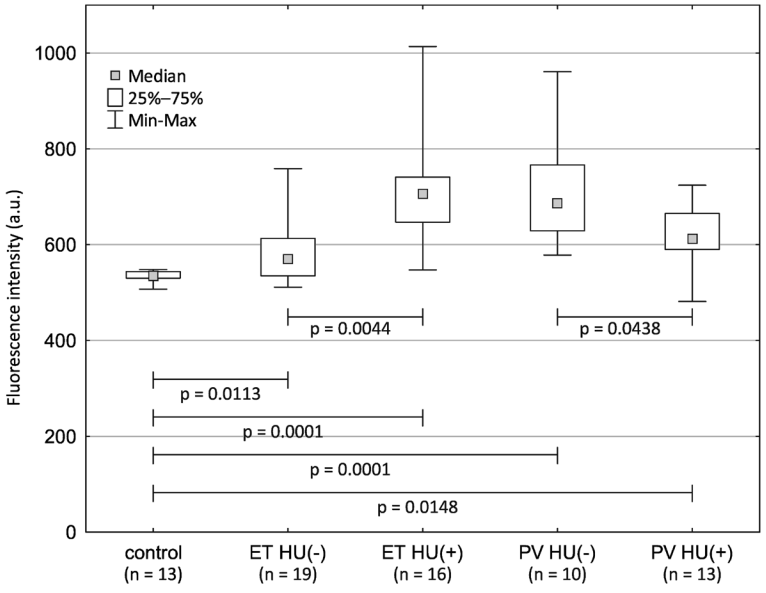

Fig. 1. Flow cytometry assessment of calpain activity (CMAC fluorescence) in erythrocytes drawn from ET and PV patients without and with hydroxyurea treatment (HU (-) and HU (+), respectively) and healthy volunteers; n = number of subjects in each category

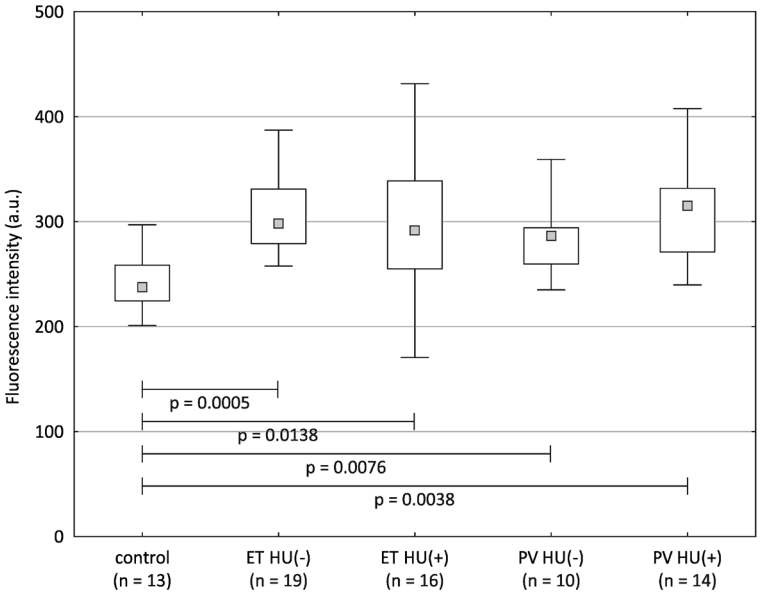

Fig. 2. PS externalization of erythrocytes drawn from ET and PV patients without and with hydroxyurea treatment (HU (-) and HU (+), respectively) and healthy volunteers evaluated Annexin-V binding by flow cytometry; n = number of subjects in each category

tly, substantially higher FSC was observed in the HU(+) PV patients than HU(-) PV patients, while no significant differences were observed between the HU(+) and HU(-) ET patients. Interestingly, no significant differences in PS translocation were observed between the HU(+) and HU(-) patients in either the ET or PV groups.

JAK2V617F mutational status in ET and eryptosis

No significant differences in PS exposure, calpain activity, FSC and SSC were found between the V617F-positive and V617F-negative ET patients.

DISCUSSION

Although a number of reports implicate impairment of HSC apoptosis as the main mechanism involved in the pathogenesis of Ph-negative MPNs [9, 19, 20, 25, 31], the role of the apoptosis at the level of circulating mature functional blood neoplastic cells remains poorly researched, and the present study is the first to confirm the presence of enhanced eryptosis in ET and PV.

We observed a markedly higher percentage of RBCs positive for active calpain as well as PS externalization in HU-naïve patients with both ET or PV than in healthy blood donors. Both increased activity of calpain and externalization of PS are two important links in the course of eryptosis process. The following basic sequence of molecular events takes place in erythrocytes during eryptosis signaling [10, 18]: mechanical/physicochemical stressor -> prostaglandin E2 release –>calcium cation (Ca^{2+}) channel opening –> inflow of Ca^{2+} ions into erythrocytes. The latter results in the initiation of three pro-

Table 2. Flow cytometric analysis of erythrocyte volume and shape using forward-scattered (FSC) and side-scattered (SSC) light in ET and PV patients without and with hydroxyurea treatment (HU (–) and HU (+), respectively) and healthy volunteers

Parameter	Control Median (range 25–75%) n = 13	ET HU(–) Median (range 25–75%) n = 21	ET HU(+) Median (range 25–75%) n = 16	PV HU(–) Median (range 25–75%) n = 11	PV HU(+) Median (range 25–75%) n = 13
FSC (a.u.)	33393 (31380–33798)	35164 (30468–37224)	34112 (31629.5–37887)	32455 (29120–32542)	35342 (33169–38220) *p = 0.0266 #p = 0.0344
SCC (a.u.)	32318 (30051–32842)	37560 (32403–42564) *p = 0.0193	39165 (36440–46965) *p = 0.0005	41972 (37435–43351) *p = 0.0104	39252.00 (36686–43849) *p = 0.0001

* p value relative to the control, # p value relative to PV HU(–); n = number of subjects in each category; a.u. = arbitrary units of fluorescence

cesses: 1) calpain activation leading to degradation of the RBC cytoskeleton and cell membrane blebbing 2) the opening of Ca^{2+}-sensitive potassium (K+) channels, leading to loss of potassium chloride from the RBC, loss of water and cell shrinkage resulting in subsequent platelet activating factor (PAF) liberation, PAF-mediated sphingomyelinase activation with ceramide generation and – as a result – ceramide-dependent scramblase activation with consequent PS externalization 3) Ca^{2+}-sensitive scramblase activation with consequent PS externalization. Our findings are consistent with those obtained by previous studies evaluating hypercoagulable status in PV and ET, which revealed a significant increase in PS translocation, thought to be a stimulator of the coagulation system, among PV and ET RBCs [12, 26, 30].

The mean life expectancy of PV RBCs is known to be shorter than or comparable to that of RBCs collected from healthy volunteers, thus excluding the participation of potential neoplastic erythrocyte "immortality" in the etiopathogenesis of PV [1, 2, 21, 23]. The presence of enhanced eryptosis among PV RBCs in the current study seems to confirm these observations. Our observations indicate that in patients with PV, the cause of increased RBC mass is not the accumulation of "immortal" RBCs, but rather the overproduction of bone marrow due to impaired apoptosis of hematopoietic bone marrow progenitor cells. The obtained results indicate that in PV, RBCs move from an antiapoptotic (typical for HSCs) to a proapoptotic profile (present in mature enucleated erythrocytes), as the neoplastically-changed cells of the erythroidal pathway mature. This change may represent a biological attempt to reduce erythrocytosis and its negative consequences in the course of the disease.

The observed enhancement of eryptosis in both PV and ET supports the hypothesis of common biological continuum existing between ET and PV dating back to the times of William Dameshek – the creator of the term "myeloproliferative disorders" [6]. It is an indirect evidence that in ET the neoplastic process affects not only the dominant pathologically changed megakaryocytic cell line, which determines the diagnosis of this disease – but also erythtrocytic line.

The main goal of therapy in PV and ET is to prevent thrombohemorrhagic complications. Current recommendations indicate that only high-risk PV patients and intermediate- and high-risk ET patients require cytoreductive therapy [27, 28]. The first-line drug of choice for cytoreductive therapy in both ET and PV is HU. HU is an antimetabolite that kills dividing cells in the S-phase of cell division via inhibition of the ribonucleoside diphosphate reductase needed for synthesis and repair of DNA, thus increasing antineoplastic activity and reducing the production of hematopoietic cells [22, 34]. HU can also exhibit cytotoxicity by inducing reactive oxygen species generation [8]. In the present study, Annexin-V staining, calpain activity, FSC and SSC were examined in fourteen patients with PV and sixteen patients with ET treated with HU. It was found that the activity of calpain in patients receiving cytoreductive therapy and in naïve patients differed depending on whether they were suffering from ET or PV. While calpain activity was significantly higher among treated patients than non-treated ones in ET, calpain activity was significantly lower in treated patients than non-treated ones in PV.

Theoretically, there may be several reasons for this observed difference in calpain activity following HU treatment: the complex mechanism of action of the drug, the different clinical and biological characteristics of the two diseases, and the unknown impact of HU on calpain activity without overall influence on eryptosis, as no significant differences were found regarding PS translocation between HU-treated versus HU-non-treated groups in either the ET or the PV groups.

Firstly, it is possible that in addition to its antimitotic or antiproliferative effect on HSCs, HU could also exert an oxidative and cytotoxic influence on circulating mature functional RBCs. HU treatment – by its induction of free radical production – can lead to oxidative stress in RBCs, which is recognized as an important direct trigger of eryptosis [10]. Additionally, the cytotoxic effect of HU on RBCs may be related to its ability to convert - in radical mechanism - hemoglobin to methemoglobin, which has been found to be capable of degrading band 3 of the RBC membrane, resulting in clearance of the RBCs from circulation (indirect proeryptotic action of HU) [14]. HU metabolites, including nitric oxide, have been found to react with hemoglobin to form methemoglobin and nitric oxide-hemoglobin in animal models [16, 24].

Secondly, while PV is characterized by negative effects mainly on the erythroid lineage, in ET the megakaryocyte lineage is affected and the erythroid remains relatively untouched. Hence, the main eryptotic trigger in PV could be increased HCT, acting via mechanical cell stressors such as hyperviscosity and impaired microcirculation, with abnormal interactions occurring between RBCs and between RBCs and the walls of microvessels [17]. Through its antiproliferative properties, HU reduces HCT and thus the degree of eryptosis. It seems that the cytoreductive and, consequently, antieryptotic effect of HU on bone marrow outweighs its proeryptotic properties resulting from oxidative action, which leads to HU displaying a net inhibition of eryptosis in patients with PV, as observed in the study group. Tan et al. [26] also demonstrated antieryptotic potential of HU treatment in PV. Their study, however, concerned the role assessment of PS exposure on erythrocyte and platelet membranes in the hypercoagulable state in PV. This effect of HU was also confirmed in patients with sickle cell disease [13, 15]. In ET, where HCT is usually normal, HU works in two ways: firstly, as an antimitotic affecting neoplastically changed stem cells proliferating mainly in the megakaryocytic direction and, secondly, as an oxidizing agent acting on circulating mature erythrocytes, inducing oxidative stress in RBCs and the formation of

potentially proeryptotic methemoglobin. It is, therefore, possible that the enhanced eryptosis observed in ET may be a consequence of oxidative stress and an increase of methemoglobin concentration, both of which have been induced by HU. Additionally, our analysis demonstrated that FSC, a marker of cell volume and cell shrinkage, was markedly higher in the HU-treated PV patients than HU-naïve group. No significant differences were observed in the other studied parameters.

The findings regarding calpain activation and FSC changes suggest that HU inhibits eryptosis in PV patients. The influence of HU on eryptosis in ET remains not fully understood; our data showed an increase in eryptosis after HU treatment in ET patients via an unknown mechanism. Interestingly, no changes in SSC were observed in HU (+) patients compared to HU-naïve patients in either the ET or the PV group. SSC parameter increased in both the PV and ET naïve patients was elevated at a similar level also in analogous HU-treated groups, which may suggest that the changes occurring in the RBC membrane during eryptosis are irreversible: the changed shape of the erythrocyte may be so permanent that even a change in cell volume caused by HU treatment does not influence SSC. The mechanism behind the influence of HU on eryptosis, as well as the variability in its effect on different cell types, requires further study and elucidation.

Our study suggests that the presence of the JAK2V617F mutation has no influence on eryptosis in ET. In Ph-negative MPN bone marrow progenitor cells, JAK2V617F mutation results in constitutive activation of the JAK2/STAT pathway, leading to nuclear transcription of a variety of the genes involved in cellular proliferation, differentiation and resistance to apoptosis [32]. No impact of the JAK2V617F mutation on eryptotic status of ET RBCs seems to be a natural consequence of the loss of the cell nucleus by mature erythrocytes, and thus the interruption of the JAK2/STAT pathway signaling at the stage of its endpoint.

CONCLUSIONS

In summary, the enhanced eryptosis observed in PV patients may be a form of systemic compensation for the pathological bone marrow overproduction of RBCs; this plays a crucial role in the removal of excessive erythrocytes in circulation and preventing the negative consequences associated with increased HCT. In addition, in ET, in which HCT is usually normal, enhanced eryptosis appears to indicate subclinical inclusion of the erythroidal lineage into the neoplasmatic proliferation. HU, the basic cytoreductive drug used in ET and PV, may affect eryptosis in PV and ET in different ways depending on the disease; this may suggest the drug has a complex and multidirectional mechanism of action, and that significant differences exist in the pathophysiology of both disease entities, and HU may be marker of them. These observations require further research.

JAK2V617F mutation, which protects HSCs from apoptosis by transcriptional deregulation of pro- and antiapoptotic genes, does not affect eryptosis in enucleated mature RBCs of patients with ET. Our study the population was limited in size and, therefore, our results should be interpreted with caution. Further large, well-designed studies are now needed to clearly determine the role of eryptosis in the pathogenesis of ET and PV and the impact of HU-treatment on eryptosis in theses neoplasms.

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